Protein tyrosine phosphatase 1B regulates metabolic, oncogenic, and hematopoietic function

Nadia Marie-Noël Dubé

Department of Biochemistry

McGill University

Montreal, Quebec, Canada

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Abstract

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed enzyme that is involved in multiple signaling pathways. Biochemical and substrate trapping studies have implicated PTP1B in the dephosphorylation of various tyrosine kinases, including the EGFR, PDGFR, IR, IGF-IR, JAK2, p210Bcr-Abl, and Src. Of particular interest, gene-targeting studies in mice have established PTP1B as a critical physiological regulator of metabolism by attenuating insulin and leptin signaling. Indeed, PTP1B null mice exhibit resistance to diet-induced diabetes and obesity. Although PTP1B is involved in signaling pathways that contribute to oncogenesis, PTP1B null mice do not develop spontaneous tumors. Therefore, my doctoral research focuses on identifying the physiological significance of PTP1B in these pathways. Our laboratory has previously demonstrated that PTP1B modulates leptin signaling via the tyrosine kinase JAK2. Accordingly, I have shown that PTP1B dephosphorylates JAK2 in a growth hormone (GH)-dependent manner, thus negatively regulating GH signaling and downstream effectors such as STAT3 and STAT5. Consequently, mice lacking PTP1B remain sensitive to GH action after starvation. In addition, I showed that the absence of PTP1B could improve glycemia during streptozotocin-induced type 1 diabetes. In the second part of my research, I have elucidated the mechanism for the previously reported decreased ERK activation in PTP1B null fibroblasts. I demonstrated that Ras activity is reduced in these cells, which is due to increased p120RasGAP expression and p62Dok hyperphosphorylation. Both of these molecules negatively regulate Ras activity by promoting the intrinsic GTPase activity of Ras, leading to decreased ERK activation. Finally, I developed a mouse model of cancer to study the role of PTP1B in tumorigenesis. Since the majority of cancers harbor mutations in p53, I generated p53/PTP1B double null mice. In the absence of p53, PTP1B heterozygous and null mice display decreased survival rates. An increased

proportion of the double null mice develop lymphomas compared to p53 null mice, suggesting that PTP1B may play a role in hematopoiesis. In fact, in mice lacking PTP1B, there is an increase in the absolute number of B cells in the bone marrow and lymph nodes. Hence, my work revealed a novel and important role for PTP1B in immune and oncogenic function, as well as underlining its involvement in metabolic homeostasis.

Résumé

La protéine tyrosine phosphatase 1B (PTP1B) est une enzyme exprimée de façon ubiquitaire et elle est impliquée dans de multiples voies de signalisation. Des études biochimiques et de "substrate trapping" ont démontré que PTP1B déphosphoryle différents récepteurs à activité tyrosine kinase (RTK), comme EGF, PDGF, IR, et IGF-1, ainsi que des kinases cytosoliques comme JAK2, p210Bcr-Abl, et Src. Des études d'inactivation de gène chez la souris ont établi que PTP1B est un régulateur physiologique important du métabolisme en modulant négativement la signalisation de l'insuline et de la leptine. En conséquence, l'absence du gène PTP1B rend les souris résistantes à l'induction du diabète et de l'obésité par une diète riche en gras. Malgré le rôle de PTP1B dans la signalisation oncogénique, les souris déficientes en PTP1B ne développent pas de tumeurs. Ainsi, mes travaux de doctorat se sont orientés vers la compréhension de la signification physiologique de PTP1B dans ces voies.

Notre laboratoire a préalablement démontré que PTP1B régule la voie de signalisation de la leptine via la tyrosine kinase JAK2. Par conséquent, j'ai montré que PTP1B déphosphoryle JAK2 de façon dépendante de l'hormone de croissance, ce qui régule négativement les molécules en aval de cette voie comme STAT3 et STAT5. Ceci a pour effet de maintenir la sensibilité à l'hormone de croissance des souris déficientes en PTP1B après une période de jeûne. De plus, j'ai démontré que l'absence de PTP1B améliore la glycémie de souris rendues diabétiques de type 1 par injection de streptozotocine. Dans la seconde partie de mes travaux, j'ai découvert un mécanisme qui explique la diminution de l'activation de ERK malgré l'hyperactivation des RTK dans les fibroblastes déficients en PTP1B, résultat récemment rapporté par notre laboratoire. J'ai établi que la réduction de l'activité de Ras en absence de PTP1B est causée par l'augmentation de l'expression de p120RasGAP et

l'hyperphosphorylation de p62Dok. Ces deux molécules régulent négativement l'activité de Ras en promouvant l'activité GTPase intrinsèque de Ras, conduisant à une baisse de l'activation de ERK. Finalement, j'ai développé un modèle de cancer chez la souris afin d'étudier la fonction de PTP1B dans la tumorigénèse. Puisque la majorité des cancers présentent des mutations dans le gène p53, j'ai généré des souris déficientes en p53 et PTP1B. En absence de p53, les souris hétérozygotes et homozygotes pour PTP1B voient leur survie diminuée. Les souris doubles mutantes développent des lymphomes dans une proportion accrue comparée aux souris déficientes en p53, suggérant un rôle pour PTP1B dans l'hématopoïèse. En effet, les souris déficientes en PTP1B montrent une augmentation du nombre absolu de cellules B dans la moelle et les ganglions lymphatiques. En somme, mes travaux ont révélé une fonction nouvelle et importante de PTP1B dans l'hématopoïèse et l'oncogénèse, en plus de souligner son implication dans l'homéostasie du métabolisme.

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Chapter 1

List of abbreviations

A	Alanine
AB	Acidic box
AgRP	Agouti-related peptide
ALS	Acid labile subunit
AKT/PKB	Protein kinase B
ASO	Antisense oligonucleotide
BCR	B cell antigen receptor
BM	Bone marrow
C	Cysteine
CAP	Cbl-associated protein
CIS	Cytokine-inducible SH2-containing protein
CLP	Common lymphoid progenitor
CML	Chronic myelogenous leukemia
CNS	Central nervous system
CRD	Cysteine-rich domain
CREB	Cyclic Amp response element binding protein
CSF-1R	Colony stimulating factor-1 receptor
CV	Conduction velocity
D	Aspartic acid
DM	Diabetes mellitus
DN	Diabetic neuropathy
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EphR	Ephrin receptor
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FACS	Fluorescence activated cell sorting
FABD	Focal adhesion-binding domain
FAS	Fatty acid synthase
FGFR	Fibroblast growth factor receptor
FNIII	Fibronectin III like domain
FRET	Fluorescence resonance energy transfer technique
GAPs	GTPase-activating proteins
GDP	Guanosine diphosphate
GEFs	Guanine nucleotide exchange factors
GH	Growth hormone
GHR	Growth hormone receptor
GK	Goto-Kakizaki insulin resistant type 2 diabetic rats
GLUT4	Glucose transporter 4
Grb2	Growth factor receptor binding protein-2
GSK3	Glycogen synthase kinase 3
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HGFR	Hepatocyte growth factor receptor

hGH	human growth hormone
HLA	Human Leukocyte Antigens
HSC	Hematopoietic stem cell
IgD	Immunoglobulin-like domain
IGF-1	Insulin growth factor-1
IGF1-R	Insulin growth factor receptor
IL-7	Interleukin-7
Ins	Insulin
IR	Insulin receptor
IRS	Insulin receptor substrate
ITTs	Intraperitoneal insulin tolerance tests
JAK	Janus kinase
JMML	Juvenile myelomonocytic leukemia
KO	Knockout
LN	Lymph nodes
LPL	Lipoprotein lipase
LRD	Leucine-rich repeat
MAPK	Mitogen activated protein kinase
MEFs	Murine embryonic fibroblasts
MOG	Myelin oligodendrocyte glycoprotein
mRNA	messenger RNA
NGFR	Nerve growth factor receptor
NRPTKs	Non receptor protein tyrosine kinases
NRPTPs	Non receptor protein tyrosine phophatases
ob/ob mice	Leptin null obese mice
ObR	Leptin receptor
OGTTs	Oral glucose tolerance tests
P, pro	Proline
p62Dok	Downstream of tyrosine kinase;
p120RasGAP	Ras GTPase activating protein
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PI3K	Phosphatidylinositol 3- kinase
PH	Pleckstrin homology
PIAS	Protein inhibitor of activated STATs
PLCg	Phospholipase C gamma
PMEFs	Primary murine embryonic fibroblasts
POMC	Pro-opiomelanocortin
PPARgamma	Peroxisome proliferator-activated receptor gamma
PRS	p210Bcr-Abl responsive sequence
Pseudo PTK domain	Kinase-like domain
PSTPs	Protein serine/threonine phosphatases
PTB	Phosphotyrosine binding
PTK domain	Kinase domain
PTKs	Protein tyrosine kinases
PTP1B	Protein tyrosine phosphatase 1B
PTPs	Protein tyrosine phosphatases
RTKs	Receptor tyrosine kinases
Q	Glutamine
S, Ser	Serine
SC	Stroma cells

SCID	Severe combined immune deficiency
SH2	Src homology 2
SH3	Src homology 3
SNP	Single-nucleotide polymorphism
SOCS	Supressors of cytokine signaling
SOS	Son of sevenless
SREBP-1	sterol regulatory element binding protein-1
STAT	Signal transducer and activator of transcription
STZ	Streptozotocin
T, Thr	Threonine
TA domain	Transcription activation domain
SV40TAg	SV40 Large T antigen
TCR	T Cell antigen receptor
Type 1 diabetes	Insulin-dependent diabetes mellitus
Type 2 diabetes	Non-insulin-dependent diabetes
VEGFR	Vascular endothelial growth factor receptor
WT	Wild-type
Y, Tyr	Tyrosine
YB-1	Y box-binding protein-1
ZDF/Drt-fa/fa	Zucker diabetic fatty rats

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Preface

The Faculty of Graduate and Postdoctoral Studies at McGill University has issued the following guidelines concerning thesis preparation.

As an alternative to the traditional thesis format, the dissertation can consist of a collection of papers of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following:

a table of contents;
a brief abstract in both English and French;
an introduction which clearly states the rational and objectives of the research;
a comprehensive review of the literature (in addition to that covered in the introduction to each paper);
a final conclusion and summary;
a thorough bibliography;
Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the

task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

6. When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the publishers and submit these to the Graduate and Postdoctoral Studies Office with the final deposition, if not submitted previously. The candidate must also include signed waivers from any co-authors of unpublished manuscripts.

7. Irrespective of the internal and external examiners reports, if the oral defense committee feels that the thesis has major omissions with regard to the above guidelines, the candidate may be required to resubmit an amended version of the thesis. See the "Guidelines for Doctoral Oral Examinations," which can be obtained from the web (http://www.mcgill.ca/fgsr), Graduate Secretaries of departments or from the Graduate and Postdoctoral Studies Office, James Administration Building, Room 400, 398-3990, ext. 00711 or 094220.

8. In no case can a co-author of any component of such a thesis serve as an external examiner for that thesis.

My thesis has been written according to these guidelines, with the following organization: chapter 1 consists of an overview of the current literature and introduction; chapters 2-5 detail my research work presented in the form of manuscripts; and chapter 6 consists of a general discussion of all my findings, as well as summary and perspectives.

Publications arising from work of the thesis

First or co-first author publications

1. Gu F., **Dubé N.**, Kim J.W., Cheng A., Ibarra-Sanchez MdJ, Tremblay M.L., Boisclair Y.R. 2003. Protein tyrosine phosphatase-1B attenuates growth hormone-mediated JAK2-STAT signaling. Mol Cell Biol. 23: 3753-62. **Co-first author**; Chapter 2

2. **Dubé N.**, Rados M., Padjen A.L., Tremblay M.L. The absence of Protein Tyrosine Phosphatase 1B delays the onset of hyperglycemia in a model of streptozotocin diabetic mice. In preparation; Chapter 3

3. **Dubé N.**, Cheng A., Tremblay M.L. 2004. The role of protein tyrosine phosphatase 1B in Ras signaling. Proc. Natl. Acad. Sci. USA 101: 1834-1839. **Co-first author**; Chapter 4

4. **Dubé N.**, Bourdeau A., Heinonen K., Cheng A., Lee Loy A., Tremblay M.L. Genetic ablation of Protein Tyrosine Phosphatase 1B accelerates tumorigenesis and modifies the tumor spectrum of p53 null mice: potential role for PTP1B in B cell development. In preparation; Chapter 5

Contributions of authors

1. F. Gu generated Figures 2 and 3, with my assistance on Figure 2. J.W. Kim performed the transcription reporter assay (Figure 5) and the northern blot (Figure 7). M. de Jesus Ibarra-Sanchez provided the TCPTP immortalized cell line. A. Cheng assisted on isolation of primary fibroblasts and provided the PTP1B immortalized cell lines. I generated rescue cell lines, Figure 1 and Figure 4. I performed experiments with PTP1B deficient mice as well as Figure 6, and provided the samples leading to Figure 7. Moreover, I was in charge of maintaining the mouse colony.

2. I generated Figure 2 as well as preliminary data leading to Figure 1.I assisted M. Rados on Figure 1 and provided the PTP1B null mice.

3. I established the rescue and V12Ras cell lines, and performed all experiments involving Ras (Ras activated cell lines, Ras-GTP assay, soft agar colony assay, Ras rescue cell lines, Figure 5). A. Cheng established the SV40TAg immortalized cell lines, generated Figure 1, Figure 4 a, and assisted on Figures 2 and 3.

4. A. Bourdeau assisted on FACS experiments. K. Heinonen assisted on FACS experiments, and generated Figure 5a and b. A. Cheng started the breeding of p53/PTP1B mice and assisted on Figure 1. A. Lee Loy contributed to dissection prior to FACS analysis. All other work is of my own.

Non-thesis related publications

5. Qiu W., Avramoglu R.K., **Dubé N.**, Chong T.M., Naples M., Au C., Lewis G.F., Cohn J., Tremblay M.L., Adeli K. 2004. Hepatic expression of PTP-1B is a key initiating factor underlying the induction of lipoprotein overproduction and metabolic dyslipidemia associated with insulin resistance: Evidence from Overexpression, Knockout and Gene Suppression Studies. Diabetes 53: 3057-3066.

I monitored weight gain of mice fed on high-fructose diet, and performed glucose measurements (Figure 1). I assisted on ApoB pulse-chase experiments (Figure 2). I provided the PTP1B null mice and assisted on isolation of primary hepatocytes.

6. Gu F., Nguyen D., Stuible M., **Dubé N.**, Tremblay M.L, Chevet E. 2004. Protein-tyrosine phosphatase 1B potentiates IRE1 signaling during endoplasmic reticulum stress. J. Biol. Chem. 279: 49689-49693.

I established PTP1B knockout cell lines, rescue cell lines, cloned EGFP vectors, retroviral vectors, and the virus production. (Figures 1, 2, supplementary data 1).

7. Sangwan V., Paliouras G.N., Cheng A, **Dubé N.**, Tremblay M.L., Park M. Protein Tyrosine Phosphatase 1B deficiency protects against Fas induced hepatic failure. Submitted.

I assisted on some figures and isolation of primary hepatocytes. I was responsible for the breeding and maintenance of the PTP1B mouse colony, and I supplied the authors with the PTP1B null mice.

8. Sangwan V., Paliouras G.N., Cheng A, **Dubé N.**, Tremblay M.L., Park M. The Met receptor tyrosine kinase is a substrate for PTP1B. In preparation.

I generated the PTP1B rescue cell lines and EGFP vectors.

Review articles

9. **Dubé N.**, Tremblay M.L. 2004. Beyond the metabolic function of PTP1B. Cell Cycle 3: 550-3.

10. Cheng A., **Dubé N.**, Gu F., Tremblay M.L. 2002. Coordinated action of protein tyrosine phosphatases in insulin signal transduction. Eur. J. Biochem. 269: 1050-1059.

I drew all three figures of the review.

11. Bourdeau A., **Dubé N.**, Tremblay M.L. 2005. Cytoplasmic PTPs regulation and function: the roles of PTP1B and TC-PTP. In press, Current opinion in cell biology. **Co-first author**

Book chapter

12. Blanchetot C., Chagnon M., **Dubé N.**, Hallé M., Tremblay M.L. 2005. Methods 35: 44-53.

I generated Figure 2 and assisted on Table 1.

Dedication

À mes parents, Louiselle et Jean-Pierre,

pour avoir cru en moi, même lorsque je n'y croyais pas

1 Chapter 1

1.1 Introduction

The regulation of tyrosine phosphorylation of cellular proteins plays a critical role in a variety of biological processes, including cell proliferation, differentiation, transformation, and metabolic homeostasis. As much as 90% of phosphorylation events occur on serine residues, about 10% on threonine, and less than 1% of tyrosine residues [1]. Tyrosine phosphorylation is a reversible process, and is controlled by the opposing activities of two major families of enzymes, namely the protein tyrosine kinases (PTKs) and the protein tyrosine phosphatases (PTPs). Aberrant changes to this equilibrium can lead to deleterious effects, such as deregulated cell growth, including diseased states in humans. Hence, the biological consequences of the signaling pathways controlled by PTKs and PTPs are important. For example, the levels of protein tyrosine phosphorylation are frequently elevated in cancer cells. When mutated or altered structurally, PTKs can become potent oncoproteins, causing cellular transformation. For instance, overexpression and/or activating mutations of at least thirty PTKs have been linked to malignant transformation and cancer [2]. In addition, other diseases also result from aberrant PTK signaling. The most recognized example in humans involves the deregulation of insulin signaling that leads to the development of diabetes mellitus, the world's most common metabolic disorder [3].

Therefore, both enzyme families are considered master regulators of physiological processes, but compared to their PTK counterparts, much less is known about the role of PTPs in human diseases. In this aspect, the aim of my doctoral research was to further define the physiological functions of the protein tyrosine phosphatase 1B, PTP1B, in metabolism and cancer. The following review of literature will focus on the role of various PTPs, and specifically, PTP1B, as major regulators of cell signaling.

1.2 The protein tyrosine kinase family

The PTK family constitutes an important class of molecules that mediate signal transduction. They regulate processes such as metabolism, cell growth, differentiation, cytoskeletal rearrangement and migration, apoptosis, and transcription. PTKs catalyze the transfer of the gamma-phosphate group of ATP to the hydroxyl groups of specific tyrosine residues in proteins. Their catalytic domain consists of two lobes in N-terminal, which interacts with the phosphatase groups of ATP, and the C-terminal domain, which provides substrate-binding sites for ATP. This domain includes the activation loop, and contains Tyr, Ser, and Thr residues that can be phosphorylated. The phosphorylation of these residues increases the kinase activity [4].

PTKs are divided into two groups: 1) the receptor tyrosine kinases (RTKs) that possess transmembrane and extracellular domains, and 2) the non-receptor tyrosine kinases (NRTKs). In the human genome, 58 genes encode RTKs [2, 5], whereas 32 genes encode NRTKs [4].

1.2.1 Receptor tyrosine kinases (RTKs)

Each RTK consists of an extracellular ligand binding domain, a transmembrane domain, and an intracellular tyrosine kinase domain. Based upon the structural features in their extracellular domain, RTKs have been classified into 20 different families [2]. Major families are represented on Figure 1 and their structural features and functions are detailed in Table 1 [6].

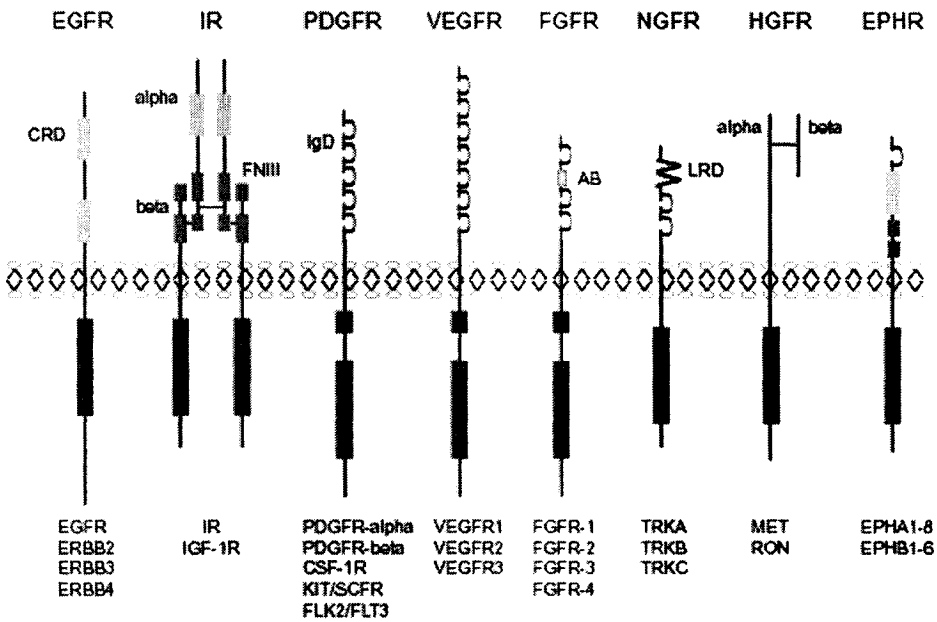

Figure 1. Schematic representation of members of the human RTKs family. The prototypic receptor for each family is indicated above the receptor, and the known members are listed below. Abbreviations: EGFR, epidermal growth factor receptor; IR, insulin receptor; PDGFR, platelet-derived growth factor receptor; VEGFR; vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; NGFR, nerve growth factor receptor; HGFR, hepatocyte growth factor receptor, EphR, ephrin receptor; AB, acidic box; CRD, cysteine-rich domain; fibronectin type III-like domain; IgD, immunoglobulin-like domain; LRD, leucine-rich domain. Adapted from Manning et al. 2002 [5].

Class	RTKs	Structural Features	Functions
I	EGFR, Neu/HER2, HER3	Cysteine-rich domain	EGFR was the first RTK identified. Important for the morphogenesis of epithelial tissues. Often amplified or activated through mutations in human malignancies.
II	IR, IGF-1R	Cysteine-rich domain;disulfide-linked heterotetramers	IR mediates metabolic effects. This family mediates important survival signals.
III	PGFR, c-Kit, CSF-1	5 Ig-like domains, kinase insert domain	PDGFR is important for the development of the connective tissue compartments of various organs, as well as for the development of smooth muscle cells of blood vessels. CSF-1 is involved in the development of hematopoietic cells, germ and neuronal cells, and macrophages.
IV	VEGFR	7 Ig-like domains, kinase insert domain	Primarily expressed on endothelial cells, thus implicated in vasculogenesis, angiogenesis, and lymphangiogenesis.
V	FGFR	3 Ig-like domains, kinase insert domain, acidic domains	Expressed on endothelial cells and are implicated in angiogenesis. Also expressed in other cell types and have important roles in the embryonal development of several organs and tissues.
VI	NGFR, TrkA, TrkB, TrkC	No or few cysteine-rich domains, NGFR has leucine-rich domain	Members of the neurotrophin receptor family (TrkA, B, and C) bind members of the NGF family of neurotrophins. Important functions during the development and maintenance of the central nervous system.
VII	HGFR (Met, Ron)	Heterodimeric like the class II, except that one of the two subunits is completely extracellular	HGFR is a proto-oncogene that was originally identified as the Met oncogene. Met and Ros undergo cleavage of their extracellular domains after their syntheses. They have important roles in regulation of cell motility and in organ morphogenesis during embryonic development.
VIII	EphR (Largest of the RTKs subfamilies)	Cysteine-rich domain, 1 Ig-like domain, 2 fibronectin III repeats	Expressed in the nervous system and also in endothelial cells; thus, they are implicated in neuronal guidance and angiogenesis.

Table 1. Description of diverse RTKs structure and functions [2, 5, 7, 8].

RTKs have intrinsic enzymatic activity, and they are capable of autophosphorylation as well as phosphorylation of other substrates. Ligand binding induces receptor homo- or heterodimerization, which brings the kinase domains close to each other. This event results in autophosphorylation in *trans* within the intracellular segment of the receptor, meaning that one subunit of the dimer phosphorylates the opposing unit. The autophosphorylation occurs on tyrosine residues located within or outside the kinase domain of the receptor [9]. Several mutations of PTK receptors cause constitutive dimerization or formation of fusion proteins between the kinase domains of the receptors and proteins that normally occur as dimers or oligomers [10]. About half of the RTKs are implicated in various human malignancies [8, 9].

1.2.2 Non-receptor tyrosine kinases (NRTKs)

Figure 2 depicts some of the NRTKs. These proteins can be localized to the cytoplasm, nucleus, or both, while several others are targeted to the plasma membrane by lipidation or protein-protein interactions, or to the endoplasmic reticulum [4].

The kinase activity of NRTKs is regulated by tyrosine phosphorylation at specific sites. For instance, there are two major tyrosine phosphorylation sites in Src-family PTKs: 1) the autophosphorylation site (Y419 of human Src), which induces autoactivation by an intramolecular process, and 2) the C-terminal negative regulatory site (Y530 of human Src). In order to get a fully activated kinase, the residue Y419 must be phosphorylated and the Y530 must be dephosphorylated. The activity of the Src-family members is regulated by various proteins that control the phosphorylation status of these residues. For example, phosphorylation of residue Y530 by the kinase Csk inhibits Src activity, and Y530 is dephosphorylated by a variety of PTPs, which then activate the kinase [4].

Autophosphorylation of tyrosine residues has two important roles: 1) as previously described, it causes activation of the kinase domain of the PTKs and 2) it creates docking sites to recruit downstream signaling molecules containing Src homology 2 (SH2) or PTB domain (which contain tyrosine phosphorylated residues). These SH2-domain containing proteins can possess intrinsic enzymatic activity that is induced by binding of the SH2 domain to the receptor, or by tyrosine phosphorylation induced by the receptor kinase. Alternatively, SH2 domain proteins lacking intrinsic enzymatic activity can be activated by the PTKs they recruited or serve as adaptors that connect the activated receptors with downstream signaling proteins (see Figure 2). These adaptor molecules often have additional domains to mediate other interactions (described in Table 3). Thus, the signaling capacity of a PTK is dependent on which SH2 domain proteins it can recruit [6].

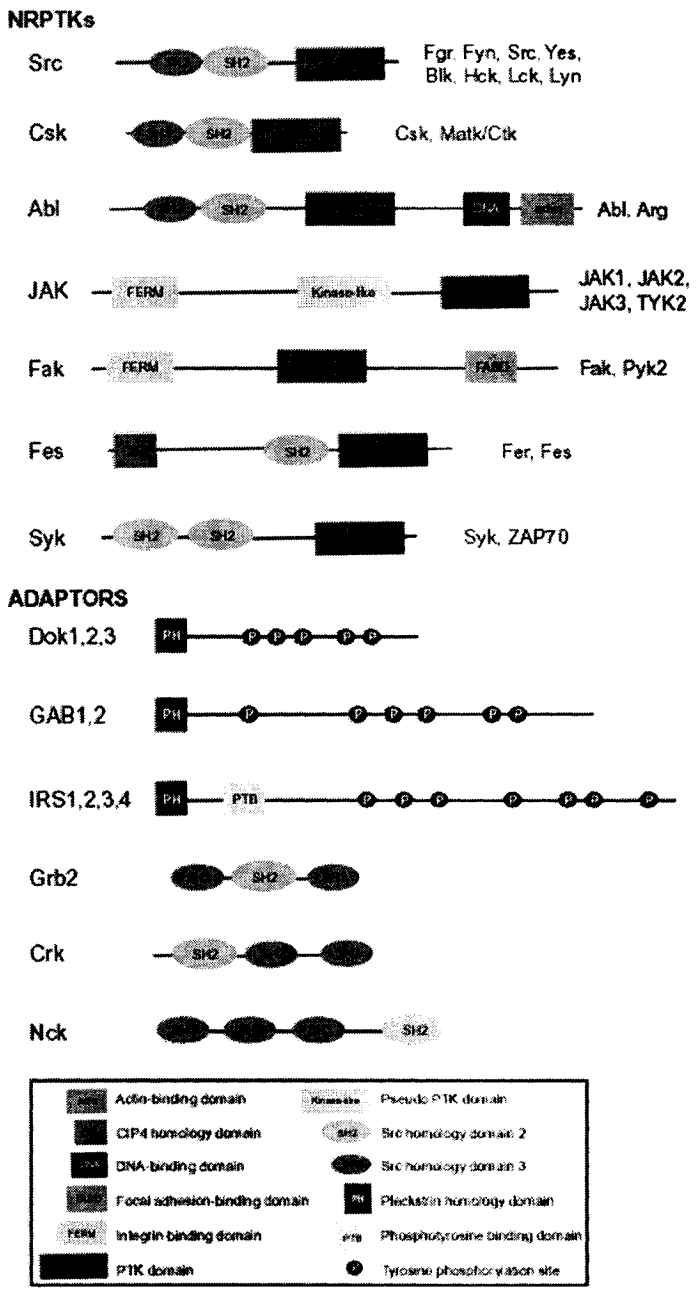

Figure 2. Schematic representation of different members of the non-receptor PTKs family and adaptor proteins. Their functions as well as the role of their structural components are described in Tables 2 and 3 respectively [4, 8].

PTK	Structural features	Functions
Src	SH3, SH2, kinase domains	Localized primarily to cellular membranes. Involved in signaling downstream of RTKs, cytoskeletal rearrangement, and immune signaling. Kinase activity is regulated by a tyrosine residue acting as an autophosphorylation site. C-terminal contains the tyrosine residue that plays an important role in the regulation of their activity. Overexpressed or truncated forms are involved in neuroblastomas, mammary, colon, and pancreatic cancers. Other members include Yes, Fyn, Fgr, Lck, Blk, Lyn, and Hck.
Csk	SH3, SH2, kinase domains	Ubiquitously expressed but predominantly in thymus and spleen. Other member: Matk (expressed primarily in brain and hematopoietic cells). Main function appears to be the negative regulation of Src-family mediated by phosphorylation of their inhibitory C-terminal tyrosine residue.
Abl	SH3, SH2, kinase domains, DNA, actin binding domains	Other member: Arg. Abl and Arg are ubiquitously expressed (highest levels in thymus, spleen and testes for Abl, and brain for Arg). Abl is involved in the regulation of stress response, integrin signaling, and growth factor receptor signaling. Its oncogenic form, BCR-ABL (product of a fusion gene between bcr and c-Abl), has been linked to the pathogenesis of chronic myeloid leukemia in human.
JAK	FERM, kinase, kinase-like domains	Members are JAK1, JAK2, JAK3, and TYK2. Ubiquitously expressed (except JAK3 which is restricted to hematopoietic cells). Localized in cytosol and/or bound to multiple membrane receptors upon ligand binding. Mediated signaling of the cytokine family of receptors (interleukins, interferons, cytokines, colony-stimulating factors, growth factors and hormones). Overexpression associated with various leukemias.
Fak	FERM, kinase, FABD domains	Ubiquitously expressed. Other member: Pyk2, primarily expressed in brain, liver, lung, kidney, and hematopoietic cells. Fak regulates the disassembly of focal adhesion, whereas the role of Pyk2 remains to be clarified. Overexpression associated with invasion and metastasis of diverse malignancies.
Fes	CIP4, SH2, kinase domains	Other member: Fer. Fes is highly expressed in cells of the myeloid lineage, endothelial, epithelial, and neuronal cells as well. Fer is expressed ubiquitously. Involved in signaling downstream of cytokine receptors as well as PDGFR. Activated forms can mediate cellular transformation. *gag-fes* (product of a fusion gene between gag and fes) is associated with diverse avian sarcomas and myeloid leukemias.
Syk	Two SH2, kinase domains	Ubiquitously expressed. Other member: Zap-70 (expressed exclusively in T and NK cells). Predominantly cytosolic proteins. Involved in immune signalling.

Table 2. Functions of various non-receptor PTKs [4, 8].

Domain	Function
Acidic box (AB)	Role in glycosaminoglycan modification
Actin-binding domain (actin)	Actin binding
CIP4 homology domain (CIP4)	SH3 and FER-like coiled-coil domain
Cysteine-rich domain (CRD)	Phospholipid binding
DNA-binding domain (DNA)	DNA binding
FERM	Integrin-binding domain
Fibronectin type III domain (FNIII)	Extracellular, protein interactions
Focal adhesion-binding domain (FABD)	Binding of focal adhesion
Immunoglobulin domain (Ig)	Extracellular, protein interactions
Leucine-rich repeat (LRP)	Ligand binding, protein interaction
PDZ/DHR/GLGF domain	Membrane targeting
Phosphotyrosine binding domain (PTB)	Binds Asn-Pro-X-Tyr motifs (NPXpY)
Pleckstrin homology domain (PH)	Signaling; phospholipid binding
PTK domain	Kinase domain
Pseudo PTK domain	Kinase-like domain
SH2 domain	Binds phosphotyrosine
SH3 domain	Binds proline-rich motifs

Table 3. Functions of various domains found in receptor and non-receptor PTKs, as well as adaptor proteins [4, 8].

As mentioned earlier, the phosphorylation events performed by RTKs are counteracted by the action of PTPs that dephosphorylate tyrosine residues. The next sections will describe the role of PTPs in signal transduction, and, in particular, the role of PTP1B in metabolism and oncogenic signaling.

1.3 The protein phosphatases family

About 150 genes in the human genome encode protein phosphatases, including up to 40 protein serine/threonine phosphatases [1]. Protein phosphatases are classified into two broad classes: 1) the protein serine/threonine phosphatases (PSTPs), and 2) the protein tyrosine phosphatases (PTPs). These two classes are not related in terms of overall and catalytic domain sequence identifiy. The major structural difference between these two families is that the PTPs are all monomeric, whereas the PSTPs are oligomeric and characterized by their association with targeting or inhibitory

subunits. Their subcellular distribution, substrate selectivities, and catalytic activities are mainly determined by these subunits [11].

1.4 The protein tyrosine phosphatases (PTPs) family

According to the sequence of the human genome, the PTPs consist of a large superfamily of 112 members [12]. Together with the RTKs, they modulate the cellular level of tyrosine phosphorylation. They participate in various signaling pathways and are critical for diverse biological functions. They have been shown to regulate many cellular events such as differentiation, cell growth, motility, and proliferation [13].

The PTP superfamily is defined by a highly conserved stretch of approximately 250 amino acids in their catalytic domain. Within this region, the sequence (H/V)**C**$(X)_5$**R**(S/T), also known as the PTP signature motif, contains the invariant cysteine residue that is critical for PTP activity [14]. Based on this motif, they can be divided into three major classes: 1) the low molecular weight (LMW) PTPs, 2) the dual specific phosphatases (DSPs), and 3) the tyrosine specific phosphatases (classical PTPs). Outside the catalytic domain, the PTPs share a low percentage of amino acid sequence identity amongst all family members, but their overall structure is similar.

The low molecular weight protein tyrosine phosphatases (LMW-PTPs) play a key role in cell proliferation control by dephosphorylating and inactivating tyrosine kinase receptors, such as PDGF, insulin, and ephrin receptors, as well as docking proteins, such as beta-catenin, endowed with both adhesion and transcriptional activity [15]. LMW-PTP is frequently overexpressed in transformed cells, human tumors, and its overexpression is sufficient to induce transformation of non-transformed epithelial cells [16]. In addition, overexpression of LMW-PTP strongly potentiates the stability of cell-cell contacts at the adherens junction level, suggesting that LMW-PTP may also contribute to cancer invasiveness. LMW-PTP-transfected NIH3T3 fibroblasts engrafted

in nude mice induce the onset of larger fibrosarcomas, whereas opposite effects have been obtained with a dominant-negative form of LMW-PTP [17]. Yet, the physiological function of LMW-PTPs remains elusive.

The DSPs dephosphorylate tyrosine, serine, and threonine residues, and some members of this family also dephosphorylate inositol phospholipids. Despite low sequence identity between classical PTPs and DSPs, they share a highly conserved signature motif. Members of this subfamily include, among others, Cdc25, MAPK phosphatases (MKPs), and VHR-like phosphatase, and the tumor suppressor PTEN [18]. PTEN encodes a dual specific phosphatase/lipid phosphatase that modulates signal pathways involving second messengers. PTEN is one of the most commonly mutated genes in human cancer. Its loss of function results in formation of tumors in different tissues and is involved in glioblastoma, endometrial carcinoma, and prostate cancer [19, 20].

The classical tyrosine specific PTPs can be subdivided into non-receptor (NRPTPs) and receptor PTPs (RPTPs) (Figure 3). A brief overview of the various classical RPTPs and NRPTPs will illustrate their involvement in several signaling pathways related to oncogenesis and cancer, metabolism, immunology, and development.

1.4.1 Receptor PTPs (RPTPs)

The RPTPs consist of an extracellular variable N-terminal domain, a transmembrane domain, and an intracellular region containing one or two PTP domains separated by 50-100 amino acids [21]. The first PTP domain (D1) adjacent to the membrane provides the catalytic activity, while the second PTP domain (D2) is suggested to have a regulatory role by being involved in the control of enzyme activation, protein-protein interaction, substrate specificity, and presentation of

substrates to the D1 PTP domain. The RPTPs are further classified into eight main types on the basis of their extracellular domains [22]. The following summary of the various types of RPTPs will illustrate the diversity of biological function of these enzymes.

1.4.1.1 CD45

CD45 (PTPRC) is a type I RPTP that is heavily glycosylated and has a cysteine-rich region next to a fibronectin III (FNIII)-like domain in the extracellular domain. It was identified as the first RPTP, and is highly expressed on hematopoietic cells [23]. CD45 was found to be required for T Cell antigen Receptor (TCR) signaling [24], as well as T and B cell activation [25]. Hence, examination of CD45 mutant cell lines, CD45-deficient mice, and samples from CD45-deficient human severe combined immune deficiency (SCID) patients has shown that CD45 is required for signal transduction through antigen receptors [25]. To date, no ligand has been identified for CD45, although dimerization has been shown to inhibit its PTP activity [24].

CD45 can operate as a positive as well as a negative regulator of Src-family kinases. First, CD45 dephosphorylates a negative regulatory tyrosine residue in the C-terminal of Lck, thus activating this Src-family kinase member [26], and positively regulating antigen receptor signaling. However, CD45 can also dephosphorylate the phosphotyrosine residue in the catalytic domain of Src-kinase and therefore, downregulate the activity of Src-kinases in thymocytes and during integrin-mediated adhesion in macrophages [27-29]. Thus, CD45 can act as a positive as well as a negative regulator of Src-family kinases and Src-mediated cellular responses, depending of the cell type and of the cellular context [25]. Furthermore, genetic and biochemical evidence have shown that CD45 is a JAK tyrosine phosphatase that can directly dephosphorylate and inactivate JAK family kinases [30], thus regulating

differentiation, proliferation and antiviral immunity of hematopoietic cells [25]. Accordingly, targeted disruption of the *cd45* gene leads to enhanced cytokine and interferon receptor-mediated activation of JAKs and STATs [30].

1.4.1.2 RPTPmu, RPTPkappa, RPTPlambda, RPTPpsi

The type IIA RPTPs contain two intracellular phosphatase catalytic domains, with external domains comprising FNIII-like and Immunoglobulin-like (Ig) domains, suggesting a potential role in cell adhesion by analogy with the N-CAM superfamily of adhesion molecules [31, 32]. In addition, they present a N-terminal meprin/A5/PTPmu (MAM) domain. RPTPmu (PTPRM) is located at the cell surface and is a member of the immunoglobulin (Ig) superfamily of adhesion molecules [33]. Since its intracellular juxtamembrane domain contains a region that is homologous to the conserved intracellular domain of the cadherins [34], RPTPmu mediates adhesion by binding homophilically and associates with cadherins. For instance, normal prostate expresses RPTPmu, whereas prostate carcinoma cells have lost RPTPmu expression, suggesting that RPTPmu might act as a tumor suppressor [35]. The human RPTPkappa (PTPRK) gene is also considered as a tumor suppressor since it localized in a chromosomal region frequently deleted in primary central nervous system lymphomas [36, 37].

1.4.1.3 LAR family of RPTPs

The LAR (leucocyte antigen-related) type IIB family of RPTPs includes, LAR, RPTPdelta, and RPTPsigma [21]. Their extracellular segment is composed of both Ig-like domains and FNIII-like domains, and they are possibly mediating heterophilic interactions with ligands [38]. Their intracellular domain consists of PTP domains, a hydrophobic transmembrane stretch, and a short extracellular segment linked to the extracellular domain [21]. The extracellular domains of these enzymes share structural features with the neural adhesion molecule N-CAM, suggesting a role during migration

and adhesion [21]. Moreover, these enzymes are predominantly expressed in the nervous system, in a distinct but overlapping spatiotemporal pattern [21, 39, 40]. Although no substrates of these RPTPs have been identified to date, knockout mouse models have clearly implicated these enzymes in the development of the nervous system [41-44] (Table 4), and suggest the possibility that these RPTPs may have overlapping roles in neural development. Furthermore, the human LAR gene is considered as a putative tumor suppressor gene, since it localized in a chromosomal region frequently deleted in human neuroblastoma [45]. In contrast, LAR is overexpressed in thyroid carcinomas [46], and in breast cancer cell lines and tissues [47].

LAR is also expressed in diverse tissues, with highest levels found in a variety of neural tissue types, liver, skeletal muscle, and adipocytes [48, 49]. LAR is thus expressed in insulin-sensitive tissues, and acts as a negative regulator of insulin signaling in cell and animal models [50-52] (described in section 1.8.2).

1.4.1.4 RPTPbeta, DEP-1, SAP-1, GLEPP1

The structure of the extracellular domain of the type III family of RPTPs represented by RPTPbeta (PTPRB) consists of multiple FNIII-like domains, and one PTP domain. This extracellular domain functions as a ligand for neuronal receptor complexes, interleukin, and growth hormones [53]. In humans, both DEP-1 (Density-enhanced phosphatase-1, PTPRJ) and GLEPP1 (Glomerular epithelial protein, PTPRO) [12] genes map in regions frequently deleted in several cancer types, including colorectal cancer for DEP-1 [54, 55]. DEP-1 has also been implicated in the regulation of cell growth, differentiation, and transformation leading to aberrant cell growth [56].

The stomach cancer-associated protein-1 (SAP-1, PTPRH) has been implicated as a negative regulator of integrin-mediated signaling [57], and its

overexpression induces apoptosis in NIH 3T3 fibroblasts [58]. SAP-1 is mainly expressed in the brain and liver, and at a lower level in the heart and stomach, but is not detected in normal pancreas or colon. In contrast, SAP-1 is highly expressed in pancreatic and colorectal cancer cells, and is frequently overexpressed in human colorectal cancers [59, 60]. Since the activity of Src kinase is elevated in colon cancers [61], the increased level of SAP-1 is hypothesized to contribute to the malignant transformation of these cells through the dephosphorylation of the C-terminal inhibitory tyrosine residue of Src.

1.4.1.5 RPTPalpha, RPTPepsilon

RPTPalpha is a glycoprotein found in most tissues, with highest expression in brain and kidney, suggesting that this RPTP has a critical function in the physiology of all cell types [62]. This type IV RPTP is characterized by an extremely short extracellular domain and heavily glycosylated external segment. Like other RPTPs, RPTPalpha is able to form inactive hetero and homodimers, which are important for the downregulation of its phosphatase activity. Therefore, its catalytic activity is inhibited by dimerization [63], a state that is reversibly modulated by stimuli such as oxidative stress [64]. In a similar manner to CD45, RPTPalpha positively regulates the activity of Src kinases through dephosphorylation of the C-terminal regulatory site [65], thereby controlling neuronal differentiation and cellular transformation [65, 66]. In accordance, Src and Fyn activation are diminished in fibroblasts lacking RPTPalpha [67, 68]. On the other hand, overexpression of RPTPalpha in fibroblasts causes cellular transformation via activation of Src kinases [65]. In humans, RPTPalpha is overexpressed in colorectal tumors [69], and in ovarian cancer cell lines transfected with HER2/neu/ErbB2 [70]. On the opposite, high RPTPalpha protein levels correlated significantly with low tumor grade and positive estrogen receptor status in primary

human breast cancer, suggesting that it could act as a tumor suppressor in this specific context [71].

RPTPepsilon is N-glycosylated in a tissue-specific manner. There are four protein forms of RPTPepsilon all produced from the single RPTPepsilon gene, and all forms share the same catalytic domain but have a unique amino acid terminus, which determines their individual subcellular locations and physiological roles [72-74]. For instance, *in vivo* evidence suggests that RPTPepsilon supports transformation of mammary epithelium since its high expression is correlated in murine mammary tumors initiated by c-neu and v-Haras [75]. Genetic evidence confirmed that cells derived from mammary epithelial tumors induced by activated Neu in mice genetically lacking RPTPepsilon appeared morphologically less transformed and exhibited reduced proliferation [76]. The rationale for this observation was that RPTPepsilon activates Src, a known collaborator of Neu in mammary tumorigenesis, and that lack of RPTPepsilon reduced Src activity, and altered Src phosphorylation in tumor cells. Granot-Attas et al. have further reported that RPTPepsilon is also a physiological activator of two additional Src family kinases, Yes and Fyn. Their kinase activities are both inhibited in mammary tumor cells lacking RPTPepsilon, and phosphorylation at their C-terminal inhibitory tyrosines is increased [77]. On the contrary, the cytosolic isoform of RPTPepsilon has a suppressive role in tumorigenesis by inhibiting terminal differentiation of murine M1 myeloblastic leukemia cells [78].

1.4.1.6 RPTPgamma, RPTPzeta/beta

The type V RPTPs, RPTPgamma and PTPzeta/beta, are characterized by an N-terminal carbonic anhydrase-like domain, together with a single FNIII-like domain [79]. Similar to the type II RPTPs, RPTPzeta functions as a ligand for neuronal receptor complexes. However, the precise biological function of these RPTPs is unknown. RPTPgamma (PTPRG) is suggested to be a candidate tumor suppressor gene since it

is located in a region frequently deleted in certain types of renal and lung cancers [80, 81]. RPTPzeta is only expressed in the nervous system [38], and it seems to be important for the development of the brain since high level of RPTPzeta were detected in the developing brain of mice [82]. In addition, RPTPzeta was detected in human primary and metastatic cutaneous melanomas [82], as well as in glioblastoma [83].

1.4.1.7 HePTP

HePTP (LC-PTP, PTPN7) is a lymphoid-specific PTP, which is exclusively expressed in thymus and spleen [84]. This type VII RPTP contains one catalytic domain. Overexpression of HePTP in NIH 3T3 cells caused altered cell morphology, disorganized growth, anchorage independent colony formation, and small differences in the pattern of tyrosine phosphoproteins compared to control cell lines [85]. Therefore, amplification and overexpression of HePTP appeared to be important factors contributing to abnormal myeloid cell growth [86]. Accordingly, the human gene is located on chromosome 1q32.1, a site that is frequently amplified and overexpressed in myeloid malignancies such as non-hodgkin lymphomas [85].

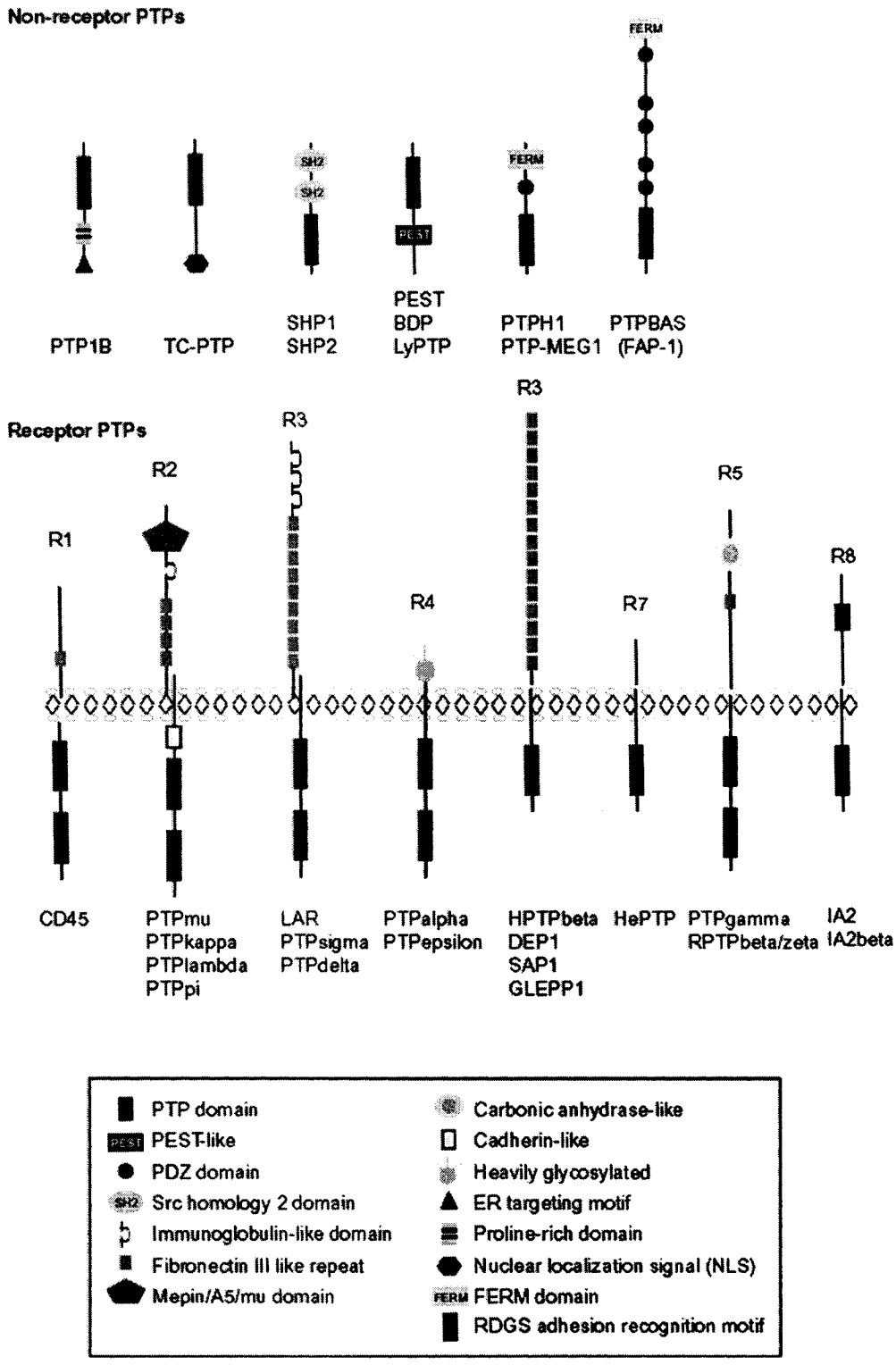

Figure 3. Overview of the classical PTP family. Adapted from Andersen et al. 2001 [22].

1.4.1.8 RPTPIA2/IA2beta

Type VIII RPTPs are characterized by a unique N-terminal extracellular domain with four cysteines, one intracellular PTP domain, and are expressed in the brain and pancreas [87]. Noteworthy, RPTPIA2 and its homologue RPTPIA2beta are important autoantigens associated with type 1 diabetes (insulin-dependent diabetes mellitus) [87-90].

1.4.2 Non-receptor PTPs

The non-receptor PTPs are cytosolic enzymes characterized by a conserved catalytic domain (PTP domain), followed by various types of motifs that account for their subcellular localization, as well as interaction with other proteins and substrates.

1.4.2.1 SHP-1

SHP-1 is a SH2 domain-containing PTP that is primarily expressed in hematopoietic cells and regulates intracellular phosphotyrosine levels in lymphocytes [91]. Chromosomal deletions in the SHP-1 gene have been associated with several diseases, but most particularly with leukemogenesis [92-95]. A significant correlation of the SHP-1 gene silencing exists with the onset of lymphomas/leukemias [96]. SHP-1 downregulates RTKs (c-kit, CSF-1, TrkA, and EGF) [97-101], cytokine receptors (EpoR, IFNalpha/beta R, IL-3R, and IL-2R) [102-105], and receptors involved in the immune response such as the TCR complex and CD45 [103, 106-108].

Hence, SHP-1 is a disease-causing gene as demonstrated by the leukemia-like phenotypes of SHP-1 deficiencies in mouse models (motheaten and viable motheaten), which exhibited several hemopoietic abnormalities such as splenomegaly, runting, and autoimmune disease [104, 109, 110]. Moreover, involvement of SHP-1 defects in human hematopoietic diseases is suggested by the reduction or loss of SHP-1 expression in various leukemia and lymphoma cell lines [111, 112].

1.4.2.2 SHP-2

SHP-2 (PTPN11, Syp, PTP1D, PTP2C), a widely expressed non-receptor PTP that contains two SH2 domains, a carboxyl-terminal catalytic domain, and a C-terminal segment containing two tyrosine phosphorylation sites [113]. The SH2 domains recruit SHP-2 to many activated growth factor receptors such as EGF, PDGF, HER2-neu, and kit-SCF [100, 114, 115], and tyrosine phosphorylated molecules such as insulin receptor substrate-1 (IRS-1) [116]. In contrast to many other PTPs, SHP-2 does not dephosphorylate growth factor receptors despite its association with various RTKs. It rather acts as an adaptor molecule to couple signaling pathways, by relaying signals from activated growth factor receptors to Ras and other signaling molecules.

The generation of SHP-2 knockout mice has demonstrated that this PTP is necessary for early embryonic development since mice die at embryonic stage day 8.5-10 of defect in mesoderm patterning [117-120]. However, its exact role and physiological functions remain to be determined. Importantly, it was recently reported that inherited and somatic mutations that result in SHP-2 gain-of-function cause Noonan syndrome, a developmental disorder characterized by cardiac and skeletal defects, as well as myeloid malignancies [121-125].

1.4.2.3 PTP-PEST, LyPTP

PTP-PEST (PTPG1, PTPN12) contains proline (P), glutamic acid (E), serine (S), and threonine (T) - rich domains (PEST). It is expressed ubiquitously and is found in higher amounts in cells of the hematopoietic lineage [86, 126]. This PTP provides proline-rich binding sites for SH3 domain containing protein such as p130cas [127], and is involved in the regulation of cell migration and adhesion. Fibroblasts lacking PTP-PEST show migration defects compared to wild-type counterparts [128, 129]. It was previously reported that the expression level of PTP-PEST is crucial to cell

migration and adhesion [130]. In addition, aberrant transcripts of PTP-PEST are detected in colon cancer [131].

LyPTP (Lymphoid PTP, LYP, PTPN22) is mainly expressed in lymphoid tissues. It associates with the Cbl oncogene [132] and Grb2, association that negatively regulates T-cell signaling [133]. In addition, its chromosomal location is associated with rearrangements in solid and hematopoietic tumors [132]. Recently, a single-nucleotide polymorphism (SNP) in the gene (PTPN22) encoding LyPTP has been related to type 1 diabetes [134].

1.4.2.4 PTP-BAS

PTP-BAS (FAP-1, PTPL1, PTPN13) is a PTP capable of interacting with the cytosolic domain of Fas, a cell surface receptor controlling a signal transduction pathway that leads to cell death by means of apoptosis [135]. Its expression is highest in tissues and cell lines that are relatively resistant to Fas-mediated cytotoxicity, and correlates with resistance to Fas-induced apoptosis in human cancer cells [135-137]. Human tumor cell lines become resistant to Fas-mediated apoptosis when transfected with FAP-1, indicating that FAP-1 functions as a negative regulator in Fas-mediated death signaling [137, 138]. However, FAP-1 can trigger apoptosis in human breast cancer cells MCF-7 independently of Fas, by inhibiting the IRS-1/Phosphatidylinositol 3-kinase (PI3K) pro-survival pathway [139].

1.4.2.5 PTPH1, PTP-MEG

Both of these PTPs have cytoskeleton-associated regulatory motifs that allow their binding to the interface between plasma membrane and cytoskeleton structures [140]. Deletions and duplications of the PTPH1 human gene are associated with cancers, suggesting that it is a tumor suppressor [12]. Accordingly, in vitro studies have shown that overexpression of PTPH1 inhibits growth of NIH/3T3 cells [141]. In contrast,

Wiener et al. have shown that overexpression of ErbB2/HER2 in ovarian cancer cells induces an increased in PTPH1 [70].

1.4.2.6 TC-PTP

T Cell PTP (PTPN2) is a widely expressed 45kDa protein that mainly localizes to the nucleus [142, 143], although TC-PTP expression is elevated in lymphoid type cells [144]. Gene-targeting studies revealed that TC-PTP deficient mice are born normal but die between three to five weeks with a multitude of phenotypes, including defects in hematopoiesis and immune function [144]. Accordingly, TC-PTP negatively regulates cytokine signaling by dephosphorylating the kinases JAK1 and JAK3 [145]. Recently, biochemical studies using null fibroblasts established TC-PTP as a modulator of insulin receptor phosphorylation [146, 147], but genetic evidence to support this role is still lacking. Furthermore, it was shown that overexpression of a truncated form of TC-PTP lacking the nuclear localization sequence into rat 2 cells stably transformed by murine v-fms which abolished the transformed phenotype of the cells and the capacity to form tumors in nude mice [148]. Although TC-PTP and PTP1B are 74% identical in the catalytic domain [149], their localization, substrates, as well as mouse knockout phenotypes are remarkably different. The role of PTP1B in various signaling pathways will be described in the subsequent sections.

RPTP	Knockout / mutated phenotype	Healthy, normal	References
CD45	Lack T cells, immature B cells	X	[150, 151]
RPTPmu	No obvious phenotype	X	Cited in [40]
RPTPkappa	No obvious phenotype Frequently deleted in primary CNS lymphomas	X	[152] [36, 37]
LAR	Mammary gland defect; reduced plasma glucose and insulin levels Frequently deleted in human neuroblastoma	X	[51, 153] [45]
RPTPsigma	Pituitary displasia, defects in olfactory lobes, reduction in CNS size and cell number Localized at 19p13.3, a region deleted in human cancers		[44] [12]
RPTPdelta	Growth retardation, early mortality, posture and motor defects	X	[43]
DEP-1	Die at mid-gestation with severe defects in vascular organization; Frequently deleted in colorectal cancer		[55]
GLEPP1	Reduced renal filtration surface area Chromosomal aberrations in cancer	X	[154] [12]
RPTPalpha	No obvious phenotype Overexpressed in colorectal tumors	X	[68] [69]
RPTPepsilon	Hypomyelination Absence of RPTPepsilon decreases mammary tumor formation in Neu mice	X	[155] [76]
RPTPzeta/beta	No obvious phenotype, but fragility of myelin suggested Impaired recovery from EAE induced by MOG peptide; role in oligodendrocyte survival	X	[156] [157]
HePTP	No obvious phenotype	X	[158]
RPTPIA2	Abnormal glucose-stimulated insulin secretion Important autoantigen associated with type 1 diabetes	X	[159] [87]

Table 4. Phenotypes of knockout and cancer associated RPTPs. Adapted from van Huijsduijnen et al. 2002 [160]. Abbreviations: EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein.

PTP	Knockout / mutated phenotype	Healthy, normal	References
SHP-1	Motheaten (me) mice lethal. Hematopoietic deregulation, splenomegaly, runting, autoimmune disease Chromosomal deletions associated with leukemogenesis		[104, 109, 110] [92-95]
SHP-2	Embryonic lethal (E8.5-10), defect in mesoderm patterning Somatic mutations associated with Noonan syndrome and myeloid malignancies (JMML)		[117-120] [121-125]
PTP-PEST	Embryonic lethal (E9.0), defective neural fold closure, lack primary hepatocytes Aberrant transcripts in colon cancer		Tremblay M.L., personal communication [131]
TC-PTP	Born normal, die between 3-5 weeks, defects in hematopoiesis and immune function		[144]
PTP1B	Enhanced insulin and leptin sensitivity Resistance to diet-induced diabetes and obesity	X	[161-164]

Table 5. Phenotypes of knockout and cancer associated cytosolic PTPs. Adapted from van Huijsduijnen et al. 2002 [160]. Abbreviations: JMML: juvenile myelomonocytic leukemia.

1.5 Mechanism of enzymatic action and substrate trapping

As mentioned in the previous section, the PTPs are defined by the presence of a PTP signature sequence motif, (H/V)**C**$(X)_5$**R**(S/T). Within this motif, the cysteine (C; Cys) and the arginine (R; Arg) residues are invariant and essential for catalysis. Insight into the mechanism of enzymatic action of PTPs was obtained by the crystal structure of PTP1B [165] along with mutational analysis data. Hence, all PTPs share a common feature, which is the basis of their catalytic mechanism that involves the formation of a phospho-cysteinyl enzyme intermediate, using the conserved cysteine, arginine, and general acid/base aspartate residue. The PTP catalysis consists of a nucleophilic attack initiated by the invariant cysteine (C215) on the phosphate group, forming a

transient substrate/PTP intermediate called the cysteinyl-phosphate intermediate (Figure 4). The arginine binds the phosphate and stabilizes the transition state. Then, hydrolysis of the substrate/phosphate (P-O) bond is achieved by the aspartate (D181), which functions as a general acid to facilitate the cleavage of the P-O bond. Finally, a water molecule completes the removal of the phosphate group [166].

A significant step in the elucidation of the physiological functions as well as the signaling pathways modulated by the PTPs is the identification of their substrates. In this respect, the laboratory of Dr. Tonks developed a method called "substrate trapping" [167]. A substrate trapping mutant has three general characteristics: 1) it does not exhibit any or very low catalytic activity, 2) it retains its normal substrate binding specificity, 3) it forms a relatively stable complex with the substrate to permit the isolation of the complex [168]. Briefly, they based their approach on PTP1B, in which mutation of specific residues in the catalytic domain would reduce or abolish the catalytic activity of the enzyme but would not modify its affinity for a substrate. Therefore, they mutated the conserved cysteine to serine (C215S) or an aspartate to alanine (D181A) within the catalytic domain of PTP1B. By assessing the k_{cat}, they found that PTP1B C215S had no detectable activity, or very low activity in the case of PTP1B D181A. As yet, the substrate trapping method has been extensively utilized to identify novel substrates, and has been applied to other PTPs.

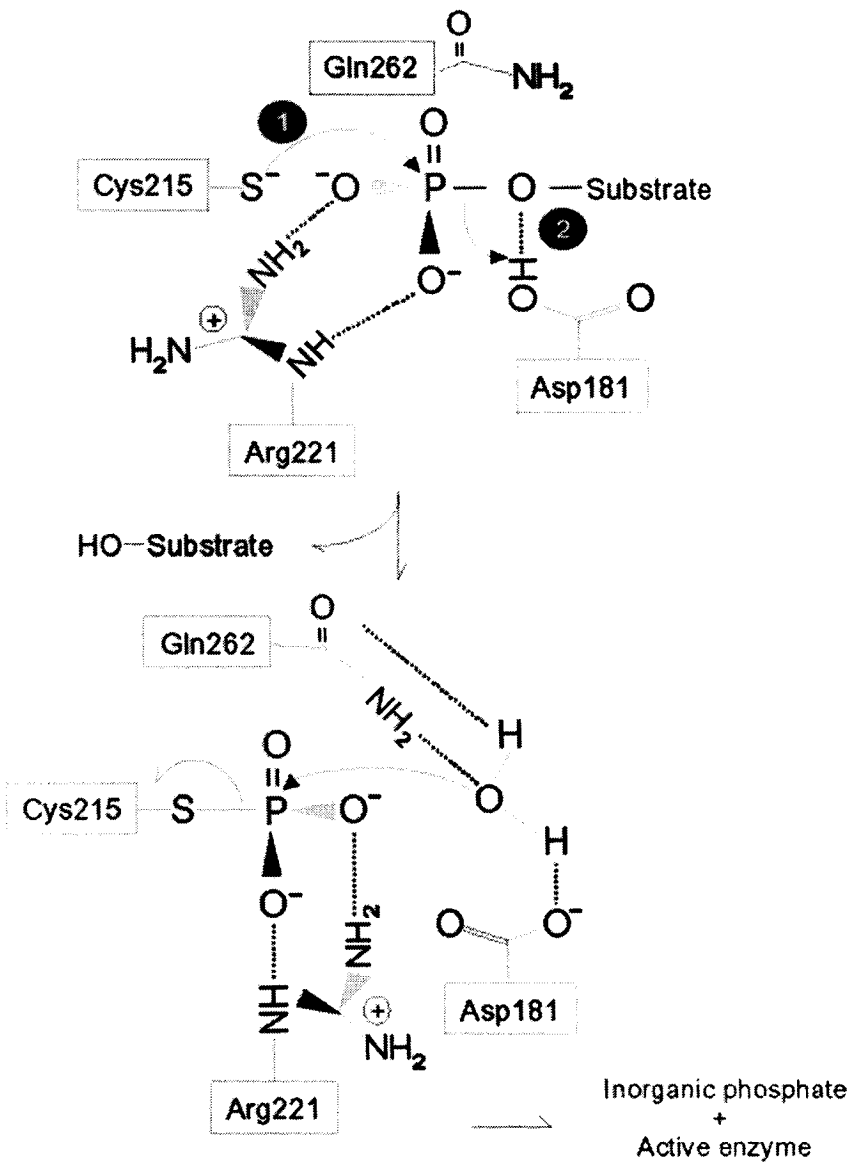

Figure 4. Chemical mechanism for the two-step PTP1B-catalyzed dephosphorylation reaction. 1) Cys215 acts as a nucleophile to attack the phosphate of p-Tyr substrate (formation of the cysteinyl-phosphate intermediate). 2) Asp181 functions as a general acid to facilitate cleavage of the pTyr P-O bond in the substrate (hydrolysis of the cysteinyl-phosphate intermediate). Adapted from Zhang et al. 2003 [169].

1.6 Protein tyrosine phosphatases and human diseases

Although the PTPs are best known as downregulators of PTKs signaling, their functions and regulation are recently beginning to be elucidated. On the other hand, the PTPs can also potentiate, rather than oppose, the action of RTKs. Therefore, deregulation of the PTP activity can lead to aberrant signaling that can contribute to the development of various diseases in human, such as cancer, diabetes, inflammation, autoimmunity, and infectious diseases, as described in Tables 6 and 7 [170, 171].

Numerous biochemical and genetic evidence suggest an emerging role for the PTPs in transformation and cancer, either acting as oncogenic or tumor suppressor PTPs [172-174]. Recently, a mutational analysis of the PTP gene superfamily in human cancers has identified 83 somatic mutations in six PTPs, affecting 26% of colorectal cancers and a smaller fraction of lung, breast, and gastric cancers [175], suggesting that the mutated tyrosine phosphatases are tumor suppressors, thus regulating cellular pathways that may have potent benefits in therapeutic intervention. Alternatively, various studies have shown that PTPs can also act as oncogenes depending on their substrate specificity and the signal pathways they regulate. As yet, it is not clear which PTPs positively or negatively regulate tumorigenesis.

Recently, there was significant progress made in identifying small molecules that enable specific inhibition of individual PTPs [169]. For instance, over 14 pharmaceutical companies are involved in PTP1B inhibitor discovery, in order to treat type 2 diabetes and obesity [171].

Disease / Function	PTP	References
Autoimmune diseases, multiple sclerosis	CD45	[151]
Acute myeloid leukemia (AML)	SHP-1	[92-95]
Noonan syndrome, Juvenile myelomonocytic leukemia (JMML)	SHP-2	[121-123, 125]
Infectious disease	SHP-1, SHP-2	[176, 177]
Inflammation	RPTPbeta, RPTPepsilon	[178]
Type 1 diabetes	RPTP2A, LyPTP	[87, 88, 134]
Type 2 diabetes	PTP1B, LAR, RPTPalpha	[52, 161, 162, 179-181]
Obesity	PTP1B, SHP2	[161-164, 182]
Osteoporosis	RPTPepsilon, GLEPP1	[183, 184]
Neurodegeneration, Traumatic neuronal injury	LAR, RPTPsigma, PTP-SL/STEP	[185-189]
Bubonic plague (Yersinia essential virulence factor)	Yersinia PTP (YopH)	[190]
Foodborne diseases (Salmonella infection and thypoid fever)	Samonella PTP	[191]

Table 6. Overview of classical PTPs associated with human diseases. Adapted from van Huijsduijnen et al. 2002, and Zhang et al. 2001 [170, 171].

PTP	Type of cancer	References
LyPTP	Chromosomal location is associated with rearrangements in solid and hematopoietic tumors	[132]
HePTP	Gene amplification and overexpression in myeloid malignancies (1q32.1)	[85]
LAR	Gene is frequently deleted in human neuroblastoma; overexpressed in thyroid carcinomas, and in breast cancer cell lines and tissues	[45-47, 192-195]
RPTPgamma	Canditate tumor suppressor gene (3p.21) Associated with lung and breast cancers	[80, 81, 196, 197]
RPTPalpha	Increased mRNA level in colon carcinoma Overexpressed in colorectal tumors, and in ovarian cancer cell lines transfected with HER/neu/ErbB2	[65, 69-71, 198]
RPTPepsilon	Expressed in mouse mammary tumors initiated by Ha-ras or Neu. RPTPepsilon expression reduces tumorigenicity of murine leukemia cells	[54, 75-78]
RPTPmu	Lost of expression in prostate carcinoma cells	[35, 199]
RPTPkappa	Gene localized at chromosomal region frequently deleted in primary CNS lymphomas	[36, 37, 200]
SAP-1	Overexpressed in pancreatic and colorectal cancer, as well as in human colorectal cancers	[59, 60]
DEP-1	Map to a region frequently deleted in colorectal cancer	[12, 55, 56, 201]
GLEPP1	Chromosomal aberration in cancer	[12]
PTP-BAS	PTP-BAS expression confers resistance to apoptosis to human cancer cells. Can trigger apoptosis of human breast cancer cells MCF-7	[135-137, 202]
PTP-PEST	Aberrant transcripts in colon cancer	[131]
PTPH1	Deletions and duplications associated with cancers Overexpression of HER/neu/ErbB2 in ovarian cancer cells induces PTPH1 expression	[70, 141, 203]
PTP1B	Overexpressed in cell line derived from a CML patient, and in human ovarian and breast carcinomas Decreased expression in oesophageal cancer.	[203-209]

Table 7. Overview of classical PTPs associated with cancer. Adapted from van Huijsduijnen et al. 2002, and Zhang et al. 2001 [170, 171].

1.7 PTP1B: the prototypic PTP

Protein tyrosine phosphatase 1B (PTP1B) is the prototype for the superfamily of PTPs, and has been the most extensively studied within the group. It was the first mammalian PTP identified and to be purified to homogeneity [210]. PTP1B was originally isolated from human placenta as a cytosolic PTP of 321 amino acids (37 kDa) [210-212]. In human platelets, proteolytic cleavage and release of a 42kDa form has been shown [213, 214]. Furthermore, an alternative splicing is able to generate a PTP1B isoform with an altered C-terminus [215]. However, subsequent cloning studies revealed that PTP1B exists as 435 amino acids form of 50 kDa (full length) [216].

Its structure consists of an N-terminal catalytic domain (PTP domain) followed by two tandem proline-rich motifs that allow the interaction with SH3 domain containing proteins (Figure 5). The first proline rich motif has been shown to potentially bind to the SH3 domains of the adapter proteins Crk1, Grb2, and p130cas [217], but the functional significance remains poorly understood. As yet, no molecule has been associated with the second proline domain (P2). This phosphatase is widely expressed and localizes predominantly to the endoplasmic reticulum (ER), through a small hydrophobic stretch, which has been shown to be necessary and sufficient to localize the enzyme to the ER [218]. Cleavage of this segment appears to release the enzyme from the ER and increase its specific activity [213], suggesting that subcellular localization may in part, regulate the accessibility of PTP1B to its substrates in a spatial and temporal manner. Several biochemical studies using the substrate trapping technique have demonstrated that PTP1B is involved in the attenuation of various PTKs signaling pathways [10].

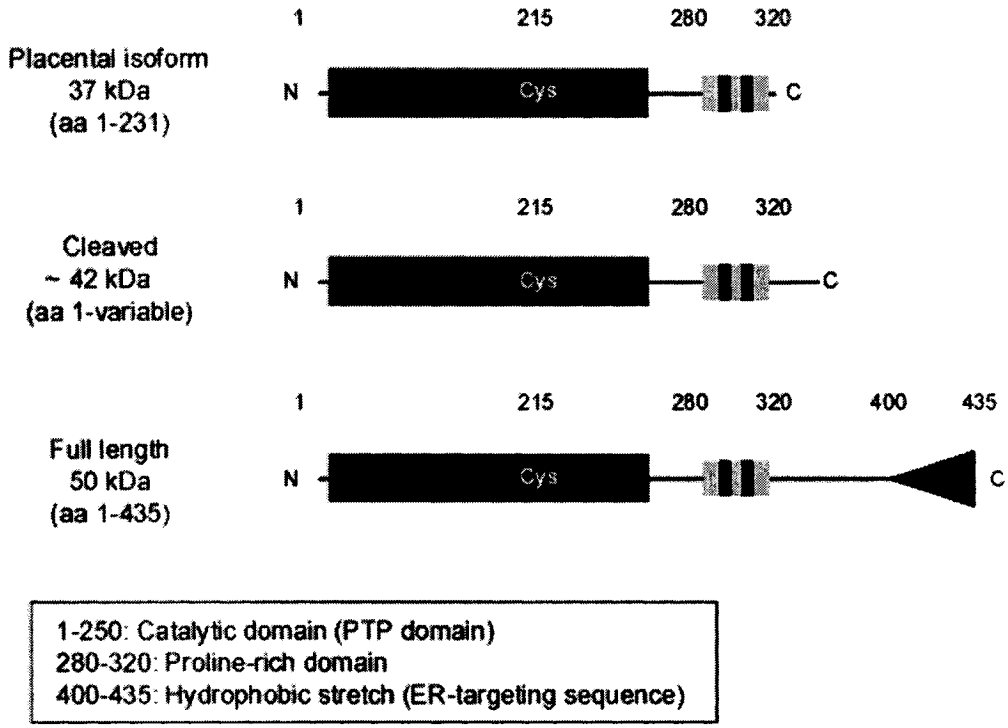

Figure 5. Various isoforms and structure of the PTP1B enzyme.

Although it is widely expressed in most cell types, PTP1B is one of the few identified PTPs that are found in the major tissues controlling insulin mediated glucose metabolism, namely the liver, muscle, fat, and brain. In the early days of the PTP field, this phosphatase was initially thought to indiscriminately dephosphorylate phosphotyrosine residues, and thus performing "housekeeping duties" [14]. The following sections will describe the role of this enzyme in multiple signaling pathways and show how PTP1B exerts specific functions related to metabolism.

1.8 Insulin and diabetes

Insulin is an anabolic hormone synthesized as a prehormone in the beta cells of the islets of Langherans, where secretion is principally regulated by plasma glucose levels [219]. The majority of cell types are responsive to insulin, the main tissues being the liver, muscle, adipose tissue, and brain. The primary biological effect of insulin is to maintain glucose homeostasis by controlling glucose uptake and storage. These effects are the response of the activation of the insulin receptor (IR) pathway, which consist of specific phosphorylation events that lead to an increase in the uptake of glucose in muscle and adipose tissue, with a concomitant decrease in hepatic glucose production (inhibition of gluconeogenesis) [220]. In addition to its role in regulating glucose metabolism, insulin stimulates lipogenesis, amino acid transport into cells, growth, as well as DNA and protein synthesis. Consequently, deregulation of insulin secretion and/or signaling through the IR leads to the development of diabetes [221].

Diabetes mellitus is now recognized as the world's most common metabolic disorder [3]. According to recent epidemiological studies, the North America Region has the highest prevalence of diabetes in the world, with 21.4 million people, or 7.8% of the adult population [222]. Diabetes is a heterogeneous clinical disorder, classified in two main categories: insulin dependent diabetes mellitus (type 1 diabetes) and non-insulin-dependent (type 2 diabetes). Type 1 diabetes is characterized by absolute lack of insulin due to beta cells destruction, and accounts for 5-10% of all diagnosed diabetes cases. Type 2 diabetes is more prevalent (90-95% of diabetic patients) and is characterized by hyperglycemia, mainly caused by insulin resistance and/or insulin deficiency. A post-receptor defect of insulin signaling is mainly thought to underlie the basis of insulin resistance [3]. The hyperglycemia that characterized diabetes is associated with severe complications such as retinopathy (blindness), nephropathy

(renal failure), neuropathy (sensory and digestive problems), and coronary artery disease (2-4x increase risk of heart attack).

1.8.1 Overview of insulin signaling

To exert its effects, insulin requires a great number of signaling events that must be coordinated to achieve the proper metabolic and mitogenic responses, in order to maintain normal glucose homeostasis. The IR, a disulfide-bonded glycoprotein, is a transmembrane receptor that exhibits intrinsic tyrosine kinase activity (RTKs). Reversible tyrosine phosphorylation plays an essential role in activation of the IR as well as at post-receptor sites in the insulin signaling pathway (Figure 6). Insulin generates its metabolic effects by binding to the alpha subunit of its tetrameric receptor within the plasma membrane of muscle, adipose and liver cells, thereby stimulating the kinase activity of the beta subunit. This in turn catalyzes the autophosphorylation of specific tyrosine residues of the beta subunit (Y1146, Y1150, Y1151), further enhancing the tyrosine kinase activity of the receptor toward other protein substrates, such as the insulin receptor substrates (IRS 1-4) [223]. Tyrosine phosphorylated IRS subsequently recruit several effector proteins containing SH2 domains, such as SHP-2 (a PTP), as well as the SH3 domain of the adaptor molecule Grb2. Activated Grb2 recruits SOS1 that, in turn, activates the Ras signaling pathway and gene transcription. IRS also activates phosphoinositide 3-kinase (PI3K) through its SH2 domain.

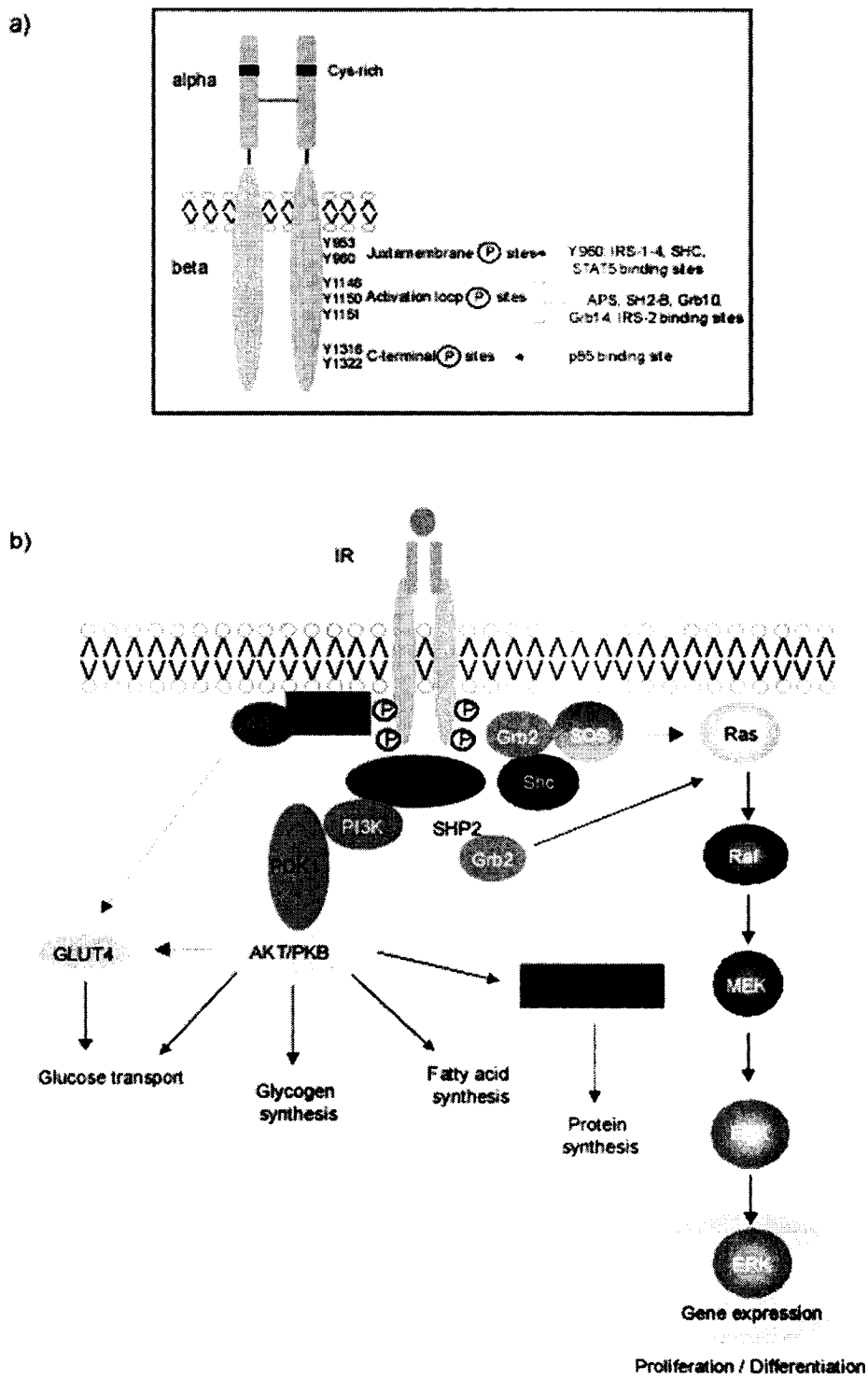

Figure 6. Signaling through the insulin receptor pathway. a) Structural features of the IR; b) Insulin signaling pathway. See text for details.

This, in turn, activates phosphatidylinositol phosphate-dependent kinase-1 (PDK1) that subsequently leads to activation of the serine/threonine kinase protein kinase B (AKT/PKB). This results in the translocation of the glucose transporter 4 (GLUT4) from cytoplasmic vesicles to the cell membrane. Because total glucose uptake into insulin-sensitive tissues is, in general, proportional to the amount of GLUT4 molecules at the cell surface, this translocation process is usually considered as the major mechanism of insulin action on glucose transport [221]. In addition, formation of a CAP-Cbl complex as well as tyrosine phosphorylation of Cbl leads to the translocation of the CAP-Cbl to lipid rafts, further enhancing GLUT4 translocation [224, 225]. Interestingly, reduced GLUT4 protein expression is a common characteristic of adipose tissue from animal models of diabetes and insulin resistance [226]. Thus, signaling through the insulin pathway is critical for the regulation of intracellular blood glucose levels and the avoidance of diabetes.

1.8.2 PTPs regulating insulin signaling

From the variety of PTPs found in insulin-sensitive tissues, ongoing work from a number of laboratories has provided biochemical evidence for a role of PTPs as key regulators of the IR signaling pathway, by attenuating insulin signaling. These potential candidates include RPTPalpha and RPTPepsilon [227], leucocyte antigen-related (LAR) [50-52, 228], TC-PTP [146, 147], and PTP1B [146, 161, 162, 180, 229-233] (Table 8). The level and the extent of phosphorylation of the IR and its downstream substrate reflect the balance between insulin induced-IR autophosphorylation, which stimulates the receptor kinase activity, and the PTPs that dephosphorylate the IR or downstream substrates.

These PTPs appear to act at least in part at the level of the insulin receptor itself. With this in mind, it has been postulated that inhibition of such PTPs will result in

prolonged insulin signaling, and that this would counteract type 2 diabetes associated insulin resistance. For instance, LAR null mice exhibit increased insulin sensitivity, with lower plasma levels of insulin and glucose in fasting state [51]. Moreover, Zabolotny et al. showed that overexpression of LAR in muscle causes whole-body insulin resistance, most likely due to dephosphorylation of specific regulatory phosphotyrosines on IRS proteins [52]. Despite significant progress towards understanding the molecular mechanisms by which these PTPs negatively regulate the IR phosphorylation and attenuate the insulin action, this mechanism is still poorly understood.

PTP	Methods / Evidence	Comments	References
RPTPalpha, RPTPepsilon	Overexpression in BHK-IR cells	Screening for rescue from effect of IR hyperactivity	[227]
LAR	Antisense		[228]
	Overexpression, LAR-neutralizing antibodies	Association by IP demonstrated	[50, 234]
	Mouse gene knockout	Low insulin and glucose levels	[51]
	Transgenic mouse overexpressing in muscle	Whole-body insulin resistance (through IRS deP)	[52]
TC-PTP	Substrate trapping mutant	Screening approach Association demonstrated	[146, 147]
PTP1B	Microinjection in oocytes	Decreased IR phosphorylation	[229]
	PTP1B-neutralizing antibodies	Increased IR phosphorylation	[230]
	Overexpression (WT, C/S mutant)	CoIP IR beta-subunit	[231, 232]
	Substrate trapping mutant	Association demonstrated	[146, 233]
	Mouse gene knockout	Increased insulin sensitivity and IR phosphorylation	[161, 162]
	Transgenic mouse overexpressing in muscle	Whole-body insulin resistance	[180]

Table 8. Protein tyrosine phosphatases identified as negative regulators of IR signaling.

1.8.2.1 PTP1B as a *bona fide* IR phosphatase: biochemical evidence

PTP1B is ubiquitously expressed, and also found in all insulin-responsive tissues. The first evidence that PTP1B had a physiological role in the regulation of IR tyrosine phosphorylation was shown by microinjection of PTP1B protein into xenopus oocytes [229]. Consequently, tyrosine phosphorylation of proteins corresponding to the molecular mass of the IR was markedly decreased, and the kinase activities of protein activated early in response to insulin such as S6 Kinase were impaired, and insulin induced meiotic cell division was retarded. These studies were corroborated by Ahmad et al. who showed that osmotic loading of PTP1B antibodies into rat KRC-7 hepatoma cells decreased insulin induced IR, IRS-1 phosphorylation, PI3-kinase activity, as well as DNA synthesis [230].

Overexpression studies by Kenner et al. using a wild-type and a C215S catalytically inactive mutant of PTP1B have shown that upon insulin stimulation, expression of the wild-type enzyme decreases IR phosphorylation and glucose incorporation into glycogen. As expected, expression of the C215S mutant resulted in the opposite observations (increased IR phosphorylation and glucose incorporation into glycogen) [231]. In addition, Chen et al. have shown that transient overexpression of PTP1B WT in primary culture of rat adipose cells reduced glucose uptake and GLUT4 translocation to the cell surface [235].

Using the substrate trapping method, Seely et al. first demonstrated a direct association between the insulin receptor and PTP1B [233]. Using a catalytically inactive GST-PTP-1B fusion protein, they were able to precipitate the activated IR from whole cell lysates. Moreover, using a ^{35}S-methionine labeled GST-PTP1B fusion protein, they demonstrated that this association occurs in a specific manner and may be displaced in a concentration dependent fashion by the addition of unlabelled fusion proteins. Independently, the direct association between IR and PTP1B was also

demonstrated in intact cells overexpressing the C215S trapping mutant of PTP1B [232]. In insulin-stimulated cells, immunoprecipitation of PTP1B results in coprecipitation of a 95kDa tyrosine phosphorylated protein that was identified by immunoblotting as the beta subunit of the IR. Conversely, immunoprecipitation of the IR results in coprecipitation of a phosphorylated 50kDa protein that was indeed identified by immunoblotting as PTP1B. Walchli et al. also reported PTP1B as a phosphatase that dephosphorylates the IR using the substrate trapping method [146]. In addition, IRS-1 was shown to be a substrate of PTP1B using recombinant proteins in vitro [236].

Additional studies have also shown that a previously undefined region in the N-terminal, catalytic half of PTP1B contributes to IR binding. Point mutations within this region of PTP1B disrupt IR binding but do not affect the phosphatase catalytic activity. The binding-defective mutant of PTP1B does not efficiently dephosphorylate the IR in cells and, consequently, does not effectively inhibit IR signaling [237]. Finally, evidence that IR is a substrate of PTP1B was further supported by crystallographic, kinetic, and peptide binding studies that showed that PTP1B recognizes a specific consensus substrate recognition motif X-pY-pY-X in the activation loop of the IR [238].

1.8.2.2 PTP1B as a *bona fide* IR phosphatase: genetic evidence

The first physiological evidence for a role of PTP1B in insulin signaling comes from the target-disruption of the PTP1B gene in mice from our laboratory [161]. Despite its involvement in a variety of signaling processes, PTP1B is surprisingly not required for embryonic development and, consequently, PTP1B null mice grow and develop normally with comparable lifespan to wild-type littermates. However, injection with insulin revealed an increased and prolonged phosphorylation of the IR in liver and muscle tissues respectively. The increase in IR phosphorylation seemed to result in

increased activity of the receptor, as IRS-1 was also hyperphosphorylated in muscle tissues. Physiologically, this correlated with enhance insulin sensitivity in PTP1B null mice. In the fed state, compared to wild-type mice, PTP1B null mice require half the level of insulin to maintain similar blood glucose concentrations. Furthermore, PTP1B null mice demonstrated enhanced performances in both oral glucose tolerance tests (OGTTs) and intraperitoneal insulin tolerance tests (ITTs), suggesting that they are more insulin sensitive and exhibit improved glucose clearance compared to wild-type littermates.

Since insulin resistance, diabetes and obesity are closely associated metabolic disorders, the role of PTP1B in obesity-induced insulin resistance was also investigated. When subjected to a high-fat diet, PTP1B deficient mice were resistant to weight gain and remained insulin sensitive, while wild-type mice rapidly gained weight and became insulin resistant. Using an independently generated mouse line, these results were further confirmed and extended by the laboratory of Dr. Neel [162]. In particular, compared to wild-type mice, the obesity resistance PTP1B null mice was correlated with increased energy expenditure without a concomitant alteration in uncoupling protein expression. PTP1B knockout (KO) mice showed a marked reduction in fat cell mass, the insulin-stimulated whole-body glucose disposal was enhanced significantly, and the increased insulin sensitivity is tissue specific.

Therefore, the regulation of insulin signaling by PTP1B appears to be tissue specific. As observed in the PTP1B KO tissues, phosphorylation of the IR was increased in the muscle and liver, but not in the adipose tissue of mice injected with insulin. This suggests that other PTPs might complement the regulation of IR in insulin sensitive tissues, particularly in adipose tissue. The following studies support this hypothesis. First, overexpression of PTP1B WT in L6 myocytes (muscle) and Fao (hepatocytes) cells blocked insulin-stimulated tyrosine phosphorylation of the insulin receptor and IRS-1 by more than 70%, and resulted in a significant inhibition of the

association between IRS-1 and the p85 subunit of PI3-K and AKT phosphorylation as well as mitogen-activated protein kinase (MAPK) phosphorylation. Moreover, insulin-stimulated glycogen synthesis was also inhibited by PTP1B overexpression in both cells [239]. When PTP1B is overexpressed in 3T3-L1 adipocytes, insulin stimulated IR and IRS-1 phosphorylation, PI3-K, as well as MAPK activation were reduced. However, when downstream events of PI3-K activation were investigated, neither AKT activation nor glucose transport seemed to be affected. Thus, it is possible that PTP1B regulates insulin mediated mitogenic, as opposed to metabolic events in this cell type [240].

1.9 PTP1B in type 2 diabetes and obesity

1.9.1 PTP1B gene polymorphisms and insulin resistance / diabetes

The human PTP1B locus maps to chromosome 20 in the region q13.1-q13.2 [241], and its mouse ortholog maps to the syntenic H2-H3 region of chromosome 2 [242]. Interestingly, this region was also identified as a quantitative trait locus linked to insulin and obesity [243]. Consistent with a role for PTP1B in insulin resistance, single nucleotide polymorphisms have been found within the coding [244, 245] or the 3' UTR [246] region that are associated with diabetic parameters.

1.9.2 Expression of PTP1B is modified in diabetic and obese subjects

Several studies have examined PTP1B expression in rodents and humans with insulin resistance and/or diabetes. Many reports showed increased expression of PTP1B in insulin-resistant states, most notably in obesity. One of the first observations made was in skeletal muscle of insulin-resistant obese (fa/fa) and diabetic (ZDFfa/fa) Zucker diabetic fatty rats, which showed an increase in *in vitro* dephosphorylation of IR correlating with higher PTP activity. The changes in PTP activity were associated with an increase in specific immunoreactivity of LAR, PTP1B, and SH-PTP2 [247]. The

same group also observed an elevated PTP activity towards the IR in adipose tissue of obese human subjects, the most prominent PTP being LAR. Accordingly, the increased abundance of LAR accounts mainly for the IR dephosphorylating activity [248]. They further observed elevated PTP activity in skeletal muscle particulate fraction of obese nondiabetic human subjects. In contrast, PTP activity is decreased in the skeletal muscle from obese type 2 diabetic subjects. Immunoblot analysis determined that LAR and PTP1B expression were augmented in the fraction of obese nondiabetic subjects [249]. Moreover, they have obtained subcutaneous adipose tissue from obese subjects before and after loss of 10% of body weight. These subjects improved their sensitivity to insulin and the weight loss was accompagnied by reduced PTP activity, as well as expression of LAR and PTP1B in adipose tissue [250].

Cheung et al. have further reported that PTP1B protein levels are 3-fold higher in abdominal adipose tissue of obese subjects than in lean control, while obese diabetic subjects exhibited a 5.5-fold increase [251]. Moreover, PTP activity was assayed by measuring the dephosphorylating activity toward a ^{32}P synthetic peptide. In accordance with previous studies in the skeletal muscle, they also observed increased PTP1B protein levels, but a marked reduction in the PTP1B activity per unit of PTP1B protein (by 71% and 88%) in adipose tissue of obese nondiabetic and obese diabetic subjects. This suggested an increased expression of a catalytically impaired PTP1B in adipose tissue of these subjects. These results were recently confirmed by Wu et al., as they reported that the increase in total cellular PTP activity, but not the activity of PTP1B, is higher the adipose tissue in more obese subjects [252]. In addition, they observed that the increased PTP activity is negatively associated with insulin-stimulated glucose transport. In summary, obese human subjects have increased PTP activity in adipose tissue that can dephosphorylate and inactivate the IR.

Increase in PTP1B activity and expression was observed in skeletal muscles isolated from non-obese, Goto-Kakizaki insulin resistant type 2 diabetic (GK) rats.

Insulin-stimulated IR autophosphorylation and IRS-1 tyrosine phosphorylation were significantly inhibited in GK skeletal muscles compared to non-diabetic control rats, suggesting that elevated PTP1B activity may lead to impaired glucose tolerance and insulin resistance in GK rats [253]. In addition, it was recently shown that PTP1B protein level is increased in fructose-fed hamster model of insulin resistance [254].

1.9.3 PTP1B antisense studies

ISIS Pharmaceutical Inc. (Carlsbad, CA, USA) was the first pharmaceutical industry that reported the development of PTP1B antisense oligonucleotide (ASO) and to use it in mouse model of diabetes *in vivo*. Rondinone et al. provided the first evidence that treatment with PTP1B ASO (ISIS-113715) downregulates PTP1B expression and adiposity in ob/ob mice (leptin null obese mice) [255]. They further reported that PTP1B inhibition using the ASO had an effect on whole body glucose and insulin metabolism [256]. In the last two years, several papers reported their interesting findings.

This ASO was used to further support that inhibition of PTP1B is an efficient mean in the treatment of hyperinsulinemia. Treatment of ob/ob mice with PTP1B ASO normalized plasma glucose levels, postprandial glucose excursion, reduced hyperinsulinemia and improved insulin sensitivity. PTP1B protein and mRNA levels were reduced in liver and fat, but unchanged in skeletal muscle. IRS-2 and PI3-K regulatory subunit p50alpha were increased, and PI3-K p85 alpha was decreased in liver and adipose tissue, changes that correlated with increased phosphorylation of insulin-stimulated AKT/PKB [256]. Moreover, they showed that FAO rat hepatoma cells transfected with a specific PTP1B ASO caused a 50-70% reduction in PTP1B protein expression (measured by Western blot analysis). Insulin stimulation increased

phosphorylation of IR and IRS, as well as increased phosphorylation of AKT/PKB and glycogen synthase kinase (GSK3) [257].

Gum et al. have demonstrated that injection of ob/ob mice with PTP1B ASO decreased PTP1B expression by 60% in the liver. Moreover, the PTP1B ASO-treated ob/ob mice stimulated with insulin showed increased tyrosine phosphorylation of the IR and the IRS-1 and -2 by three to four-fold. IRS-2 associated PI3-K activity, AKT/PKB, and GSK3 phosphorylation were also augmented. Despite the fact that PTP1B expression was unchanged in the muscle, peripheral insulin signaling was increased in PTP1B ASO treated mice. These results suggest that the reduction of hepatic PTP1B expression is sufficient to increase insulin-dependent metabolic signaling and improve insulin sensitivity in a diabetic animal model [258]. In addition, treatment of ob/ob mice with PTP1B ASO result in reduced phosphorylation of p38MAPK and ERK, but not JNK in the liver, and also normalized plasma glucose while reducing plasma insulin. Furthermore, the PTP1B ASO treatment decreased TNFalpha protein levels and phosphorylation of the transcription factor cyclic AMP response element binding protein (CREB) in the liver, both events that can occur through decreased phosphorylation of p38MAPK and which have been implicated in insulin resistance or hyperglycemia. Reduction of PTP1B protein using ASO reduced activation of p38 and its substrates TNFalpha and CREB in liver of diabetic mice, which correlates with decreased hyperglycemia and hyperinsulinemia [259].

All together, these studies constitute a solid body of evidence that PTP1B inhibitors will potentially normalize blood glucose in type 2 diabetic patients, and could lead to weight loss in obese human subjects.

1.9.4 PTP1B regulates specific gene expression

Additionally, ISIS reported that treatment of ob/ob mice with PTP1B ASO reduced adiposity and correlated with a decrease of PTP1B protein level in fat. Triglyceride content in adipocytes was reduced, correlating with a downregulation of genes encoding for proteins involved in lipogenesis, such as sterol regulatory element-binding protein-1 (SREBP-1) and downstream targets (spot14 and FAS; fatty-acid synthase). In addition, other adipogenic genes such a lipoprotein lipase (LPL) and peroxisome proliferator-activated receptor gamma (PPARgamma) were reduced [255]. Treatment of ob/ob mice with the same PTP1B ASO resulted in downregulation of liver gluconeogenic enzymes (phosphoenolpyruvate carboxykinase and fructose-1,6-biphosphatase) [256].

Accordingly, Shimizu et al. also reported that insulin-resistant rats fed with high-fructose diet showed increased PTP1B level, with strong expression of SREBP-1 mRNA in the liver. Overexpression of PTP1B in rat hepatocytes leads to increased mRNA content and promoter activity of SREBP-1, as well as increased mRNA of FAS, one of the SREBP-1-responsive lipogenic genes [260].

Using DNA microarrays and by combining all their results, ISIS further showed that treatment with PTP1B ASO resulted in the downregulation of genes involved in lipogenesis both in fat and liver, as well as downregulation of genes involved in adipocyte differentiation in fat [261].

Evidence suggests that these changes in PTP1B expression might be regulated by the transcription factor Y box-binding protein-1 (YB-1). There is an enhancer sequence within the PTP1B promoter that serves as a binding site for YB-1. Overexpression of YB-1 in Rat 1 fibroblasts and in HepG2 cells increases the level of PTP1B protein. Inversely, depletion of YB-1 by specific antisense construct led to a 70% decrease in PTP1B expression, and resulted in increased insulin sensitivity in Rat 1 fibroblasts. Moreover, enhanced signaling through the cytokine receptor gp130,

which signals through JAK2, a substrate of PTP1B, was also observed. Finally, a positive correlation was established between the expression of PTP1B and that of YB-1 in cancer cell lines and animal model of type 2 diabetes [262].

1.9.5 Regulation of PTP1B activity by insulin

Evidence suggests that PTP1B levels and activity can be regulated by insulin. For example, treatment of L6 skeletal muscle cells with insulin increased PTP1B mRNA and protein levels, suggesting the possibility of a negative feedback loop that desensitized insulin signaling [263]. Furthermore, high glucose treatment of Rat 1 fibroblasts expressing human IR also increased mRNA levels of PTP1B, corresponding to an increase in total cytosolic PTP activity. Pioglitazone, an insulin-sensitizing agent (a thiazolidinedione), reduces cytosolic PTP activity through reduction of cytosolic PTP1B content, but it had no effect on its mRNA levels [264]. Accordingly, Sell and Reese reported that *in vivo* levels of chronic hyperinsulinemia modulate transcription of the PTP1B gene, which results into two alternative splice variants of PTP1B mRNA [265].

PTP1B does not only act on and modulate the IR, but recent evidence suggests that the reverse is also true. Insulin stimulated phosphorylation of PTP1B on both serine and tyrosine residues, although their effects on PTP1B are not entirely clear. One of the first observations came from a coimmunoprecipitation of the IR and tyrosine phosphorylated PTP1B in insulin-stimulated Rat1 fibroblasts overexpressing human IR. Insulin stimulation resulted in tyrosine phosphorylation of PTP1B on three sites (Tyr66, Tyr152, Tyr153), which were found to be crucial for its binding to the IR [232]. Furthermore, insulin-mediated tyrosine phosphorylation of PTP1B by the IR at these tyrosine residues also increases its phosphatase activity [266], creating a negative feedback loop to downregulate insulin signaling.

On the other hand, *in vivo* insulin stimulation of skeletal muscle and adipose tissue also resulted in PTP1B tyrosine phosphorylation; however, this seemed to decreased PTP1B activity [267]. Moreover, it seemed that the phosphoserine content of PTP1B is also regulated by insulin stimulation. Insulin mediated decrease in PTP1B activity also occurs via Ser50 phosphorylation by AKT/PKB, and impairs its ability to dephosphorylate the IR [268], suggesting that phosphorylation on residue Ser50 by AKT may negatively modulate its phosphatase activity, thus creating a positive feedback mechanism for insulin signaling [268] opposite to the one created by the IR-induced tyrosine phosphorylation. PTP1B is also phosphorylated on other serine residues (Ser352, Ser 378, and Ser386) by enzymes such as PKA, PKC or p34 cdc2 cyclinB [267, 269]. However, the potential effect of serine phosphorylation at these sites has not been identified yet. It is possible that tyrosine and serine phosphorylation regulate PTP1B in a temporal fashion depending on the stimulus and/or growth factor involved.

In addition, the activity of PTPs is tightly regulated *in vivo* by oxidation and reduction reactions involving the invariant cysteine in the catalytic domain of the PTPs [166]. In insulin-sensitive hepatoma and adipose cells, insulin stimulation generated a burst of intracellular hydrogen peroxide (H_2O_2) that is associated with reversible oxidative inhibition of cellular PTP activity [270]. Accordingly, PTP1B activity is also strongly inhibited following insulin stimulation [271]. All together, these results suggest that PTP1B is a major modulator of insulin sensitivity and fuel metabolism, and clearly point to PTP1B as a potential therapeutic target for the treatment of type II diabetes and obesity.

1.10 Leptin and obesity

1.10.1 Role of leptin in maintenance of body weight

The prevalence of obesity is increasing worldwide, reaching epidemic proportions in industrialized societies [272]. Obesity results in an increase in the number and the size of adipocytes [273], and is associated with health complications, including diabetes and coronary heart disease. Both environmental and genetic factors are thought to play a role in the development of this disorder. It is now well established that the hormone leptin and its receptor regulate energy metabolism and body weight [274-276]. Leptin is mainly secreted by white adipose tissue [277] and acts via hypothalamic receptors to suppress feeding and increase energy expenditure [278].

Spontaneous mutations in leptin or its receptor result in marked hyperphagia leading to obesity in both mice and human [277, 279-281]. The complete lack of leptin in ob/ob mice results in severe hyperphagia leading to morbid obesity as well as diabetes. Chronic administration of leptin to these mice induces weight loss [282-284].

At the time of its discovery, leptin was considered a miracle drug for treatment of obesity [275, 277]. Generally, the rate of leptin production is related to adiposity [285]. However, obese individuals often have elevated leptin levels, and leptin administration showed very limited effects on body weight [286], suggesting that resistance to leptin action may underlie most types of obesity [287]. Recent findings indicate that leptin resistance can be caused by a defect in the transporter system of leptin through the blood-brain barrier, and/or through abnormalities at the level of leptin receptor activation and/or signal transduction in the hypothalamus [288-290].

1.10.2 Leptin signaling through JAK2 kinase

The receptor for leptin (ObR) belongs to the class of Type I cytokine receptors that utilize Janus Kinase (JAK) to transmit signals to downstream molecules [291]. Leptin binding to the extracellular domain of the ObR dimer in the hypothalamus activates the JAK2 tyrosine kinase that associates with ObR via the proline-rich "Box 1" motif. The leptin receptor has three conserved tyrosine residues in its cytoplasmic domain. Activated JAK2 tyrosine phosphorylates itself and tyrosine residues 985, 1077, and 1138 on the intracellular tail of the ObR. Phosphorylated Y1138 binds and mediates the phosphorylation dependent activation of the Signal transducers and activators of transcription 3 (STAT3) on Y705 (Figure 7). STAT3 homodimerizes and translocates to the nucleus to activate transcription of SOCS3, [292-294], as well as POMC (anorexigenic neuropeptide), and inhibit the transcription of AgRP (orexigenic neuropeptide) [285].

The membrane-proximal Y985 and Y1077 are involved in the regulation and attenuation of the leptin signal [276, 294]. SOCS3 is part of feedback loop inhibiting leptin signaling by binding to both tyrosine residues. PTP1B, which is localized at the endoplasmic reticulum, negatively regulates leptin receptor signaling through dephosphorylation of JAK2 [163, 164].

Other signaling components of the leptin receptor include SHP2, which is recruited at position Y985 and activates MAPK pathway through the adaptor protein Grb2, ultimately inducing c-fos expression. Signals mediated via unidentified tyrosine phosphorylation sites of ObR include the tyrosine phosphorylation of IRS proteins and a minor component of ERK activation [285].

1.10.3 Biochemical studies implicating JAK2 and TYK2 as substrates of PTP1B

Previous crystallographic, kinetic, and peptide binding studies have shown that PTP1B recognizes a specific consensus substrate recognition motif X-pY-pY-X in the activation loop of the IR [238]. Interestingly, this motif was also identified in the kinase JAK2, suggesting JAK2 as a potential substrate of PTP1B. Myers et al. have indeed demonstrated a stable interaction between the D181A substrate trapping mutant of PTP1B, and two members of the JAK kinase family, JAK2 and TYK2, in response to interferon stimulation [295]. They showed that mouse embryonic fibroblasts deficient in PTP1B display increased phosphorylation of JAK2, STAT1, and STAT3 upon interferon treatment, supporting the fact that JAK2 and TYK2 are substrates of PTP1B. In addition, they further demonstrated that (E/D)-pY-pY-(R/K) is a consensus substrate recognition motif for PTP1B.

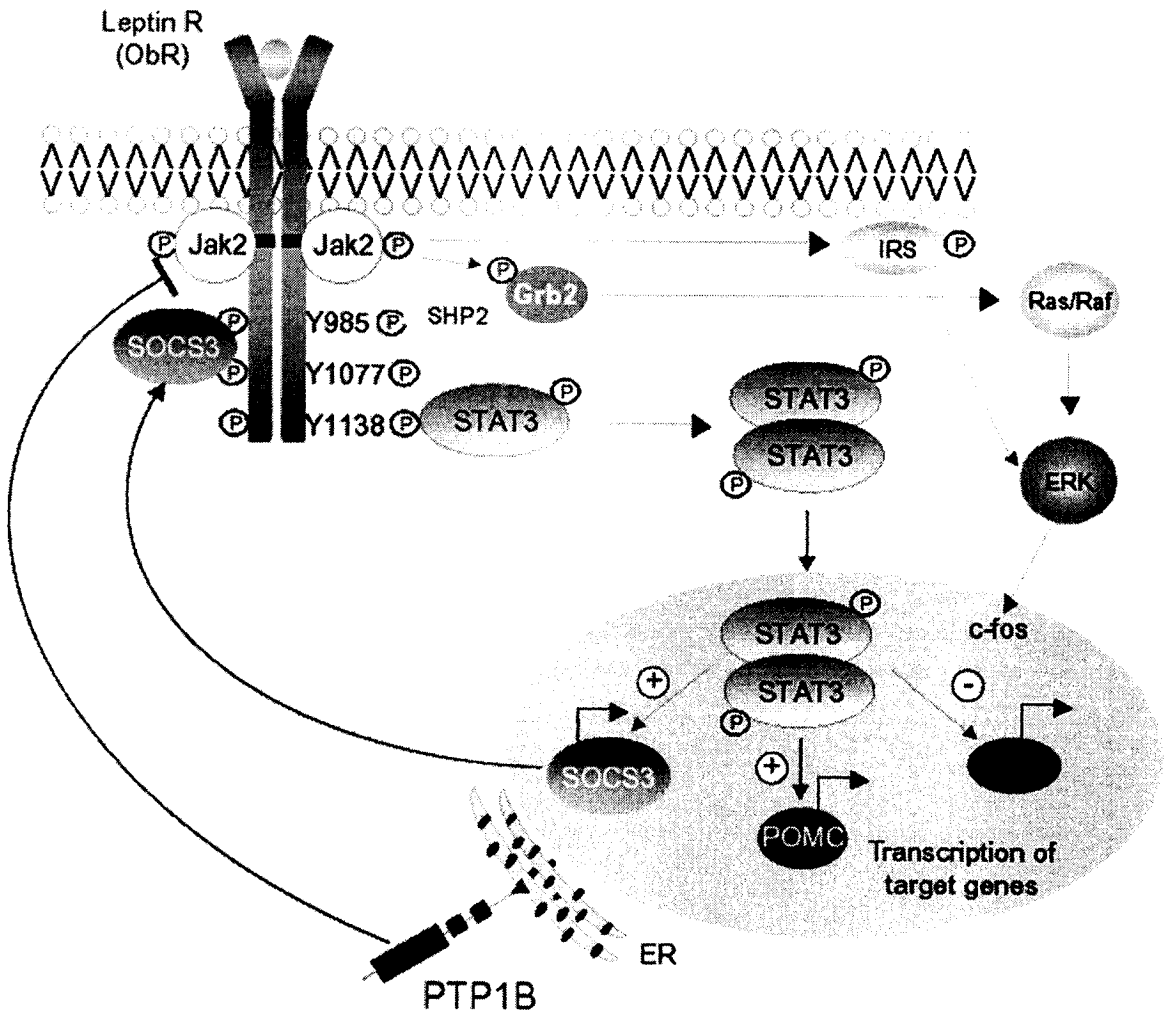

Figure 7. Leptin signaling pathway. Binding of leptin activates JAK2, which in turn phosphorylates the receptor and the transcription factor STAT3. Tyrosine phosphorylation of STAT3 allows it to homodimerize, and leads to its subsequent translocation to the nucleus where it can mediate transcription of target genes, such as the suppressors of cytokine signaling-3 (SOCS3), and anorexigenic peptides such as pro-opiomelanocortin (POMC), or inhibit the transcription of orexigenic neuropeptides such as agouti-related peptide (AgRP) [285].

1.10.4 Genetic and biochemical evidence involving PTP1B in leptin signaling

Our laboratory and others previously demonstrated that PTP1B deficient mice are resistant to diabetes and diet-induced obesity [161, 162] and displayed lower leptin levels. Interestingly, although serum leptin in these mice are lower compared to wild-type counterparts, they do not exhibit hyperphagia, suggesting that perhaps PTP1B deficient mice are leptin hypersensitive. Thus, our laboratory investigated the relationship between PTP1B and leptin in modulating obesity. We introduced the PTP1B null mutation into the leptin deficient background of ob/ob mice and studied if the loss of PTP1B could modulate leptin signaling to effect obesity resistance.

The complete lack of leptin in ob/ob mice results in severe hyperphagia leading to morbid obesity as well as diabetes. Interestingly, in ob/ob mice lacking PTP1B weight gained was significantly decreased, and an increase in resting metabolic rate was observed. Analysis of fat pads suggested that most of the difference in weight was due to a decrease in adipose tissue. This means that in the complete absence of leptin, loss of PTP1B is able to attenuate weight gain without any change in food intake [163, 164].

Furthermore, PTP1B deficient mice show an enhanced response towards leptin mediated weight loss and suppression of feeding. Hypothalami from these mice also display markedly increased leptin induced STAT3 phosphorylation, pointing out that administration of exogenous leptin also reveals that PTP1B deficiency leads to enhanced leptin sensitivity. Importantly, loss of PTP-1B in ob/ob mice increases their sensitivity towards leptin mediated hypothalamic action (feeding inhibition) as well as signaling (STAT3 phosphorylation) [163, 164].

Finally, substrate trapping experiments using the catalytically inactive PTP1B D181A demonstrate that leptin activated JAK2 is a substrate of PTP1B. This is in agreement with the results obtained by Myers et al., and provides an *in vivo* relevance

to the fact that JAK2 is a substrate of PTP1B. These results, in accordance with the results of Zabolotny et al., suggest that PTP1B negatively regulates leptin signaling, and provide one mechanism for the obesity resistance of the PTP1B null mice.

Another study reported that a two-day leptin treatment of ob/ob mice improved fasting but not postprandial glucose homeostasis. Enhanced hepatic insulin sensitivity was observed by *in vivo* IR activation in the liver, but not in skeletal muscle or adipose tissue. Liver PTP1B expression was increased by leptin to levels similar to lean controls, whereas levels in muscle and fat remained unchanged. In HepG2 cells, leptin can augment liver IR activation as well as PTP1B expression. However, overexpression of PTP1B in HepG2 cells led to diminished insulin-induced IR phosphorylation, supporting the role of PTP1B as a negative regulator of IR activation in hepatocytes [296]. These results suggest that leptin acutely improved hepatic insulin sensitivity *in vivo* with concomitant increased in PTP1B expression, possibly counterregulating insulin action. This reflects the complexity of the effects of leptin on IR signaling.

Since *in vitro* and *in vivo* evidence supports JAK2 as a substrate of PTP1B, I was interested to investigate if the role of PTP1B in the regulation of JAK2 could be extended to another important metabolic pathway, namely the modulation of growth hormone-mediated signaling. The results are presented in Chapter 2.

1.11 Genetic and biochemical evidence for other substrates of PTP1B and interacting molecules

In addition to the IR, IRS-1/2, and JAK2, other kinases, either RTKs or cytosolic, were identified as potential substrates of PTP1B (Figure 8 and Table 9). These studies are mainly based on the substrate trapping method or overexpression of wild-type or catalytically inactive enzyme, whereas a few of them rely on genetic evidence.

1.11.1 EGFR and PDGFR

A few reports have shown that PTP1B has a potential function in the dephosphorylation of EGFR and PDGFR. These conclusions have been reached mainly from overexpression studies and substrate trapping approaches [167, 297-299]. However, Haj et al. have shown that not only EGFR and PDGFR are hyperphosphorylated in response to growth factor stimulation in PTP1B null fibroblasts [300], but that the PTP1B D181A catalytically inactive mutant interacts with EGFR and PDGFR upon growth factor stimulation using fluorescence resonance energy transfer technique (FRET) [301]. PTP1B interacts with these RTKs in punctate structures, corresponding to the surface of the ER. As the FRET was more efficient 30 min after stimulation, it suggests that PTP1B must interact with RTKs that have been internalized. Thus, they provided the first evidence that PTP1B-catalyzed dephosphorylation required endocytosis of the receptors and occurred at specific sites on the surface of the ER, and that RTK activation and inactivation are spatially and temporally partitioned within cells.

1.11.2 IGF-1R

Previous overexpression studies have implicated PTP1B in IGF-1 signaling [231]. By virtue of similarity between the IR and IGF-1R, this seems quite expected. In particular, the tandem-phosphorylated tyrosines found in the IR and JAK2 are also found in the IGF-1R [238]. Our laboratory has provided genetic evidence for a role of PTP1B in the dephsphorylation of the IGF-1R. In collaboration with the laboratory of Dr. O'Connor (National University of Ireland, Cork, Ireland), we have shown that the loss of PTP1B in immortalized fibroblasts leads to increased IGF-1 mediated receptor phosphorylation, AKT activation, as well as increased cell survival under apoptotic stress [302].

1.11.3 p210Bcr-Abl

The p210 Bcr-Abl protein tyrosine kinase that is responsible for the initial manifestations of chronic myelogenous leukemia (CML) is also a substrate of PTP1B, as demonstrated by substrate trapping experiments [208]. In addition, expression of PTP1B is enhanced in various cells expressing p210 Bcr-Abl, including a cell line derived from a patient with CML. Furthermore, expression of either wild-type PTP1B or PTP1B D181A in p210Bcr-Abl-transformed Rat-1 fibroblasts diminished the ability of these cells to form colonies in soft agar, to grow in reduced serum, and to form tumors in nude mice [303]. In addition, expression of PTP1B is induced specifically by p210Bcr-Abl. Accordingly, a p210Bcr-Abl responsive sequence (PRS) has been identified in the human PTP1B promoter [304]. The PRS is recognized by Egr1 and SpC_2H_2 zinc finger transcription factors, which act in a reciprocal manner to regulate the expression of PTP1B in response to the p210Bcr-Abl oncogene [304].

1.11.4 Src

One of the first evidence for a role of PTP1B in the regulation of Src was the finding that several human breast cancer cell lines with elevated c-Src specific activity also possess elevated phosphatase activity. PTP1B was identified as the phosphatase involved in the activation of Src by dephosphorylating Tyr527 in the carboxy-terminal negative regulatory domain of Src. In addition, these cell lines showed elevated levels of PTP1B protein relative to normal control breast cells [209]. Then, our laboratory employed a genetic approach using fibroblasts lacking PTP1B to establishing the physiological relevance of PTP1B is this process. Indeed, it was observed that Tyr527 was hyperphosphorylated in absence of PTP1B, implying reduced activity of Src [305]. These results were further confirmed by Dadke et al. that showed that PTP1B activates through binding of the proline-rich region of PTP1B to the SH3 domain of Src [306]. In addition, they used PTP1B antisense to show that Rat fibroblasts lacking PTP1B expression cannot activate Src.

1.11.5 JAK2 and TYK2: LepR, EpoR, IFNgamma, and IFNalpha

In addition to the IR recognition, structural studies on the PTP1B enzyme by the Barford laboratory suggested that PTP1B might preferentially recognize substrates containing two adjacent phosphorylated tyrosines (X-pY-pY-X) [238]. Soon after this report, Dr. Tremblay's lab also contributed to demonstrate that both JAK2 and TYK2 (that contain such tandem phosphotyrosines) were found to be hyperphosphorylated in PTP1B deficient fibroblasts upon interferon stimulation [295]. In addition, we and others independently found that PTP1B dephosphorylates JAK2 in the leptin pathway (as described in section 1.10.4) [163, 164]. The role of PTP1B in regulating JAK2 was also extended to another member of the cytokine receptor family, namely the erythropoietin (EPO) receptor (EpoR) [307]. This receptor also relies on JAK2 for its downstream signaling. Thus, following the binding of its ligand EPO, the receptor must recruit JAK2 to trigger the signaling cascade [308]. Cohen et al. have shown that JAK2 is a substrate of PTP1B by overexpression studies and using the substrate trapping method [307].

1.11.6 STAT5a/b

While investigating the role of PTPs in the prolactin (PRL) signaling pathway, which uses JAK2 to transmit downstream signal to activate STAT5a/b [308], Aoki et al. found that STAT5a and STAT5b were substrates of PTP1B in transfected COS7 cells [309]. Moreover, they showed that overexpression of PTP1B inhibited the nuclear translocation of STAT5a and STAT5b, as well as PRL-dependent transcriptional activation of the beta-casein gene promoter.

1.11.7 p130cas: a SH3 interacting protein or a "real" substrate?

In addition to the IR, the adapter protein p130cas was one of the first candidate substrates for PTP1B [217], implicating the phosphatase in both integrin signaling [310] and transformation [311]. It was shown that overexpression of PTP1B wild-type

decreased p130cas tyrosine phosphorylation [310]. However, Dr. Tremblay's lab provided genetic evidence that p130cas is not hyperphosphorylated in PTP1B deficient fibroblasts during fibronectin signaling [305], thus raising the possibility that this function might be redundant or non-physiological. As demonstrated previously, the presence of the proline-rich domains of PTP1B is essential for the interaction of p130cas with PTP1B, and this interaction is independent of tyrosine phosphorylation [217]. When wild-type PTP1B is overexpressed in cells, p130cas gets dephosphorylated, whereas a PTP1B proline mutant does not have this effect. Thus, overexpression of PTP1B might lead to artefactual results in this context. Alternatively, overexpression of PTP1B could activate another PTP that dephosphorylates p130cas, such as PTP-PEST.

Substrate	Method / Substrate trapping mutant	References
EGFR	Substrate trapping *in vivo*, D/A	[167]
	Substrate trapping *in vitro*, C/S, IP	[298]
	Substrate trapping *in vivo*, D/A Q/A, D/A, C/S	[299]
	Interaction detected by FRET	[301]
	Hyperphosphorylation in KO fibroblasts	[300]
PDGFR	Substrate trapping *in vitro*	[297]
	Substrate trapping *in vitro*, C/S, IP	[298]
	Interaction detected by FRET	[301]
	Hyperphosphorylation in KO fibroblasts	[300]
IR	Cell loading with PTP1B neutralizing antibodies	[230]
	Overexpression WT and C/S	[231]
	Substrate-trapping *in vitro*	[233]
	Co-immunoprecipitation	[232]
	Hyperphosphorylation in knockout tissues	[161,162]
IRS-1/2	Overexpression WT and C/S	[231]
	Hyperphosphorylation in knockout tissues	[161]
	Substrate trapping, *in vitro* assay	[236]
IGF1-R	Overexpression WT and C/S	[231]
	Overexpression WT and D/A, KO cells	[302]
JAK2	Substrate trapping *in vivo*, D/A, KO cells	[295]
	Substrate trapping *in vivo*, D/A	[163]
	Substrate trapping *in vitro* C/S, in vivo D/A	[164]
TYK2	Substrate-trapping *in vivo*, D/A, KO cells	[295]
p210Bcr-Abl	Substrate trapping *in vivo*, D/A	[208]
STAT5a/b	Substrate trapping *in vivo*, D/A	[309]
Src	Overexpression WT, C/S, *in vitro* assay	[209]
	Knockout cells	[305]
	Interaction through SH3 domains	[306]
p130cas	Substrate-trapping *in vitro*, C/S, D/A	[127]
	Overexpression WT and mutants	[217]

Table 9. Potential substrates of PTP1B. All known substrates of PTP1B are listed here according to biochemical and genetic evidence that led to their identification.

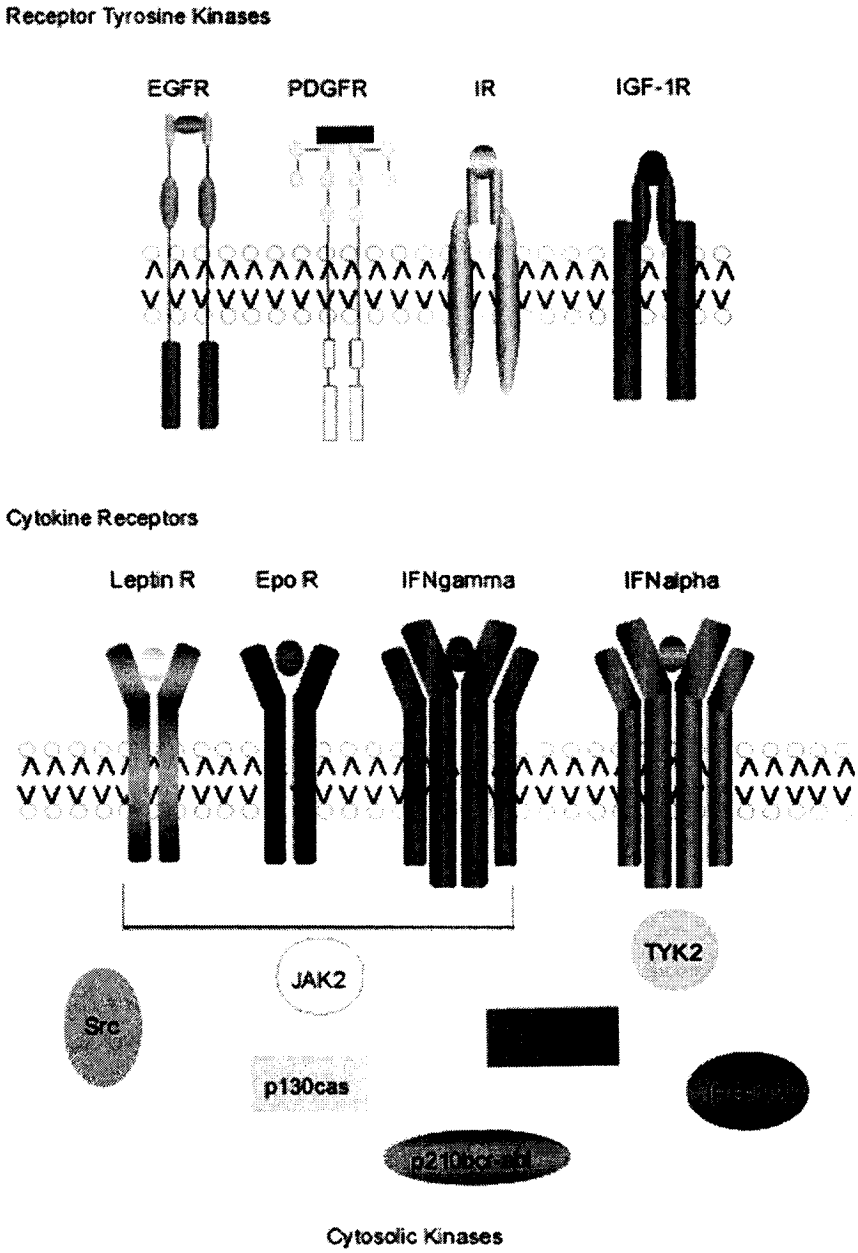

Figure 8. Substrates and signaling pathways regulated by PTP1B. By dephosphorylating a variety of RTKs and cytosolic kinases, PTP1B is involved in the regulation of a multitude of signaling pathways, such as oncogenic, metabolic, and cytokine signaling pathways.

1.11.8 Adaptor molecules: Grb2, Crk, and p130cas

The C-terminal region of PTP1B contains two proline-rich regions, which conform to the canonical class II SH3 domain binding motif, Pro-X-X-Pro-X-Arg. Liu et al. established that PTP1B interacts with Crk, Grb2, and p130cas *in vitro*, and with p130cas in intact cells [217]. Further studies revealed that the adaptor protein Grb2 strongly promotes the formation of a stable protein complex between tyrosine-phosphorylated IRS-1 and catalytically inactive PTP1B, as judged by their increased co-immunoprecipitation [236]. In addition, inclusion of Grb2 in a reaction mixture of IRS-1 and active PTP1B also increased the overall rate of IRS-1 tyrosine dephosphorylation.

Although Crk was previously identified as interacting via SH3 domain with PTP1B, Takino et al. recently reported that CrkII is dephosphorylated *in vitro* and *in vivo* by PTP1B [312]. However, overexpression studies are sometimes insufficient to confirm the identity of a potential substrate. Observation of an interaction between CrkII and PTP1B D181A substrate trapping mutant would further support this notion. As for p130cas, more studies are required to determine if it is solely an interacting molecule or a putative substrate.

In summary, as described in this section, a multitude of biochemical and/or genetic studies suggest that PTP1B is involved in the attenuation of various tyrosine kinases (RTKs and cytosolic kinases).

1.12 Potential role of PTP1B in growth hormone signaling

1.12.1 Role of growth hormone as a metabolic regulatory hormone

Growth hormone (GH) is a major growth-promoting hormone that is secreted by the anterior pituitary into the circulation, mainly regulating postnatal growth in mammals. Many of the actions of GH are mediated by the activation of IGF-1. GH acts on tissues such as liver and bone (and muscle and fat [313]) to stimulate the synthesis of IGF-1, a growth factor required for complete postnatal development [314, 315]. Deregulated levels of GH can lead to the development of clinical syndromes as such acromegaly (GH excess), and Laron syndrome (GH deficiency and GH insensitivity) [316].

More recently, the generation of genetically modified mice has further demonstrated the critical roles of GH, GH receptor (GHR), and IGF-1 in mediation of growth. First, GHR null mice display postnatal growth retardation, dwarfism, and they are only half the size of their wild-type littermates. They also exhibit lower IGF-1 levels, and increased GH concentrations, features observed in Laron syndrome. As for the IGF-1 KO mice, they present a growth deficiency of 60%. Finally, the IGF-1R KO mice die at birth from respiratory failure and exhibit a more reduced size [313]. Hence, these animal studies further support an important role for GH in the promotion of growth [317].

In addition, GH has important metabolic regulatory actions, with effects on glucose, protein and lipid metabolism (Figure 9). As a consequence, secondary disorders often occur in individuals suffering from abnormal GH secretion. For example, excess plasma GH leads to the development of insulin resistance, whereas a deficit is associated with increased adiposity. In mice, GH is involved in the development of insulin resistance and has been associated with retinal neovascularization and nephropathy, both complications of diabetes [317].

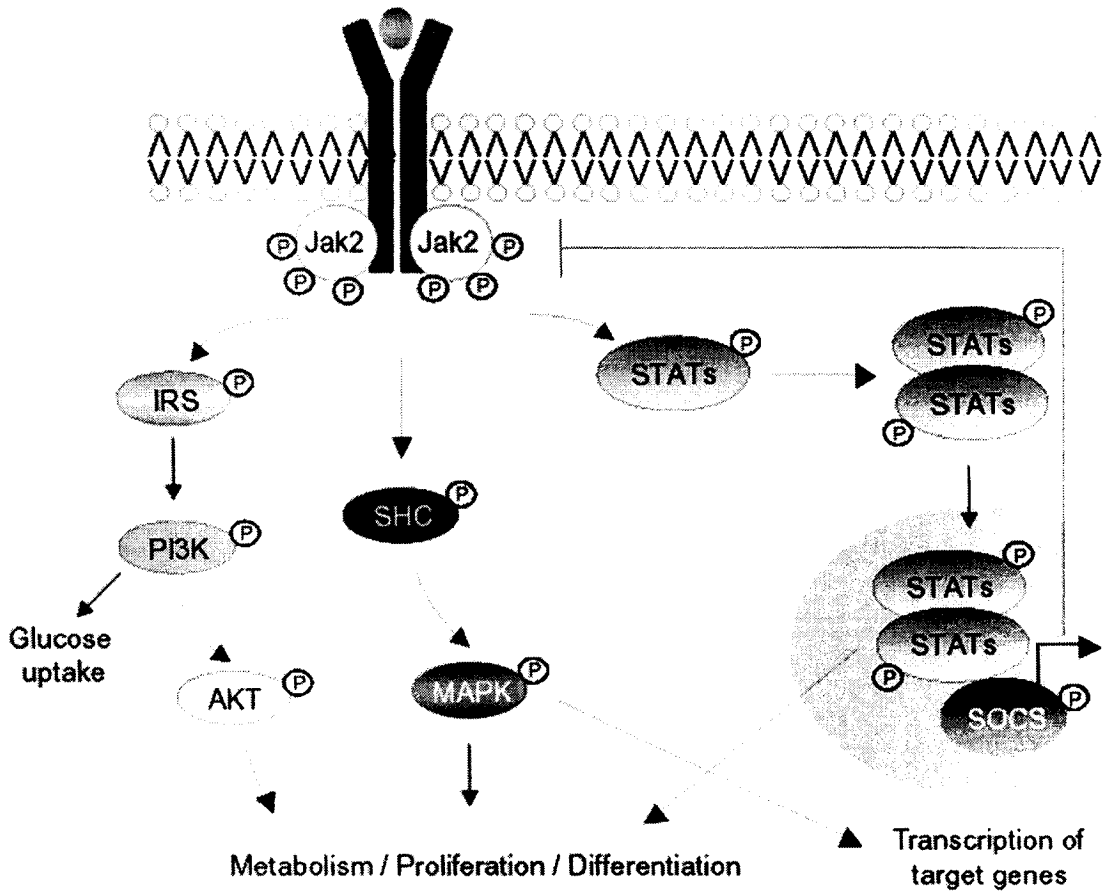

Figure 9. Growth hormone receptor signaling pathway. Some of the signaling pathways activated by GH activation of JAK2 are shown. Binding of GH induces dimerization and subsequent activation of JAK2. Several tyrosine residues on the GHR are phosphorylated by JAK2, and phosphorylated residues Y1007/1008 recruit the transcription factor STAT3 and 5 via an SH2 interaction. This allows the STATs to become phosphorylated by JAK2. Tyrosine phosphorylated STATs homodimerize and enter the nucleus where they mediate transcription of target genes. JAK2 also phosphorylates SHC, leading to activation of MAPK. MAPK and STATs are important for GH regulation of gene transcription. JAK2 phosphorylates IRS proteins, which are thought to lead to activation of PI3-K. GH activation of PI3-kinase via IRS proteins might be important for GH stimulation of glucose transport [317].

Given its role in growth and metabolism, GH can exert both insulin-like (early) and anti-insulin like (late) effects on certain target cells and tissues [316]. The insulin-like action lasts approximately 2 h, and includes transient increases in glucose and amino acid transport, lipogenesis, and protein synthesis [317, 318]. Hence, GH and insulin might activate some common signaling pathways, such as the IRS proteins and PI3-K, thus regulating glucose transport. Anti-insulin-like effects of GH occur much later (after 3 h), and include decreasing fat by increasing triglycerides breakdown and lipolysis [317, 319].

1.12.2 Growth hormone signaling through JAK2, STATs, and SOCS proteins

The GHR, similarly to the leptin receptor, is a member of the class I superfamily of cytokine receptors and lacks intrinsic kinase activity. In order to get activated, it relies on tyrosine kinase activity of the JAK2. As depicted on Figure 9, upon binding of GH and dimerization of the GHR, JAK2 autophosphorylates and phosphorylates the GHR on tyrosine residues. The proline-rich "Box1" motif of the GHR is required for JAK kinase interaction and activation. Additional less-conserved sequences such as "Box 2" are also important for JAK kinase interactions and likely function in JAK kinase isoform selectivity [308, 320]. Tyrosine phosphorylated residues on the GHR create docking sites for the SH2 domains of the STATs transcription factors, thus recruiting the STATs to the GHR-JAK complexe. JAK2 then phosphorylates the STATs, which phosphorylation at a conserved tyrosine residue induces homo- or heterodimerization. GH can stimulate tyrosine phosphorylation of STATs 1, 3, and 5a and 5b [321]. The STATs dimers then translocate to the nucleus, where they bind to specific DNA response elements to activate transcription of target genes such as the Suppressor of cytokine signaling (SOCS). This exerts a negative feedback loop on JAK activation by competing with STATs for the binding of the GHR [322].

There are four mammalian JAKs: JAK1, JAK2, JAK3, and TYK2. JAK1, JAK2 and TYK2 are ubiquitously expressed, whereas JAK3 is restricted to the lymphoid/hematopoietic tissues [316]. The JAKs have two tandem kinase-like domains, one of which is a functional catalytic tyrosine kinase domain that is located at the C-terminal end of the protein. Upstream of this domain is located an inactive kinase-like (pseudokinase) domain and a large N-terminal half that is supposed to be important for interaction with particular cytokine receptors and other signaling effector and modulator proteins (Figure 10) [316].

As mentioned above, the JAKs phosphorylate and activate a family of intracellular proteins known as STATs, which translocate to the nucleus, where it binds DNA and activate gene expression. The STATs serve as both signal transducer in the cytoplasm and activators of transcription in the nucleus. They possess a DNA binding domain, a transcription activation (TA) domain, a SH3-like domain, a conserved site of tyrosine phosphorylation, as well as a SH2 domain, which is important for their dimerization before they translocate to the nucleus [323].

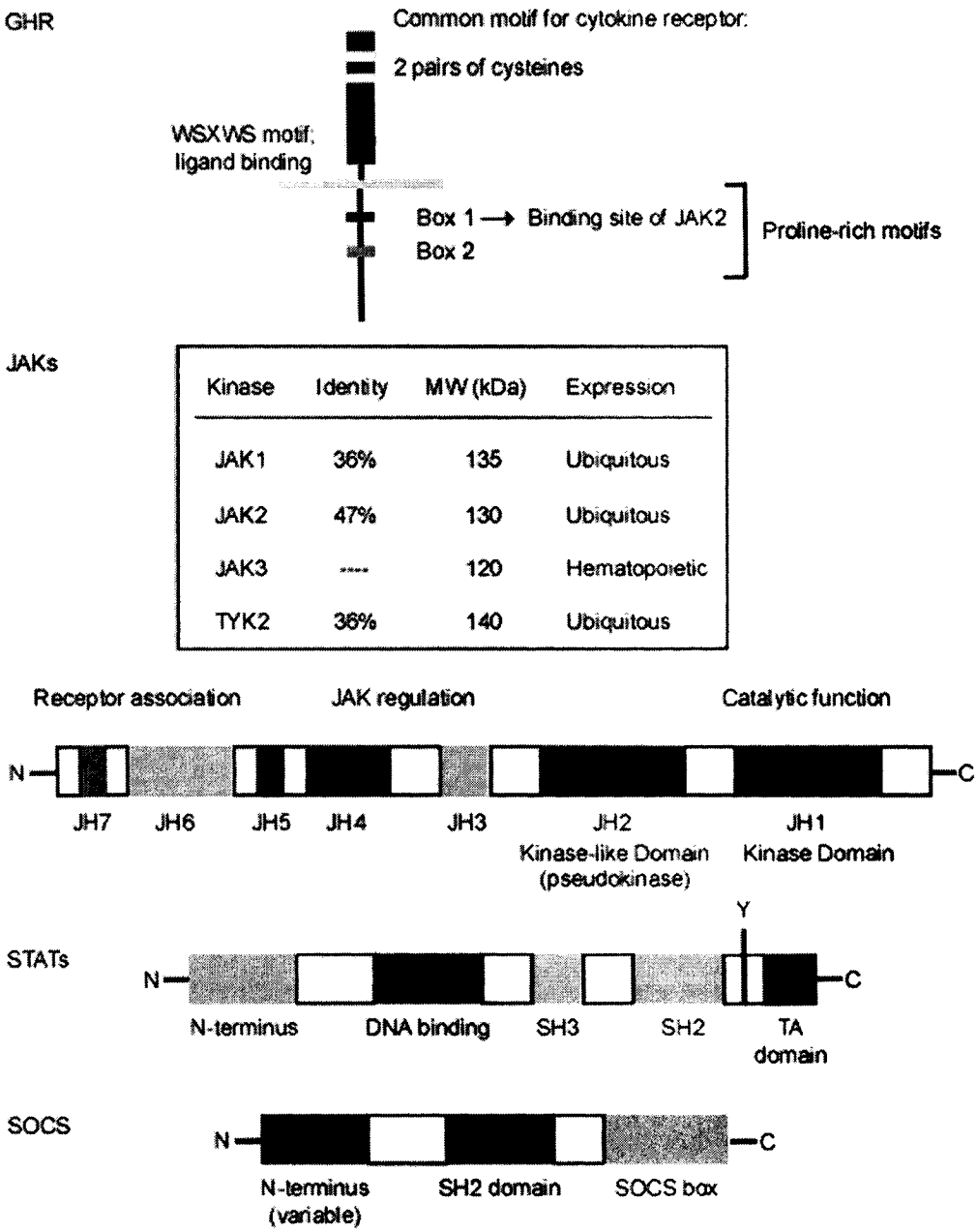

Figure 10. General structure of the GHR, JAK kinases, STATs, and SOCS proteins. The seven JAK homology domains (JH), six regions of high homology between STAT family members, as well as the domains of SOCS proteins are indicated as colored boxes. Also shown is a comparison of members of the JAK family in amino acid sequence identity, molecular weight (MW), and expression. Adapted from [316].

Equally important, but less understood, are the mechanisms limiting and terminating GHR signaling. These include internalization and degradation of the GHR, inhibition of signaling by negative regulators such as the SOCS and the Protein inhibitor of activated STAT (PIAS), and inactivation of JAK2 and downstream signaling molecules by dephosphorylation [316, 317, 324]. The SOCS are cytokine-inducible genes, which are important in the regulation of GHR-JAK2 signaling. They possess a central SH2 domain, a variable region at the N-terminus and a region with high homology at the C-terminus termed the SOCS box. SOCS transcription is activated by the STATs. GH induces expression of SOCS-1, -2, -3, and Cytokine-inducible SH2-containing protein (CIS) in rat liver to varying degrees, and with different kinetics. SOCS proteins are thought to inhibit signaling by inhibiting the kinase activity of JAKs. However, some members of the family appear to inhibit signaling by competing with STATs for a docking site on cytokine receptors [313, 317].

Given the central role of JAK2 in initiating GH signaling, identification of the phosphatases involved in deactivation is important. Candidates that have been shown to associate with JAK2 include the cytosolic tyrosine phosphatases SHP1, SHP2 [325-328], and the transmembrane tyrosine phosphatase CD45 [30]. However, the relevance of these phosphatases in the context of GH action remains uncertain. In addition, since JAK2 was identified as a substrate of PTP1B in the leptin signaling pathway, I decided to investigate if JAK2 could be an *in vivo* substrate of PTP1B in the GH pathway and whether this interaction has a physiological significance (as Chapter 2).

1.13 Potential role of PTP1B in type 1 diabetes

As previously described in section 1.8, the clinical syndrome of diabetes mellitus (DM) is common to a group of diseases characterized by high levels of blood glucose resulting from defects in insulin secretion, insulin action, or both. This section will focus on the potential involvement of PTPs, particularly PTP1B, in type 1 diabetes.

1.13.1 Type 1 diabetes and development of complications

Type 1 diabetes is characterized by insulinopenia or absolute lack of insulin due to beta cell destruction of unknown etiology but with strong autoimmune component, HLA association, and probable involvement of environmental factors or virus [329, 330]. Although type 1 diabetes represents less than 10% of the total cases of diabetes mellitus (DM), it has become evident that it is underdiagnosed in adults [331]. Symptoms of type 1 diabetes usually develop over a short period, although beta cell destruction can begin years earlier ("honeymoon period" when no insulin is required). Autoimmune, genetic, and environmental factors are recognized as contributing factors in development of type 1 diabetes [332]. As a generalized metabolic disorder, the disease is associated with long-term complications that affect almost every major system in the body. Diabetic cardiomyopathy, retinopathy, neuropathy, nephropathy, vascular diseases and other DM-associated pathological states make DM one of the major health burdens of society. Diabetic neuropathy (DN) is one of the most common of human DM complications. The prevalence of clinically overt DN is 7% in the first year of diagnosis, increasing to 50% after 25 years, and 100% if subclinical nonsymptomatic neuropathy is included [331]. DN consists of several clinical syndromes affecting motor, sensory and autonomic nerves, with distal symmetric sensory neuropathy. It is characterized by both positive and negative signs - sensory

loss, pain, paresthesiae, reduced conduction velocity - interpreted as evidence for dysfunctional ion channels in peripheral nervous system of diabetics [331].

Currently, the basic therapy for type 1 diabetes is multiple daily insulin injections. The overall goal of diabetes management is to keep blood glucose levels as close to the normal range as possible since many studies demonstrated that control of glycemia reduces the risk of developing major complications of diabetes [331]. In addition to insulin replacement, there are several experimental approaches in treatment with considerable promise. Although preservation of pancreatic beta cell so far has not been achieved, transplanting islets seems a promising experimental approach that has the potential for curing type 1 diabetes. However, there are still major problems with transplantation of islets: adequate number of viable islets to transplant and prevention of rejection of the transplanted islets by the body's immune system [333, 334]. Advances in stem cell technology seem to have the most promising future.

1.13.2 Mouse models of type 1 diabetes

Large number of models are available that mimic insulinopenia of type 1 diabetes based on selective destruction of pancreatic beta cells by toxins or viral infection. Streptozotocin (STZ)-induced diabetes in rodents has been the most widely studied model based on selective properties of STZ for beta cells [335], and produces a relatively permanent type 1 syndrome. Since mice show strain and gender dependent differential and higher sensitivity to STZ, some strains may exhibit a spontaneous remission indicating that not all beta cells were destroyed. However, it is possible to dose the level of beta cell destruction by STZ injection protocol. The detailed mechanism of STZ action is depicted in Figure 11.

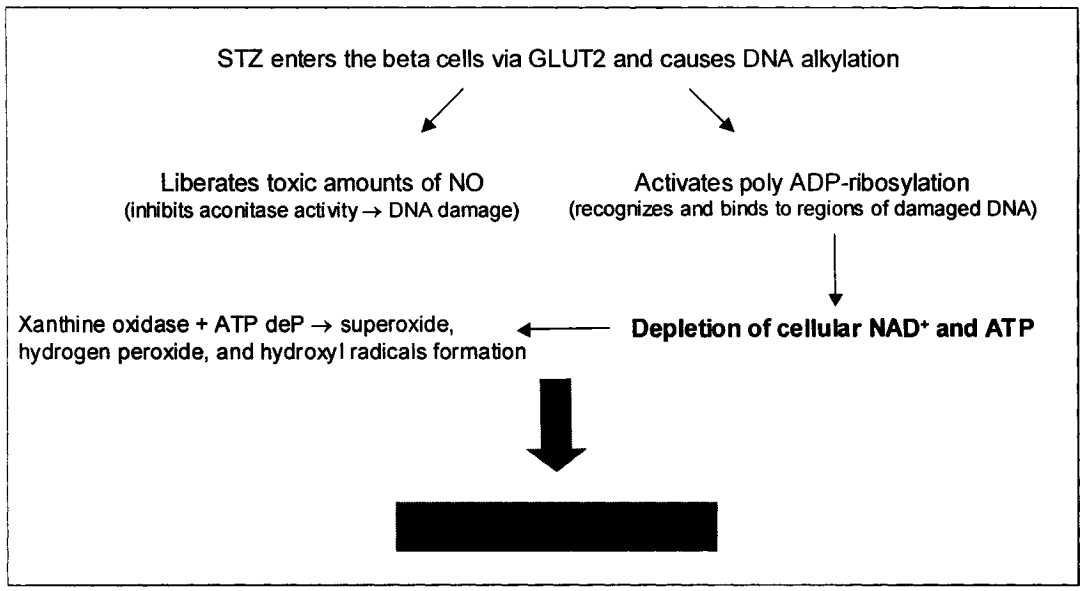

Figure 11. Mechanism of beta cells destruction by streptozotocin. STZ is an antibiotic and active agent against tumors, especially endocrine tumors of the pancreas, but is damaging to insulin-producing cells (beta cells). Abbreviations: GLUT2, glucose transporter 2; NO, nitric oxide; deP, dephosphorylation; H peroxide, hydrogen peroxide). Adapted from Szkudelski 2001[335].

1.13.3 PTPs and type 1 diabetes

One of the first insights pointing to a role for PTPs in insulin signaling comes from early studies using vanadium compounds that normalized blood glucose levels in animal models of type 1 and type 2 diabetes [336, 337]. Vanadium compounds (vanadate, vanadyl sulfate, metavanadate, peroxovanadate) possess substantial insulinomimetic activity, both *in vitro* and *in vivo*. Meyerovitch et al. showed that oral administration of vanadate normalizes blood glucose levels in STZ-diabetic rats through stimulation of glucose uptake [338]. Furthermore, in rats made diabetic by STZ treatment, hepatic cytosolic PTP activity increased to 180% of the control values after two days of diabetes and remained elevated at 30 days. Treatment of STZ diabetic rats with subcutaneous insulin or vanadate in their drinking water for 3 days reduced PTP activity in the particulate, but not in the cytosolic fraction, and was associated with

normalization of blood glucose. This suggests that insulin deficient diabetes is accompanied by significant changes in hepatic PTP activity, and that increase in PTP activity is an important factor in diabetic states [339]. The inhibitory action on PTPs and enhancement of cellular tyrosine phosphorylation appear to be the most relevant explanation for this effect. In rat adipocytes, vanadate compounds promote insulin action by three mechanisms 1) a direct insulin-mimetic action, 2) an enhancement of insulin sensitivity and 3) a prolongation of insulin biological response [338]. A recent study of human diabetics showed that administration of vanadium produces a modest increase in insulin sensitivity and decrease in insulin requirements, at the expense of undesirable toxic effects [340, 341]. Therefore, development of better insulinomimetics remains one of the objectives of therapeutic studies for type 1 diabetes, with properties that would be superior to insulin itself in the control of diabetes.

Thus, the absence of insulin production in type 1 diabetes leads to a need for exogenous insulin injection to provide IR activation and to maintain blood glucose homeostasis. Certainly, understanding the molecular events triggered by insulin has provided important clues in the treatment of both types of diabetes. One important mechanism in regulating insulin signaling is mediated by PTPs, which may either act on the insulin receptor itself and/or its substrates [221]. It is well known that the IR itself is negatively regulated by a series of PTPs, as described in section 1.7.2. Among them, PTP1B has been physiologically validated as a negative regulator of the IR [161, 162].

1.13.4 PTP1B in type 1 diabetes

Several studies have examined PTPs enzyme activity and PTPs protein levels in various models of insulin resistance. The STZ-diabetic rat has been primarily used as a disease model to study diabetes associated with pancreatic beta cell destruction.

However, it also serves as model for exploring the mechanism of insulin resistance in insulinopenic diabetes. In addition, Ahmad et al. found increased cytosolic PTP activity in particulate fractions of skeletal muscle and liver in STZ-diabetic rats. An immunoblot analysis of various PTPs in these tissues showed a significant increase in PTP1B expression [342], suggesting that downregulation of PTP1B could be a mean to decrease blood glucose concentrations in these diabetic animals. Recenlty, Kushner et al. showed that PTP1B regulates beta cells homeostasis [343]. They observed that the absence of PTP1B improved glucose tolerance and delayed the onset of diabetes in IRS2 KO diabetic mice, suggesting that the specific inhibition of PTP1B can partially compensate for the lack of insulin in the IRS2 KO mice.

In addition, the PTPs RPTPIA2 and its homologue RPTPIA2beta are important autoantigens associated with type 1 diabetes [87-89]. Morever, a single-nucleotide polymorphism (SNP) in the gene (PTPN22) encoding LyPTP, a suppressor of T-cell activation, has been recently associated with type 1 diabetes [134].

Given the central role of PTP1B in the regulation of insulin signaling, we investigated whether PTP1B deficiency could improve glucose homeostasis in a mouse model of type 1 diabetes induced by STZ (as Chapter 3). This could determine if PTP1B inhibitors could be used therapeutically, in complement with insulin, in the treatment of various stages and severity of type 1 diabetes.

1.14 Potential role of PTP1B in Ras signaling

1.14.1 Biochemical evidence for a role of PTP1B in signaling downstream of RTKs

As mentioned in previous sections, PTP1B downregulates the activity of receptor tyrosine kinases such as the EGFR, PDGFR, IGF-1R, and IR, by dephosphorylating tyrosine residues important for their catalytic activity [10]. Thus, this evidence demonstrated that PTP1B is capable of regulating RTK signaling at the level of the receptor. Recently, in collaboration with the O'Connor laboratory (National University of Ireland, Cork, Ireland), Dr. Tremblay's laboratory has demonstrated that PTP1B also regulates RTK signaling by acting downstream of the receptor [302]. Using fibroblasts lacking PTP1B, it was shown that stimulation with IGF-1 resulted in increased tyrosine phosphorylation of IGF-1R, as well as increased phosphorylation of AKT/PKB, a molecule downstream of the receptor. However, it was observed that extracellular regulated kinase1/2 (ERK1/2) phosphorylation was decreased upon IGF-1 stimulation in PTP1B null fibroblasts, suggesting that PTP1B is involved in the regulation of additional molecules downstream of RTKs. Furthermore, reduced ERK1/2 activation was also observed previously upon fibronectin stimulation, suggesting that PTP1B also regulates integrin signaling [305]. In this case, reduced Src activation in the absence of PTP1B was responsible for the lack of ERK1/2 phosphorylation.

A common feature in the activation of all these RTKs signaling pathways is the involvement of the small GTPase Ras, which serves as a molecular switch that transduces an upstream signal to downstream effectors such as the MAPK family, thus regulating cell proliferation, differentiation, and gene expression [344]. Ras has both GDP/GTP-binding activities, and exists in two interconvertible forms: guanosine diphosphate (GDP)-bound inactive and guanosine triphosphate (GTP)-bound active forms. Ras protein activity is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). Their activation is induced by a large variety

of extracellular signals, such as those that activate receptors with intrinsic or associated tyrosine kinase activity [345, 346]. The GDP/GTP exchange reaction is stimulated by GEFs, which interact with the GDP-bound form and releases bound GDP. On the other side, the GTPase activity (GTP to GDP) is stimulated by GAPs. Indeed, mutated versions of the human Ras genes have been detected in 30% of all metastatic cancers, implying an important role for aberrant Ras protein function in carcinogenesis [344]. For example, Ras gene mutations are highly prevalent in pancreatic (90%), lung (30%), colorectal (50%) carcinomas, thyroid tumors (50%), and in myeloid leukemia (30%) [347]. Since fibroblasts lacking PTP1B showed decreased activation of ERK1/2, it is possible that PTP1B plays a role in the regulation of Ras signaling.

1.14.2 Potential role of PTP1B in PDGFRb signaling

Binding of PDGF to the receptor triggers autophosphorylation of a total of 13 tyrosine residues in its cytoplasmic domain [348]. These tyrosine-phosphorylated residues create docking sites for a variety of proteins, many of which are in turn phosphorylated upon binding to the receptor, and initiate signaling cascades that result in cell proliferation, motility, and survival [349]. As depicted on Figure 12, these include enzymes (PI3K, PLCg, SHP-2, RasGAP, Src family kinases SFK; Src, Fyn, Yes), transcription factors (STATs 1, 3, and 5), and adaptor molecules (Grb2, Grb7, Grb10, Shc, and Nck) [348].

The Ras GTPase-activating protein p120RasGAP has a C-terminal RasGAP domain, and two N-terminal SH2 domains that flank a SH3 domain. In addition, p120RasGAP has a PH domain and a calcium and lipid-binding domain that may be required for interactions with the inner surface of the plasma membrane [350]. It also has an N-terminal proline-rich sequence that is essential for *in vitro* association with

members of the Src kinase family [351]. Previous studies have demonstrated that p120RasGAP associated with Tyr771 of the phosphorylated PDGFRbeta through its C-terminal GAP SH2 domain [352, 353]. Moreover, p120RasGAP also interacts with the EGFR following ligand binding [354]. Although the p120RasGAP murine knockout is embryonic lethal at E10.5, p120RasGAP deficient fibroblasts show increased and prolonged activation of the Ras/MAPK pathway [355]. In addition, tyrosine-phosphorylated p120RasGAP also interacts with tyrosine-phosphorylated p62DOK (Downstrearm of tyrosine kinase) and p190RhoGAP [356, 357]. p62DOK is involved in the negative regulation of Ras signaling [358, 359], and p190RhoGAP is a GAP for RhoA and other members of the Rho family of GTPases [360-362].

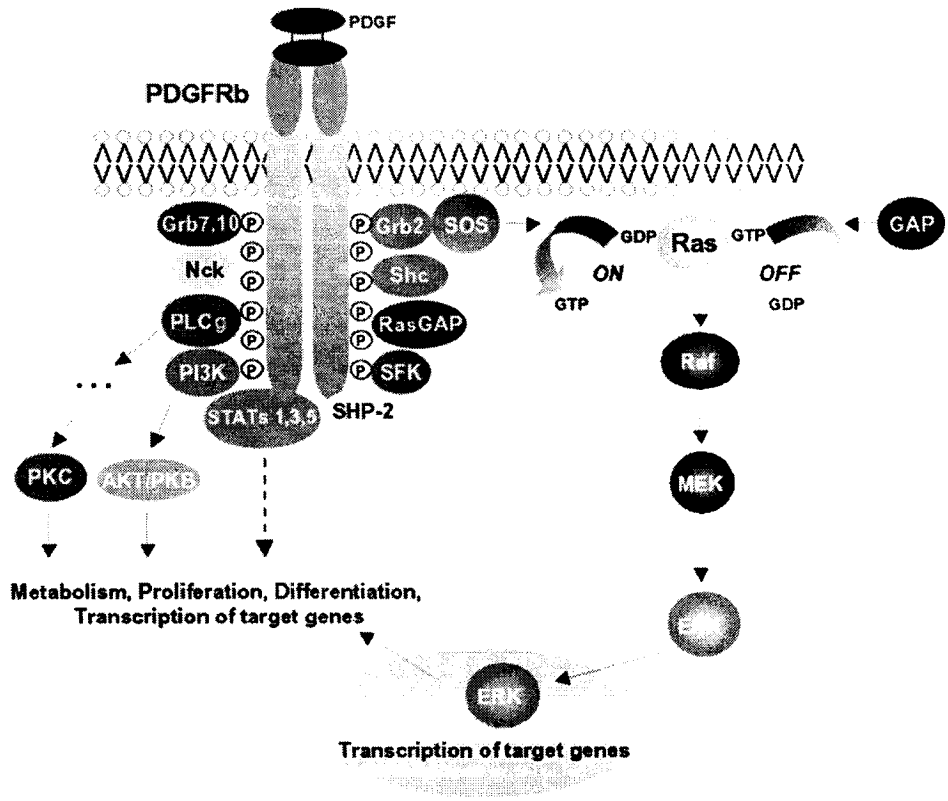

Figure 12. PDGFRbeta signaling pathway. Association of signaling proteins with tyrosine phosphorylated sites of the activated PDGFRbeta. Proteins binding with high affinity (e.g., PI3K, PLCg, RasGAP) occupy one or two phosphotyrosine residues on the receptor, whereas low-affinity binding (e.g., Nck or Shc) involves multiple sites. Adapted from Heldin et al. 1999 [348].

1.15 Potential role of PTP1B in p53 tumorigenesis

1.15.1 p53 regulates the cell cycle

p53 is a DNA-binding protein involved in regulating the expression of genes that control the cell cycle and apoptosis. For instance, when cells undergo DNA damage, p53 arrests the cell cycle at the G1/S checkpoint to permit DNA repair [363, 364], or in many cases, lead the cell to execution of apoptosis, thus suppressing abnormal cell proliferation [365]. For this reason, p53 is called "guardian of the genome" and acts as a tumor suppressor gene.

Within the cell, p53 localizes in the nucleus and its levels are normally kept very low. Once stimulated by agents that cause DNA damage for instance, p53 becomes stable and its protein levels are rapidly increased in the nucleus, along with its half-life. Thus, the regulation of p53 at the protein level is critical for its activation. An important regulator of p53 at the protein level is MDM2, which is actually a p53-responsive gene (Figure 13). When p53 becomes activated, levels of MDM2 increase, which then inactivates p53 by masking the transcriptional activation domain of p53, which promotes p53 ubiquitination and degradation by the proteasome [366-368]. On the other hand, DNA damaging agents can induce the activation of kinases such as ATM, ATR, and DNA-PK, that can phosphorylate a critical serine residue in the MDM2-binding domain of p53, thus preventing the interaction between p53 and MDM2. In addition, p53 is a transcription factor that activates the expression of genes that control the cell cycle [364, 369, 370] and apoptosis [371-373], while it represses transcription of genes involved in stimulation of cell growth but the specific mechanisms of p53 repression are not well understood [374-377].

a)

b)

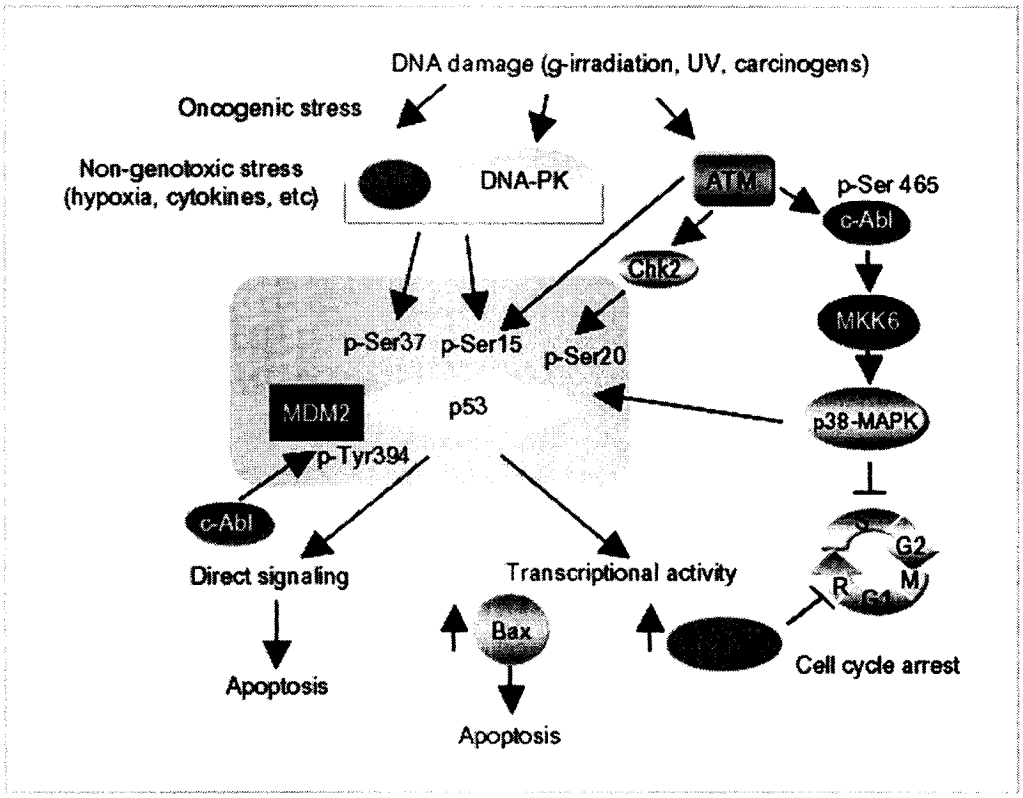

Figure 13. p53 modulates the expression of genes involved in various cellular pathways, regulating cell life and death. a) Structure of the p53 protein. Numbers above the bars are amino acid numbers. b) Signaling pathways that activate p53. Abbreviations: AD, activation domain; Basic, p53 C-terminal basic domain; DNA-binding, sequence-specific DNA-binding domain; NLS, nuclear localization signal; Oligo, oligomerization domain; PXXP, proline-rich domain where P represents proline and X any amino acid. Adapted from May et al. 1999 [365].

In human cancer studies, it was observed that chromosome 17p loss of heterozygosity (LOH) was common in a number of different tumor types, including colorectal, bladder, breast, and lung cancer [378, 379]. A detailed mapping showed that the region 17p13.1 that was lost in colorectal cancers included the p53 gene [380]. Indeed, p53 is the most frequently disrupted gene in cancer; almost 50% of human cancers contain a p53 mutation, including breast, cervix, colon, lung, liver, prostate, bladder, and skin cancers. The majority of somatic mutations in p53 are missense mutations leading to amino acid substitutions in the central portion of the protein [381]. These mutations appear to affect the capacity of p53 to bind to its cognate/related/associated DNA recognition sequence [382]. When a mutation in the p53 gene results in the substitution of one amino acid for another, p53 loses its ability to block abnormal cell growth.

Germ line mutations in the p53 gene have been observed in people affected by the Li-Fraumeni syndrome [383]. These individuals are at risk for the development of a number of tumors, including soft-tissue sarcomas, osteosarcomas, brain tumors, breast cancers, and leukemias [384-386].

1.15.2 The absence of the p53 gene predispose mice to develop tumors

Inactivation of the p53 gene in the germline of mice by gene targeting has resulted in the generation of developmentally viable mice but susceptible to early spontaneous tumors [387-391]. p53 null mice develop tumors by 10 months of age, with an average time to tumor development of 4.5 months, while half of the heterozygous mice develop tumors by 18 months [387]. The type of tumors observed in p53-deficient mice is reported in Figure 14. p53 null mice develop lymphomas, mainly T-cell type, at a very high incidence (70-75%) [387-389, 392, 393]. In contrast, heterozygous mice develop lymphomas, osteosarcomas, and soft tissue sarcomas in

equal proportions [388, 393, 394]. It was also reported that genetic background affects the type and the time of appearance of tumors of p53 deficient mice. 129/Sv p53 null mice develop tumors earlier than their counterparts on mixed background (C57BL/6 X 129/Sv), and about 50% of the male p53 null mice develop testicular teratomas, a tumor type infrequently observed in p53 deficient mice of mixed genetic background [387, 394].

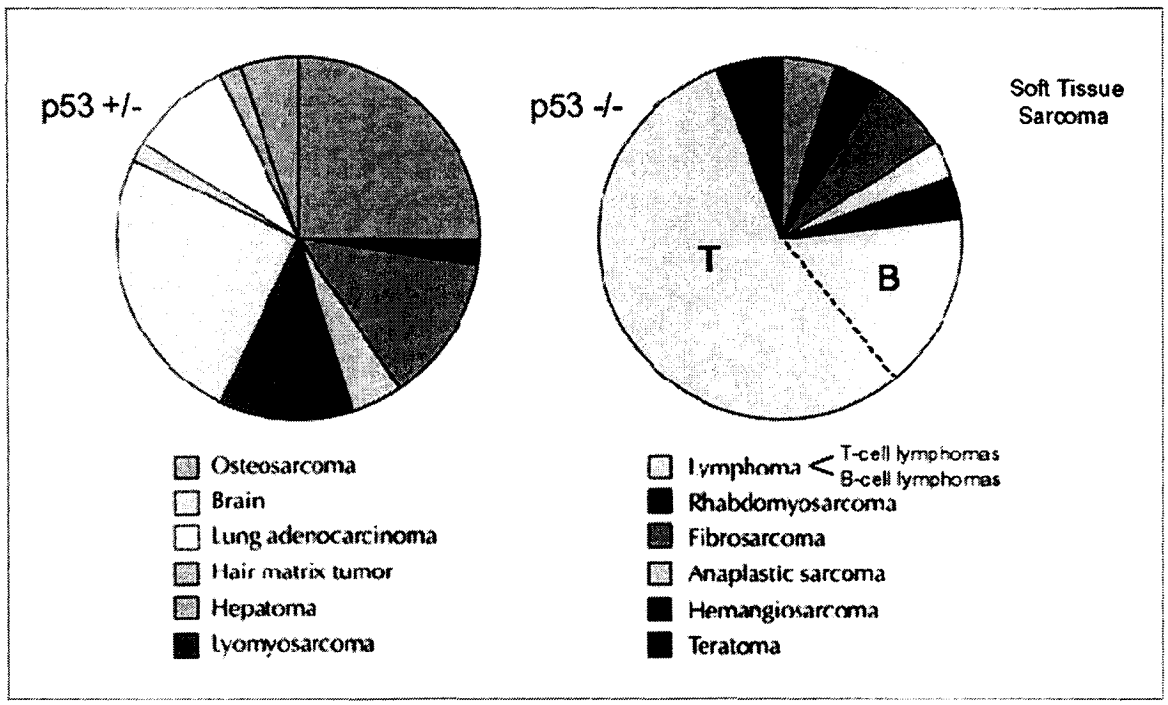

Figure 14. Tumor spectrum analysis of p53-mutant mice. Adapted from Jacks et al. [388].

Given the central role of PTP1B in the negative regulation of oncogenic signaling through the dephosphorylation of protein tyrosine kinases, it is often speculated that inhibition of PTP1B could potentially increase tumorigenesis. However, PTP1B deficient mice do not overtly develop tumors and live as long as their wild-type littermates [161, 162]. As yet, a genetic mouse model to address this question is still lacking. It is of great importance since PTP1B is a valuable target for pharmaceutical industries in the treatment of type 2 diabetes and obesity [395]. Therefore, our laboratory has introduced a PTP1B deficiency in p53 null mice in order to study the role of PTP1B in p53 deficient tumorigenesis, and to determine if the absence of PTP1B can affect tumor onset and/or progression.

1.16 Overview of thesis

To date, PTP1B has been clearly established as a critical physiological regulator of metabolism. In addition, PTP1B is involved in the downregulation of multiple tyrosine kinases signaling pathways such as epidermal growth factor, platelet derived growth factor, insulin, insulin growth factor-1, p210Bcr-Abl, as well as JAK2. The PTP1B null mice are resistant to diet induced diabetes and obesity, and most of the recent studies underlined the role of PTP1B in insulin and leptin signaling pathways. Therefore, using mice lacking the PTP1B gene, the specific objectives of my doctoral research are:

1. To understand the role of PTP1B as a regulator of JAK2 signaling in a metabolic context using the growth hormone signaling pathway;
2. To determine whether PTP1B plays a role in type 1 diabetes by inducing hyperglycemia in PTP1B null mice;
3. To establish how PTP1B regulates Ras signaling downstream of the receptor tyrosine kinases to modulate ERK activity;
4. To develop a mouse model of cancer by studying the role of PTP1B in p53 dependent tumorigenesis.

Therefore, this doctoral thesis focuses on understanding the biological functions of this enzyme in metabolism and oncogenesis, using cell based approach and mouse models. The following chapters constitute the research I have been conducting in the laboratory of Dr. Michel L. Tremblay for the past four years: chapter 2 underlines the role of PTP1B in the regulation of the kinase JAK2 in the context of growth hormone signaling; chapter 3 provides evidence that the absence of PTP1B improves glycemia of type 1 diabetic mice; chapter 4 illustrates how PTP1B regulates Ras signaling

downstream of the RTKs to modulate ERK activity; and chapter 5 describes the role of PTP1B in p53 dependent tumorigenesis. The central role of PTP1B in the regulation of multiple signaling pathways and whole body homeostasis is discussed in chapter 6. To complete this thesis, summary and perspectives are provided.

2 Chapter 2: Protein tyrosine phosphatase-1B attenuates growth hormone-mediated JAK2-STAT signaling

2.1 Abstract

Protein tyrosine phosphatase-1B (PTP-1B) attenuates insulin, PDGF, EGF, and IGF-I signaling by dephosphorylating tyrosine residues located in the tyrosine kinase domain of corresponding receptors. More recently, PTP-1B was shown to modulate the action of cytokine signaling via the non-receptor tyrosine kinase JAK2. Transmission of the growth hormone (GH) signal also depends on JAK2, raising the possibility that PTP-1B modulates GH action. Consistent with this hypothesis, GH increased the abundance of tyrosine-phosphorylated JAK2 associated with a catalytically inactive mutant of PTP-1B. GH-induced JAK2 phosphorylation was greater in knockout (KO) than in wild type (WT) PTP-1B embryonic fibroblasts, and resulted in increased tyrosine phosphorylation of STAT3 and STAT5, while over-expression of PTP-1B reduced the GH mediated activation of acid labile subunit (ALS) gene. To evaluate the *in vivo* relevance of these observations, mice were injected with GH under fed and fasted conditions. As expected, tyrosine phosphorylation of JAK2 and STAT5, occurred readily in the liver of fed WT mice, and was almost completely abolished during fasting. In contrast, resistance to the action of GH was severely impaired in the liver of fasted KO mice. These results indicate that PTP-1B regulates GH signaling by reducing the extent of JAK2 phosphorylation, and suggest that PTP-1B is essential for limiting the action of GH during metabolic stress such as fasting.

2.2 Introduction

Postnatal growth is profoundly reduced in mice and humans suffering from a deficit in growth hormone (GH) signaling [313, 396]. GH promotes growth by acting directly on tissues such as liver and bone, and by stimulating the synthesis of IGF-I, a growth factor required for complete postnatal development [314, 315, 397]. In addition, GH is a major metabolic hormone, with effects on glucose, protein and lipid metabolism [313, 397]. As a consequence, secondary disorders often occur in individuals suffering from abnormal GH secretion. For example, excess plasma GH leads to the development of insulin resistance whereas a deficit is associated with increased adiposity [313, 398]. In mice, GH is involved in the development of complications associated with diabetes such as retinal neovascularization and nephropathy [313, 317].

Molecular events involved in the transmission of the GH signal have received considerable attention in recent years. Primary events include homodimerization of the growth hormone receptor (GHR), recruitment of the tyrosine kinase JAK2 to the cytoplasmic domain of the receptor and activation of JAK2 by autophosphorylation [317, 399]. A variety of signaling proteins are then recruited to high affinity binding sites on tyrosine phosphorylated JAK2 and GHR, leading to the activation of signal transducers and activators of transcription (STATs) 1, 3 and 5b, the Ras-MAP (mitogen-activated protein) kinase pathway, and the insulin receptor substrate 1 (IRS-1)/phosphatidylinositol (PI) 3'-kinase/AKT pathway [317, 397]. Equally important, but less understood, are the mechanisms limiting and terminating GHR signaling. These include internalization and degradation of the GHR, inhibition of signaling by negative regulators such as supressors of cytokine signaling (SOCS) and protein inhibitor of activated STAT (PIAS), and inactivation of JAK2 and downstream signaling molecules by dephosphorylation [316, 317, 324].

Given the central role of JAK2 in initiating GH signaling, identification of the phosphatases involved in its deactivation is important. Candidates that have been shown to be involved include the cytosolic tyrosine phosphatases SHP-1 and SHP-2 [326, 400, 401], and the transmembrane tyrosine phosphatase CD45 [30]. Recently, the ubiquitously expressed protein tyrosine phosphatase-1B (PTP-1B) was shown to bind phosphorylated JAK2 in leptin and interferon-γ (INF-γ) treated-cells [163, 164, 295]. We now show that PTP-1B interacts with JAK2 in a GH-dependent manner, and dephosphorylates tyrosine residues present in the activation loop of JAK2. Absence of PTP-1B results in GH-dependent hyperphosphorylation of JAK2, and enhanced activation of STAT3 and STAT5, while over-expression of PTP-1B reduced GH mediated activation of a STAT5-dependent gene. PTP-1B modulation of GH signaling is physiologically relevant as shown by loss of GH resistance in the liver of fasted null PTP-1B mice.

2.3 Material and Methods

Reagents

Reagents were from Fisher Biotech unless otherwise mentioned. Rabbit polyclonal antibodies against PTP-1B were described previously [161]. Antibodies against the extracellular domain of the rat GHR were a gift of Dr. W. Baumbach (American Cyanamid, Princeton, NJ). Other antibodies were purchased from Upstate Biotechnology (JAK2), Biosource International (JAK2 $pYpY^{1007/1008}$), Cell Signaling (STAT3, STAT3 pY^{705} and STAT5 pY^{694}) and Santa Cruz Biotechnology (GST, JAK1, JAK3, and STAT5b). Horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse secondary antibodies were from Jackson Immunoresearch. Human GH (hGH) was provided by the National Hormone & Pituitary Program (Torrance, CA).

Substrate-trapping

293 cells stably expressing JAK2 (293LA [402]) were transfected with 5 μg of the empty GST vector pEBG (GST) or with pEBG expressing WT PTP-1B (GST-PTP-1B WT) or D181A PTP-1B (GST-PTP-1B D181A). D181A is a mutant with normal binding capacity but reduced catalytic activity (described in [145]). Transfections were performed using lipofectamine (Invitrogen, Life Technologies). Thirty-six hours after transfection, cells were starved overnight in serum free media and then stimulated with 1 μg/ml hGH for 10 minutes. Cells were lysed in TNE buffer (150mM NaCl, 50mM Tris-HCl and 1% NP-40) supplemented with an EDTA-free cocktail of protease inhibitor (Complete™, Roche Molecular Biochemicals). Protein concentrations of the lysates were determined and GST-tagged proteins were retrieved using 20 ul of glutathione-Sepharose beads (Pharmacia Biotech). Beads were washed in lysis buffer, boiled in 2X SDS sample buffer, resolved by SDS-PAGE and analyzed by immunoblotting.

Cells and culture conditions

Spontaneously immortalized murine embryonic fibroblasts (MEFs) lacking PTP-1B or T-cell protein tyrosine phosphatase (TC-PTP) were previously described [305, 403]. For rescue experiment, PTP-1B KO MEFs were infected with a retrovirus vector encoding myc-tagged murine PTP-1B, and stable transfectants were selected with hygromycin. Primary WT and PTP-1B KO fibroblasts (PMEFs) were isolated from E14 embryos. H4-II-E cells were established from rat liver hepatoma [404]. All cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (5 units/ml penicillin and 5 μg/ml streptomycin).

Cell stimulation, preparation of cell lysate and immunoblotting

To study the effect of GH, confluent cells were incubated overnight in serum-free media. Thirty min prior to stimulation, media was changed to fresh serum-free media

followed by treatment with 100 ng/ml hGH. At indicated times, cells were washed with ice-cold PBS and lysed in M-RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40, 0.25% Na deoxycholate) supplemented with 1mM sodium vanadate, 50 mM NaF, and Complete™ protease inhibitors. Cell lysates were cleared by centrifugation and the protein concentration of all lysates was measured by the Bradford method. After SDS-PAGE, proteins were transferred to PVDF membranes (Immobilon, Millipore). Membranes were blocked in 1% BSA when using anti-phosphotyrosine antibodies and in 5% milk for all other antibodies. HRP conjugated anti-mouse or anti-rabbit was used as secondary antibodies and Enhanced Chemi-Luminescence (ECL) was used to develop the blot. Signals were quantified by densitometric scanning and quantified with NIH image software.

Cell fractionation

WT and KO PTP-1B MEFs were treated with 100 ng/ml hGH for 10 minutes. Cells were washed on ice with cold PBS, scraped in homogenization buffer (3 mM imidazole, 8.5% sucrose) and homogenized by extrusion though a 22G needle. Nuclei and unbroken cells were removed by low speed centrifugation. The resulting supernatant was ultra-centrifuged (100,000 g for 15 minutes) for isolation of cytosolic and total membrane proteins.

Transfection of rat liver cells

Transfections of H4-II-E cells were performed exactly as described previously [405]. Briefly, each well of near confluent monolayers were exposed to 100 µl of a DNA solution (0.5 mg/ml DEAE-dextran, 0.7 µg of the firefly luciferase plasmid mALS703WT, 0.3 µg of plasmid pRL-TK and 1.5 µg of expression vector). m703ALSWT was constructed by inserting the GH-responsive mouse ALS promoter in the luciferase plasmids pGL3-basic. Expression vectors used were pcDNA 3.1 or

expression vectors encoding mouse PTP-1B (pcDNA3.1/PTP-1B, J. Wagner & M. Tremblay, unpublished), mouse TC-PTP (pcDNA4/TC-PTP, D. Simoncic & M. Tremblay, unpublished) or mouse PTP-PEST (pACTAG-2-mPTP-PEST [406]). The plasmid pRL-TK (Promega) encodes renilla luciferase and was used to correct for variation in transfection efficiency. Plasmids were purified by ion-exchange chromatography (Qiagen, Chatsworth, CA). After a 40 h recovery period in DMEM supplemented with 10 % FCS, media were changed to serum-free DMEM supplemented with or without 100 ng/ml of bGH. Twenty hours later, cell lysates were assayed for firefly and renilla luciferase by the Dual-Luciferase Reporter System (Promega).

Liver hGH stimulation

Male WT or PTP-1B KO mice are a hybrid of 129S/v and Balb/c backgrounds [161], backcrossed into a Balb/c background for three generations. At 9 or 10 weeks, they were offered ad libidum levels of a standard diet or fasted for 48 hours. After anesthesia, mice were administered saline or hGH (0.5 μg / g of body weight via the *vena cava*). Liver was removed at indicated times after injection and homogenized in M-RIPA buffer. Equal amount of lysate was resolved by SDS-PAGE and immublotting. For RNA extraction and Northern analysis, WT and KO mice were fasted for 48 hours and were administered saline or hGH (1 ug / g of body weight) intraperitoneally. Liver was collected before (0 time) and after injection (30, 60 and 90 min).

Northern analysis

Total RNA was prepared from rat liver cells by the acid guanidium thiocyanate phenol-chloroform method, and quantified by absorbance at 260 nm [405]. Total RNA (15 μg/lane) was electrophoresed on a 1.2 % agarose/formaldehyde gel, blotted onto a nylon membrane and hybridized to [^{32}P]dCTP labeled DNA probes. Probes used

included the coding region of mouse SOCS2, SOCS3 and CIS cDNA (obtained from Drs Hilton and Starr, [407]). Staining with ethidium bromide confirmed that ribosomal RNA was intact and that equal amounts of RNA were loaded in each lane.

Statistical analysis

Unless mentioned, results are reported as mean ± SEM of at least three separate experiments. Statistical analyses were performed using a two-tailed, unpaired, Student's *t*-test.

2.4 Results

2.4.1 JAK2 is a substrate of PTP-1B upon GH stimulation

Leptin and interferon-γ (IFN-γ) have recently been shown to promote the association of JAK2 with PTP-1B [163, 164, 295]. To determine whether GH could promote a similar interaction, substrate trapping was used [167]. 293LA cells were transfected with expression vectors encoding wild type PTP-1B (GST-PTP-1B WT), a catalytically inactive mutant (GST-PTP-1B D181A) or the corresponding empty vector (GST). After cell lysis, GST proteins were collected on glutathione beads, separated by SDS-PAGE and immunoblotted with an antibody specific for phosphorylated tyrosine residues responsible for activating the kinase domain of JAK2 (anti-JAK2pYpY$^{1007/1008}$). Under basal conditions, low levels of phosphorylated JAK2 were detected with GST-PTP-1B D181A perhaps due to residual level of phosphorylated JAK2 after serum starvation. More importantly, the amount of phosphorylated JAK2 associated with GST-PTP-1B D181A was increased to about 2-fold when cells were incubated for 10 min in the presence of GH (Fig 1A). A total of ~5% of phosphorylated JAK2 was found to be associated with PTP-1B D181A after GH stimulation. A smaller increase was also

visible when the blot was reprobed with anti-JAK2 antibody (Fig. 1B). As expected, no JAK2 was detected with GST-PTP-1B WT, reflecting the transient nature of interaction between catalytically-active PTP-1B and its substrates. In selected cell systems, GH has also been shown to weakly activate JAK1 or JAK3 [408, 409]. These kinases were also present in 293 cells, but were not detected in materials precipitated with the PTP1B mutant. Similarly, other components of the GH signaling cascade (GHR, STAT3 and STAT5) were also present in the cells, but were not trapped by the PTP-1B D181A mutant (data not shown). Overall, these results confirm that phosphorylated JAK2 binds to PTP-1B, and suggest a role for PTP-1B in GH signaling.

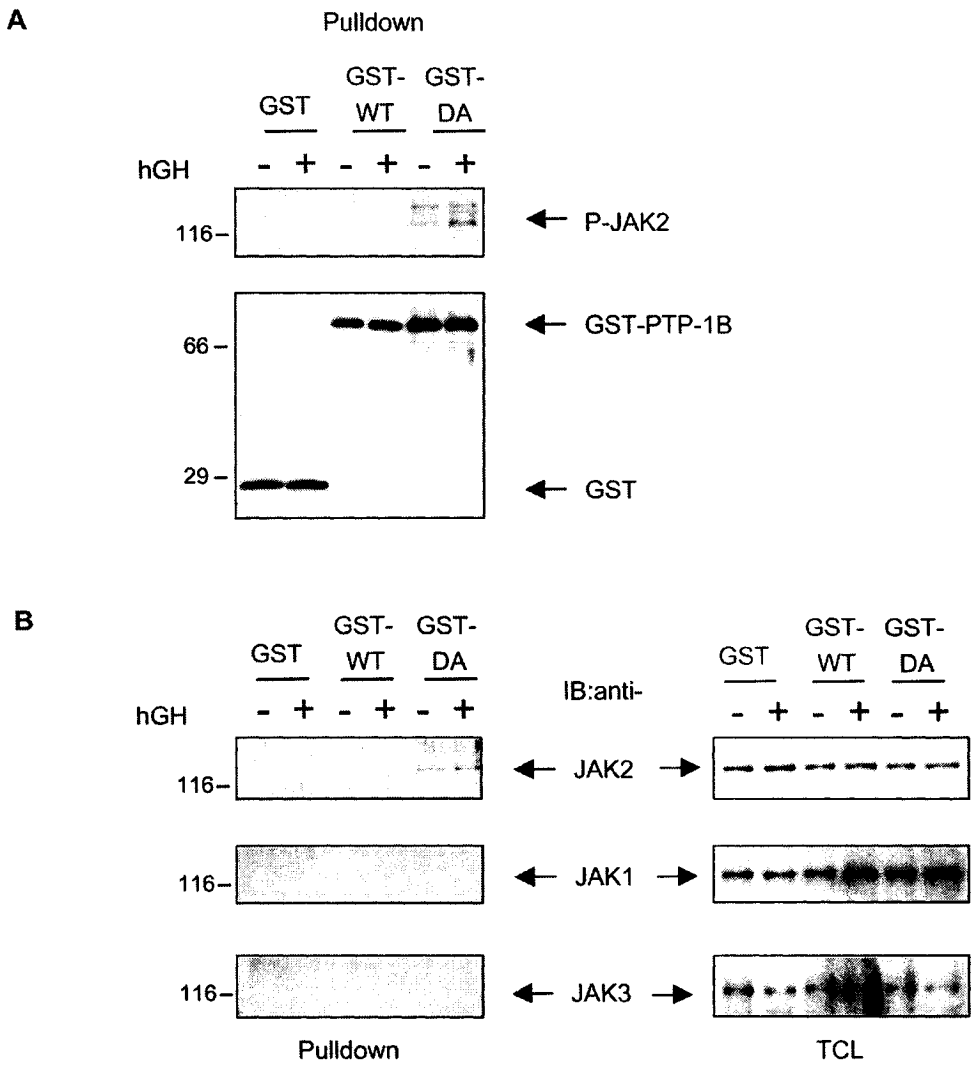

Figure 1. PTP-1B D181A binds JAK2 in hGH stimulated cells. A) 293LA cells were transiently transfected with GST vector (GST), GST-PTP-1B WT (GST-WT) and GST-PTP-1B D181A (GST-DA). Cells were incubated in the absence (-) or presence (+) of 1 μg/ml hGH for 10 min. Proteins associated with Glutathione Sepharose (GST) beads were removed by centrifugation (Pulldown). Precipitated proteins were resolved by SDS-PAGE and immunoblotted with antibodies against $pY^{1007/1008}$JAK2 (P-JAK2). Immunoblotting with the anti-GST antibody demonstrates that comparable amounts of GST containing proteins were pulled down from cells transfected with GST-PTP-1B WT and GST-PTP-1B D181A. B) Precipitated materials (Pulldown) and total cell lysates (TCL, 10 μg) were resolved by SDS-PAGE and immunoblotted with antibodies against JAK2, JAK1 and JAK3. Results shown are representative of three independent experiments.

2.4.2 GH-treated fibroblasts lacking PTP-1B have increased JAK2 phosphorylation

To determine whether JAK2 phosphorylation is altered in the absence of PTP-1B, we used spontaneously immortalized MEFs isolated from WT and PTP-1B KO mouse embryos. Cells were incubated with 100 ng/ml of hGH for various times and lysates were analyzed by SDS-PAGE and immunoblotting. As shown in Figure 2A, JAK2 phosphorylation peaked within 5 min of hGH addition in both WT and KO PTP-1B MEFs. After 5 min of hGH exposure, however, JAK2 phosphorylation, was 63% higher in KO than in WT MEFs, and remained near maximal after 10 minutes. JAK2 phosphorylation returned to basal levels in both cell lines after 60 minutes. Abundance of JAK2 and GHR did not differ significantly at any point between WT and KO PTP-1B MEFs. These results suggest that PTP-1B attenuates the ability of GH to activate JAK2, but that JAK2 dephosphorylation is not solely dependent on PTP-1B.

To confirm that GH-mediated JAK2 hyperphosphorylation was caused by the absence of PTP-1B, Myc-tagged PTP-1B was re-expressed into PTP-1B KO MEFs using retroviral infection ("rescued"). Both WT and "rescued" MEFs were stimulated with hGH, and cell lysates were analyzed for JAK2 phosphorylation. Rescued cells had lower levels of PTP-1B than WT cells, but similar levels of JAK2 and GHR. Nevertheless, this level of PTP-1B expression was sufficient to restore the profile of JAK2 phosphorylation in response to GH to that seen in WT cells (Fig. 2B).

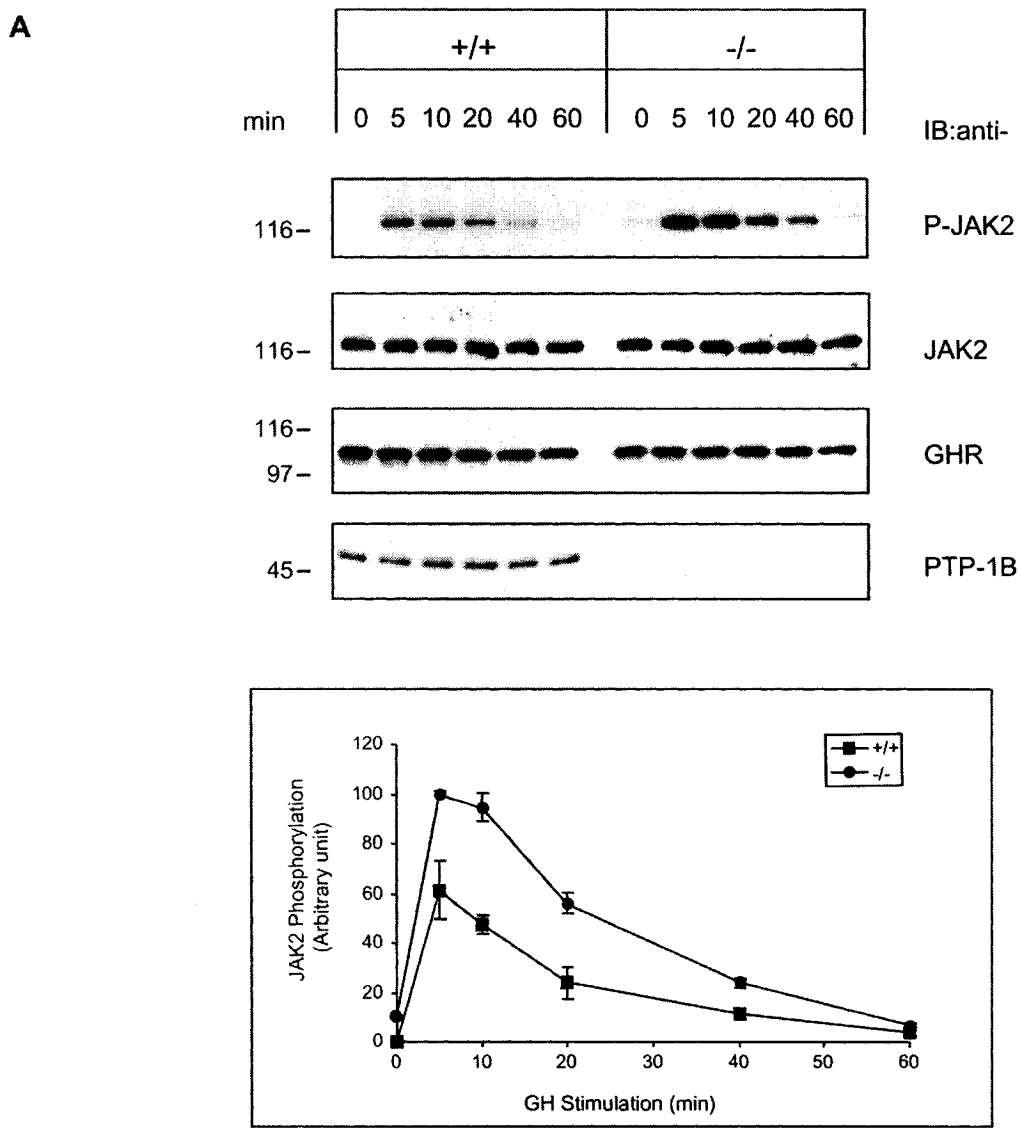

Figure 2. Hyperphosphorylation of JAK2 in PTP-1B deficient fibroblasts stimulated with hGH. A) WT (+/+) and PTP-1B KO (-/-) MEFs were stimulated with hGH (100 ng/ml) for indicated times. Total cell lysates (20 μg) were subjected to SDS-PAGE and immunoblotted using antibodies detecting specifically total JAK2, GHR and PTP1B, or tyrosine phosphorylated JAK2 (P-JAK2). P- JAK2 signals were quantified by densitometry and are reported as mean ± SEM of three independent experiments.

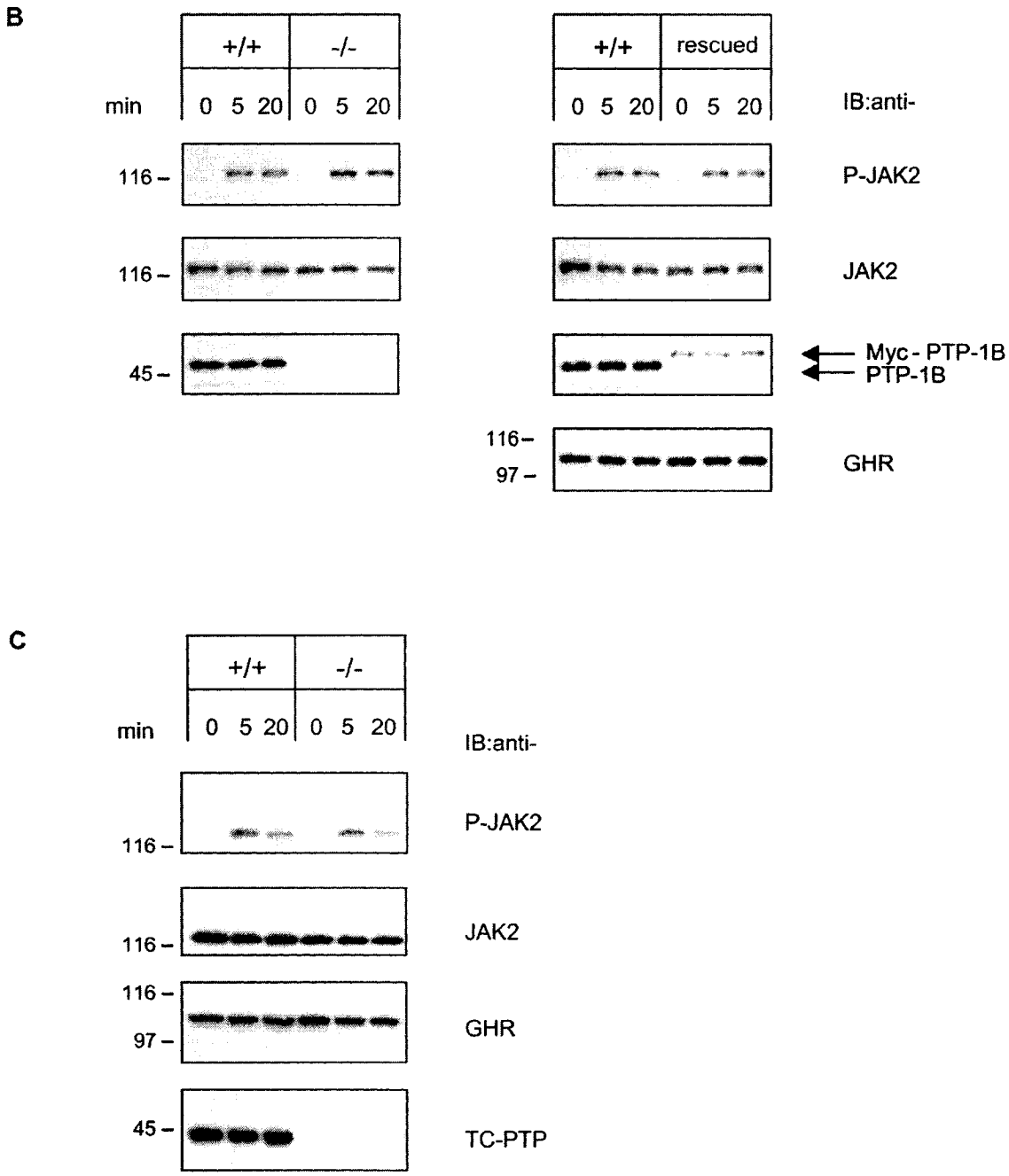

Figure 2. Hyperphosphorylation of JAK2 in PTP-1B deficient fibroblasts stimulated with hGH. Cont'd.

B) WT (+/+), PTP-1B KO (-/-) MEFs and PTP-1B KO MEFs stably expressing Myc-tagged PTP-1B (rescued) were stimulated for 5 and 20 min with 100 ng/ml hGH. Analysis of cell lysates by SDS-PAGE and immunoblotting were performed as described in A. Similar results were obtained with other "rescued" cell lines. C) WT (+/+) and TC-PTP KO (-/-) MEFs were stimulated for 5 and 20 min with 100 ng/ml hGH. Analysis of cell lysates by SDS-PAGE and immunoblotting were as described in A, with the additional detection of TC-PTP using a specific antibody. Results shown are representative of three independent experiments.

To further assess the specificity of PTP-1B in GH/JAK2 signaling, we asked whether the absence of the closely related phosphatase TC-PTP would also increase GH-mediated JAK2 phosphorylation. WT and KO TC-PTP MEFs [144, 403] were treated with hGH as before. Immunoblot analysis showed that WT and KO TC-PTP MEFs expressed the same amounts of JAK2 and GHR. More importantly, after hGH treatment, levels of JAK2 phosphorylation in KO TC-PTP MEFs were undistinguishable from those of WT TC-PTP MEFs (Fig. 2C). Overall, these results indicate that PTP-1B, but not TC-PTP attenuates GH-mediated JAK2 phosphorylation.

2.4.3 GH-mediated interactions between PTP-1B and JAK2 occur in the membrane compartment

The C-terminus of PTP-1B contains a 35 amino acids hydrophobic stretch that anchors PTP-1B to the endoplasmic reticulum (ER) [218]. Unlike PTP-1B, JAK2 is located predominantly in the cytosol and associates with the GHR in a ligand dependent manner [399]. A portion of JAK2 has also been reported to be associated with the membranes of endoplasmic reticulum (ER) [410]. Therefore, we sought to identify the cellular compartment where GH-induced interactions between JAK2 and PTP-1B occurred. WT and KO PTP-1B MEFs were stimulated with 100 ng/ml hGH for 10 minutes. Cells were homogenized in an isotonic buffer and post-nuclear supernatant was separated by ultracentrifugation into cytosol and membrane fractions, and aliquots of each fraction (total homogenate, 10%; cytosol, 20%; membrane, 50%) were analyzed by SDS-PAGE and immunoblotting. Proper fractionation was confirmed by exclusive localization of PTP-1B and insulin receptor in the membrane compartment, and predominant localization of JAK2 in the cytosol (~70% of total JAK2) (Fig 3).

As shown above, GH induced a higher level of JAK2 phosphorylation in KO than in WT PTP-1B MEFs (Fig 2). In both cell types, GH-induced-phosphorylation of JAK2 was restricted to the membrane, a compartment containing only ~ 30% of total JAK2 (Fig 3). As a consequence, JAK2 hyperphosphorylation in null PTP-1B MEFs was greater for membrane than for total JAK2. We conclude that the modulation of GH-induced JAK2 phosphorylation by PTP-1B is restricted to the membrane compartment.

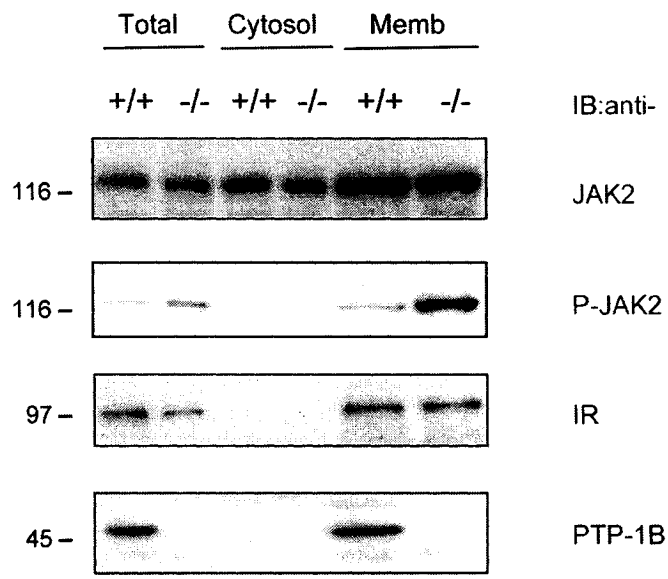

Figure 3. PTP-1B dephosphorylates membrane-associated JAK2. WT (+/+) and PTP-1B KO (-/-) MEFs were stimulated with 100 ng/ml hGH for 10 min. Cell homogenates from a single 10-cm dish (total) were separated in cytosolic (Cytosol), and membrane fractions (Memb). Aliquots of each fraction (Total, 10 %; Cytosol, 20%; Membrane, 50%) were analysed by SDS-PAGE and immunoblotting using specific antibodies against $pY^{1007/1008}$JAK2 (P-JAK2), JAK2, Insulin Receptor (IR) and PTP-1B. IR presence was measured as a control for membrane fractionation. Results shown are representative of three independent experiments.

2.4.4 Enhanced GH signaling in PTP-1B deficient primary fibroblasts

Signaling events following JAK2 phosphorylation include phosphorylation of specific tyrosine residues in the GHR and in STAT1, 3 and 5 [317, 397]. After hGH treatment, increased JAK2 phosphorylation in KO PTP-1B MEFs was associated with increased tyrosine phosphorylation of GHR, STAT3 and STAT5 (not shown). However, these cells also had higher expression of STAT3 and STAT5 than WT cells, complicating the interpretation of these results.

To better answer this question, we derived primary fibroblasts from WT and PTB-1B KO mouse embryos (PMEFs). Unlike MEFs, WT and PTP-1B KO PMEFs had identical levels of STAT3 and STAT5 (Fig 4). Primary cells were stimulated with 100 ng/ml of hGH and lysates were analyzed for JAK2, STAT3 and STAT5 phosphorylation. Absence of PTP-1B resulted in a significant increase in JAK2 phosphorylation during the first 20 minutes of hGH stimulation. JAK2 hyperphosphorylation increased tyrosine phosphorylated STAT3 after 5min, and tyrosine phosphorylated STAT5 over the entire 20 min treatment period (Fig 4). These results indicate that, by reducing the GH-dependent phosphorylation of JAK2, PTP-1B limits the activation of downstream components of the GH signaling pathway.

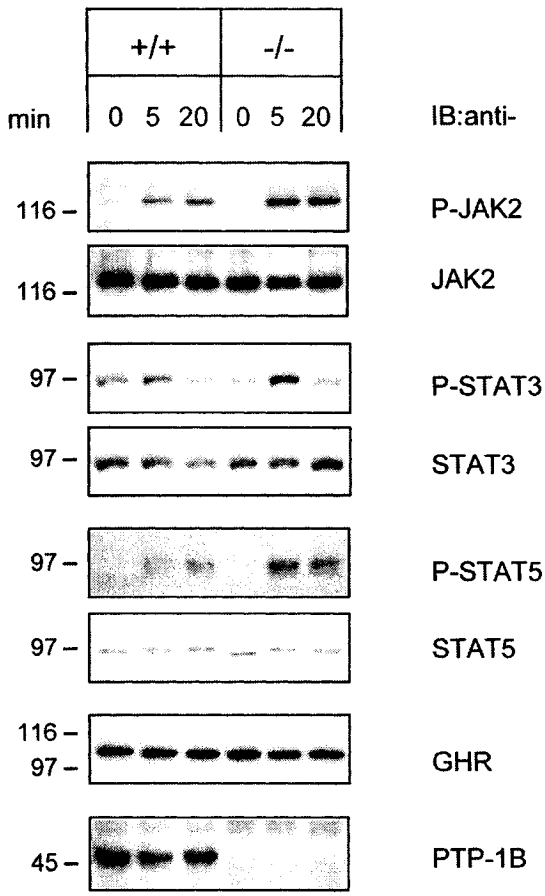

Figure 4. JAK2, STAT3, and STAT5 are hyperphosphorylated in PTP-1B deficient primary fibroblasts upon hGH stimulation. Passage four WT (+/+) and PTP-1B KO (-/-) primary fibroblasts (PMEFs) were stimulated for 5 and 20 min with hGH (100 ng/ml). Cells lysates (20 μg) were analyzed by SDS-PAGE and immunoblotting using specific antibodies against total JAK2, STAT3 and STAT5 or their tyrosine phosphorylated forms (P-JAK2, P-STAT3 and P-STAT5). Levels of the GHR and PTP-1B were also determined using specific antibodies. Results shown are representative of three independent experiments.

2.4.5 Over-expression of PTP-1B reduces GH mediated transcription in liver cells

Next, we asked whether PTP-1B could modulate GH-regulated transcription. For these studies, we used the H4-II-cells transiently transfected with mALS703WT, a luciferase construct under the control of the GH-dependent mouse ALS promoter. As shown in Fig 5, GH activates ALS promoter activity 2.8-fold in this system, an effect that we showed to be completely dependent on the binding of STAT5 to a single γ-interferon activated sequence (GAS) [411]. Over-expression of PTP-1B attenuated this stimulation by 30% ($P < 0.05$) whereas the related tyrosine phosphatases TC-PTP and PTP-PEST had no effects. These results suggest that the attenuation of GH signaling by PTP-1B is functionally significant.

2.4.6 Fasted PTP-1B knockout mice have decreased hepatic GH resistance

Results in fibroblasts and rat liver cells indicate that PTP-1B plays a negative role in GH signaling. To determine whether PTP-1B played a similar role *in vivo,* we focused on liver, a major GH target tissue in postnatal animal [314, 412]. Liver was obtained from fed WT and PTP-1B KO mice 5 or 30 min after hGH administration, and analyzed by immunoblotting with phosphospecific antibodies. As shown in Figure 6, under the fed condition, GH administration increased the amount of phosphorylated JAK2 to a similar extent in WT and KO mice, at both 5 and 30 min. STAT3 phoshporylation did not change after hGH administration whereas phosphorylation of STAT5 was slightly higher in KO than in WT liver after 5 min, but similar after 30 min. These results suggest that, under conditions associated with normal GH

responsiveness, PTP-1B has little impact on the ability of GH to activate signaling in liver.

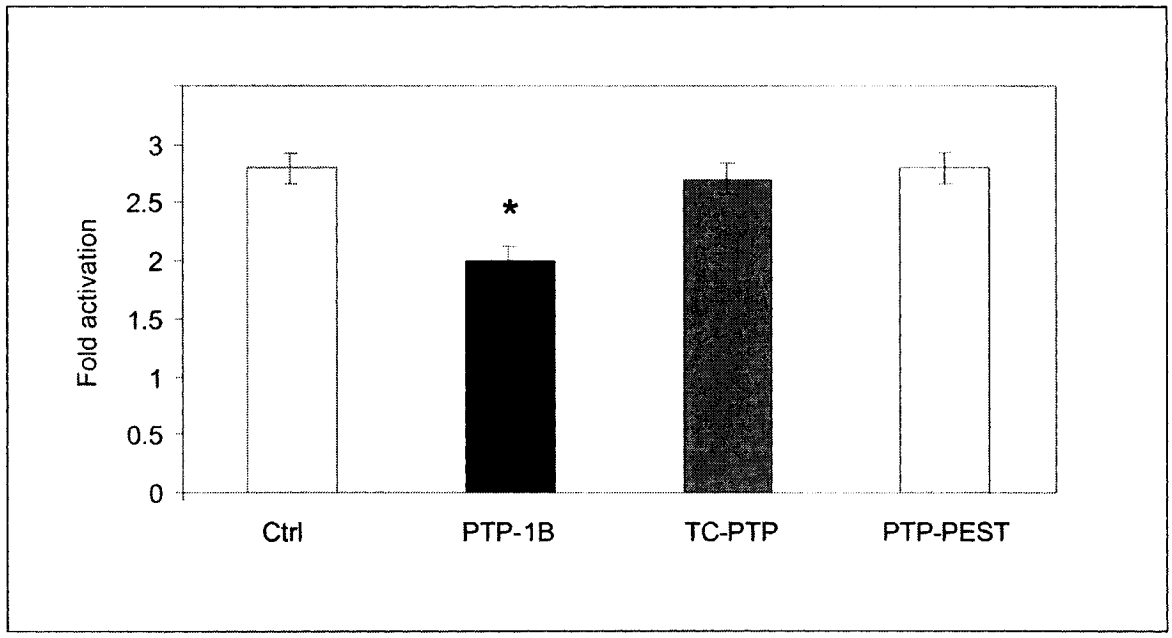

Figure 5. Overexpression of PTP-1B reduces the ability of GH to increase ALS promoter activity in H4-II-E cells. The luciferase plasmid mALS703WT driven by the mouse ALS promoter was transfected in H4-II-E cells with either the an empty expression vector (Ctrl), or with expression vectors encoding PTP-1B, TC-PTP or PTP-PEST. The plasmid pRL-TK encoding for renilla luciferase was used to correct for variation in transfection efficiency. Transfected cells were incubated in serum-free medium in the absence or in the presence of 100 ng/ml of GH. After 24 h, firefly luciferase activity were measured in cell extracts and corrected for renilla luciferase activity. The fold stimulation (mean ± se of 2 experiments) was calculated as the ratio of luciferase activity in the presence and in the absence of GH. Bar with star (*) differs at $P < 0.05$ using one-way ANOVA followed by Fisher Protected Least Significant

Next, we examined whether the same situation prevailed during fasting when a state of GH-resistance develops. In the rat, this state is characterized by reduced ability of GH to induce tyrosine phosphorylation of JAK2, GHR, and STAT5 despite the unchanged abundance of these proteins [413]. Consistent with these findings, JAK2 phosphorylation was reduced throughout the study period in the liver of WT mice

fasted for 48 hours (Fig 6). For example, 30 min after GH administration, the ratio of phosphorylated to total JAK2 (pJAK2/total JAK2) in fasted liver was only 50% of that observed in fed liver (1.01 vs 2.01 arbitrary units, lane 10 vs lane 3). Hepatic levels of PTP-1B were reduced by fasting, whereas levels of the closely related tyrosine phosphatase TC-PTP, remained unchanged.

In KO PTP-1B mice, however, GH-induced JAK2 phosphorylation was as efficient in fasted as in fed liver. Using the 30 min time point for comparison, the ratios of phosphorylated to total JAK2 were similar in fasted and fed liver (2.12 vs 1.95 arbitrary units, lane 14 vs lane 6). Increased JAK2 phosphorylation in fasted KO PTP-1B mice led to improved activation of STAT5 over the first 10 min following GH administration (P-STAT5, lanes 12-13 vs 8-9), and after 30 min, to a dramatic activation of STAT3 (P-STAT3, lanes 14 vs 10). Overall, these results suggest that in conditions where GH is decreased such as fasting, levels of PTP-1B are reduced, reflecting perhaps a lesser need for GH signal attenuation. Complete absence of PTP-1B, however, impairs the development of hepatic GH resistance.

Next, we examined whether expression of SOCS were altered. These proteins have been shown to act as a rapid negative feedback in response to GH [324]. Thirty min after GH injection, expression of CIS and SOCS3 were increased very modestly and similarly in both WT and KO mice (Fig 7). In contrast, SOCS-2 expression was increased to a greater extent 90 minutes after GH administration in KO than in WT mice. This suggests that impaired GH resistance in fasted KO mice has functional consequences, such as a compensatory increase in the expression of other negative modulators of GH signaling such as SOCS2.

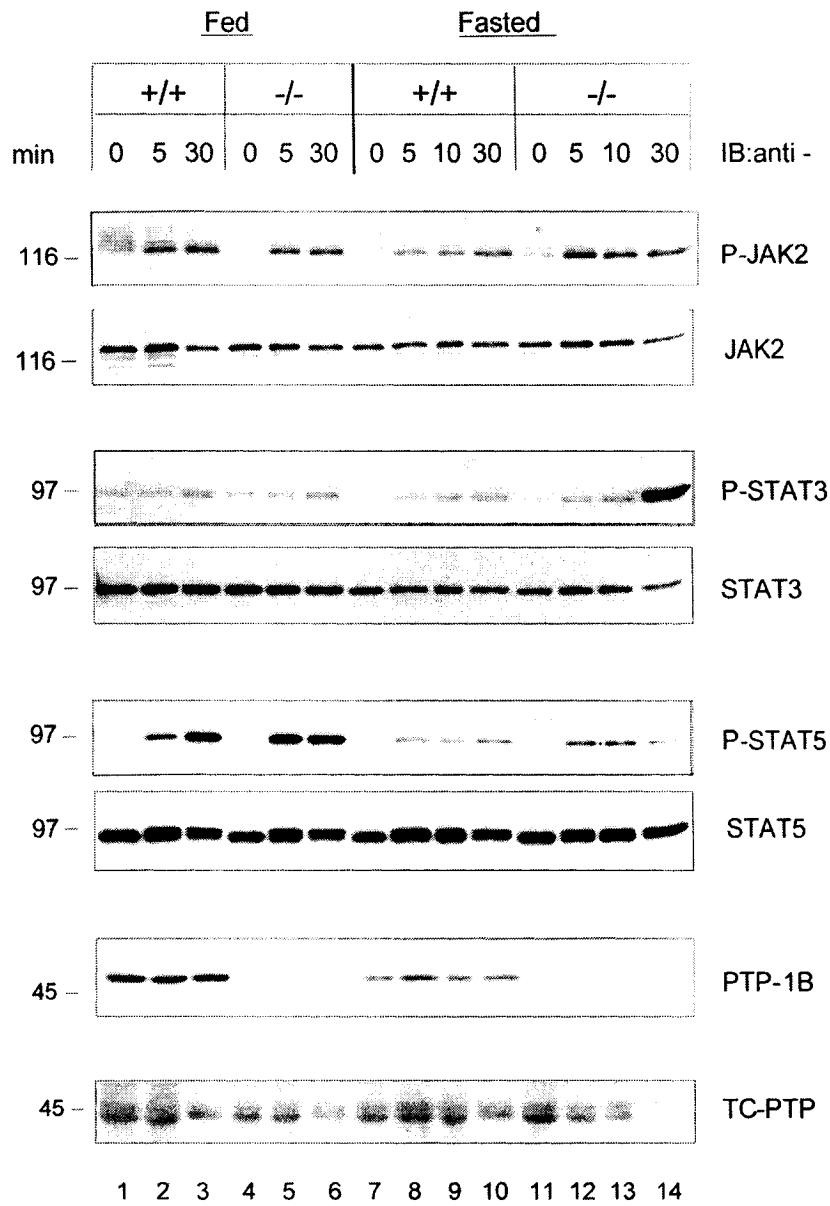

Figure 6. Fasting-induced GH resistance is impaired in the liver of PTP-1B KO mice. PTP-1B WT (+/+) and PTP-1B KO (-/-) male mice were injected with PBS or hGH (0.5 μg hGH / g body weight) when fed or after a 48 hours period of fasting. At indicated time points after hGH injection, liver was collected and extracts were prepared. Liver extracts were analyzed by SDS-PAGE and immunoblotting using specific antibodies against total JAK2, STAT3, STAT5 or their tyrosine phosphorylated forms (P-JAK2, P-STAT3 and P-STAT5), as well as PTP-1B and TC-PTP. Results shown are representative of three independent experiments.

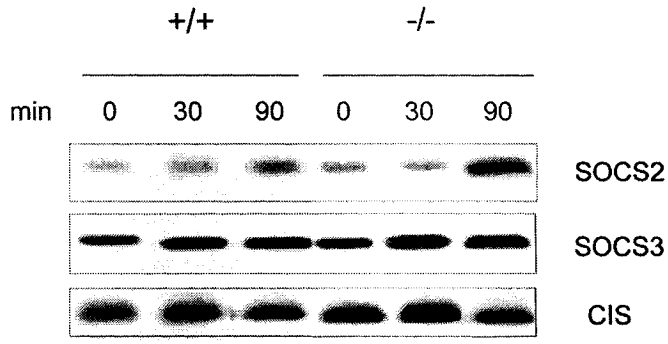

Figure 7. GH induced a higher expression of SOCS2 in fasted PTP-1B KO mouse liver. WT and PTP-1B KO mice were fasted for 48 h followed by hGH administration (1 ug/g body weight). Liver was obtained from individuals before (time 0) and after GH administration (30 and 90 min). Total RNA was isolated and analyzed by Northern blotting for the abundance of SOCS mRNA using the corresponding mouse cDNA probes. Signals detected with the various probes corresponded to a mRNA of 2.5 kb for CIS, 3.4 kb for SOCS2, and 3.2 kb for SOCS3. Each lane represents RNA from one mouse.

2.5 Discussion

Genetic and biochemical evidence that PTP-1B is a critical regulator of metabolism has emerged in recent years. Null PTP-1B mice are hypersensitive to the action of insulin and are resistant to diet-induced obesity [161, 162]. Moreover, mice lacking both leptin and PTP-1B are leaner than their leptin deficient counterpart, and are more sensitive to the anorexic and weight-reducing effects of exogenous leptin [163, 164]. PTP-1B exerts these actions by dephosphorylating tyrosine residues involved in recruiting and activating signaling molecules [14, 221]. In this paper, we demonstrate that PTP-1B also modulates the action of GH, another important metabolic hormone.

Our data indicate that PTP-1B is recruited to JAK2 in response to GH, and is consistent with a model whereby PTP-1B limits ligand-dependent signaling by dephosphorylating tyrosine residues located in the activation loop of JAK2. PTP-1B is

obviously not the only PTP capable of acting on JAK2, as shown by similar dephosphorylation kinetics of pY1007 and 1008 on JAK2 in WT and null PTP-1B fibroblasts treated with GH. Other tyrosine phosphatases shown to be involved in JAK2 dephosphorylation in GH-treated cells include SHP-1, a PTP expressed predominantly in tissues and cells of hematopoietic origin [325], and the widely expressed SHP-2 [326, 401]. In the present work, we also considered the possibility that TC-PTP, a phosphatase highly homologous to PTP-1B, could be involved. Our functional data showed that TC-PTP is not involved in JAK2 dephosphorylation in GH-treated fibroblasts, an observation consistent with the ability of TC-PTP to trap JAK1 and JAK3, but not JAK2 [145]. A role for TC-PTP in GH-signaling remains possible because we recently demonstrated that its nuclear isoform dephosphorylates STAT1 and STAT3 [414]. In contrast to Aoki et al. [415], however, we could not identify STAT5 as a physiological target of TC-PTP [414]. This finding is corroborated in the present study by inability of over-expressed TC-PTP to reduce a GH action dependent on STAT5 activation (i.e., stimulation of the mouse ALS promoter in H4-II-E cells [411]. Finally, the transmembrane tyrosine phosphatase CD45 can dephosphorylate all members of the JAK family, but any role of CD45 in GH signaling would be limited by its near exclusive expression in hematopoietic tissues [30]. Although PTP-1B is not unique in its ability to dephosphorylate JAK2, its widespread expression suggests that it could modulate the action of GH in many target tissues.

Our data do not preclude the possibility that PTP-1B dephosphorylates other components of the GH signaling pathway, such as the GHR, STAT3 and STAT5. In this context, Aoki et al. have suggested that STAT5a and STAT5b are PTP-1B substrates in prolactin-treated cells [309]. Whether STAT5 is a genuine PTP-1B substrate at physiological level remains to be determined, particularly in the context of recent studies of co-crystal between PTP-1B and the activation segment of the insulin receptor. These studies indicate that PTP-1B has a 70-fold greater affinity for tandem-

pY than for mono-pY containing peptides [238]. Moreover, specificity of this interaction is increased when an acidic and a basic residue flank the tandem pY residues, yielding E/D-pY-pY-R/K as the preferred sequence recognized by PTP-1B [295]. This motif is exactly conserved in the activation loop of JAK2, but absent in STAT5a, STAT5b, STAT3 and GHR. Consistent with being a physiologically important substrate, JAK2 has also been found to associate with PTP-1B in leptin and INF-γ signaling.

The cellular location of various tyrosine phosphatases needs to be considered to understand their role in GH signaling. For example, cytosolic SHP-1 is recruited directly to JAK2 in response to GH [400], whereas SHP-2 is associated with the GHR itself [326, 401]. However, because of its exclusive location on the cytosolic face of the ER, it is not immediately obvious how PTP-1B interacts with JAK2 present in GH receptor complexes. In this context, recent studies using fluorescent energy transfer microscopy have shown that PTP-1B dephosphorylates the EGFR and PDGFR tyrosine kinases on the ER membrane after receptor internalization [301]. Consistent with this model, we found that most of the interactions between PTP-1B and phosphorylated JAK2 occurred in the membrane compartment, and others have found that disruption of ER function results in prolongation of JAK2 phosphorylation in GH-treated cells [416]. It will be important to determine whether this model holds in the case of the GH and leptin receptors, which do not have intrinsic kinase activity, but instead rely on the recruitment of cytosolic JAK2 for signaling.

Decreased GH stimulation of ALS promoter activity in H4-II-E cells overexpressing PTP-1B suggested that PTP-1B could be an important modulator of GH action in liver. Our *in vivo* studies on liver yielded two important findings. First, hepatic expression of PTP-1B was reduced by chronic fasting, whereas expression of the related TC-PTP was unchanged. This reduction in PTP-1B may be the consequence of reduced needs for negative regulation when plasma concentrations of metabolic hormones such as GH and insulin are reduced. In this context, it has been

recently shown that over-consumption of fructose leads to increased hepatic expression of PTP-1B in hamsters [254]. Overall, these results suggest that PTP-1B is unique among tyrosine phosphatases in its ability to respond to changes in metabolic stress. Further studies are needed to determine whether this regulation extends to other important metabolic tissues, and to identify the basis for altered PTP-1B expression.

Second, our results indicate that, in the context of acute GH actions, the functional importance of PTP-1B varies according to nutritional status. Under fed conditions, absence of PTP-1B does not result in consistent changes in the GH-dependent activation of JAK2, STAT3 and STAT5. During fasting, however, null PTP-1B liver display significantly higher activation of these molecules than WT liver, indicating that the development of GH resistance normally associated with fasting is impaired [413, 417]. On the short term, this failure resulted in increased induction of SOSC-2, but not of the related SOCS3 or CIS. Greater induction of SOCS2 is interesting given that it appears to be particularly important in the attenuation of GH action in liver and in the whole animal [418-420]. Whether the absence of PTP-1B would rescue chronic anabolic actions of GH during states normally associated with hepatic GH resistance such as inflammation, burn injury and aging [421-423] remains to be studied.

2.6 Acknowledgements

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3 Chapter 3: Delayed onset of streptozotocin induced hyperglycemia in mice lacking protein tyrosine phosphatase 1B

3.1 Abstract

Aim/hypothesis. Our aim was to evaluate, in a mouse model of type 1 diabetes, if absence of protein tyrosine phosphatase 1B, a validated target for the treatment of type 2 diabetes, could improve glucose homeostasis. *Methods.* Type 1 diabetes was induced by streptozotocin injection in wild-type and PTP1B null mice. Blood glucose concentrations and insulin levels were measured following induction of type 1 diabetes. *Results.* Streptozotocin rapidly induced hyperglycemia in wild-type mice, whereas PTP1B null mice maintained significantly lower blood glucose over a period of eight days. Streptozotocin treatment drastically reduced insulin concentrations in both WT and PTP1B null mice, as further confirmed by immunohistochemical analysis of pancreas sections. *Conclusion/interpretation.* Streptozotocin can induce hyperglycemia independently of PTP1B expression. However, blood glucose levels remained significantly lower 8 days following induction of type 1 diabetes in PTP1B null mice, and up to three months. These results suggest that administration of a PTP1B inhibitor, in combination with insulin, could normalize glycemia in type 1 diabetic patients.

3.2 Introduction

The development of type 1 diabetes results from the destruction of insulin-producing pancreatic beta cells by autoimmune responses [331]. In order to maintain blood glucose homeostasis, exogenous insulin is required to provide insulin receptor (IR) activation. One important mechanism in regulating insulin signaling is mediated by protein tyrosine phosphatases (PTPs), which may act on the insulin receptor itself and/or its substrates [221]. Among them, protein tyrosine phosphatase 1B (PTP1B) has been validated as a negative regulator of the IR [161], hence identifying a potential drug target in diabetes.

Previous studies showed that vanadium compounds, which possess insulinomimetic activity, normalized blood glucose levels in animal models of type 1 and type 2 diabetes [336]. Their inhibitory action on PTPs and enhancement of tyrosine phosphorylation appear to be the most relevant explanation for this effect. Hence, it was showed that oral administration of vanadate normalizes blood glucose levels in type 1 diabetic rats treated with streptozotocin (STZ), through stimulation of glucose uptake [338]. In addition, Ahmad et al. found increased cytosolic PTP activity in particulate fractions of skeletal muscle and liver of STZ-diabetic rats. An immunoblot analysis of various PTPs in these tissues showed a significant increase in PTP1B expression [342]. These results suggest that downregulation of PTP1B could normalize glycemia in diabetic animals.

In the present study, we evaluated if removal of the PTP1B gene could normalize glycemia in STZ-treated mice. Weight, blood glucose and insulin concentrations, as well as changes in peripheral nerve pathophysiology were monitored in wild-type (WT) and PTP1B knockout (KO) mice following destruction of pancreatic beta cells. To the best of our knowledge, this is the first time that the role of PTP1B is evaluated in a model of type 1 diabetes.

3.3 Material and methods

Mice

Male WT and PTP1B KO mice are a hybrid of 129S/v and Balb/c backgrounds [161], backcrossed into a Balb/c background for three generations. All experiments were carried out under the "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985), and in accordance with the Canadian Council on Animal Care (CCAC) ethical regulations.

Induction of type 1 diabetes

5-8 weeks old male mice WT (n=8) and PTP1B KO (n=7) were treated with a total of two intraperitoneal injections of 150 mg/kg of STZ (Sigma-Aldrich, Canada) dissolved in citrate buffer (pH=6.4). Control animals WT (n=6) and PTP1B KO (n=5) were injected with citrate buffer.

Blood glucose and insulin concentrations

Blood glucose concentration was measured (MediSense, Abbott Labs) in samples drawn from tail vein. Serum insulin concentrations were determined using a radioimmunoassay (Linco, St. Charles, Mo).

Histological and immunohistochemical analysis

Sections of pancreas were histologically examined and stained with hematoxylin and eosin or with guinea pig polyclonal anti-insulin antibody coupled to horseradish peroxidase (Zymed Laboratories, South San Francisco, California).

Peripheral nerve conductivity in STZ-treated mice

Electromyography was used for monitoring progression of diabetic neuropathy. Motor and sensory fibers conduction velocity was measured in the sciatic nerve using electromyographic method as previously described [424].

Statistical analysis

Results are expressed as mean values ± SEM. Statistical analyses were performed using a two-tailed, unpaired, Student's *t*-test. The differences were considered statistically significant at $p < 0.05$.

3.4 Results and discussion

Type 1 diabetes, which accounts for 5-10% of total diabetes mellitus cases, is characterized by high levels of blood glucose resulting from absolute lack of insulin due to beta cell destruction of the pancreas. As a generalized metabolic disorder, the disease is associated with long-term complications that affect almost every major system in the body, such as diabetic cardiomyopathy, retinopathy, neuropathy, nephropathy, and vascular diseases [331]. Currently, the treatment of type 1 diabetes consists of maintaining glycemia as close to the normal range as possible, which is achieved by multiple daily insulin injections. In addition, there are several experimental promising approaches in the treatment of this disease, such as islet transplantation, but still, some major problems are encountered with this method [334]. Consequently, development of better insulinomimetics remains one of the objectives of studies for better type I diabetes therapy, with properties that would be superior to insulin itself in the control of diabetes.

Vanadium compounds, which possess insulinomimetic activity by inhibiting the activity of PTPs, were shown to normalize blood glucose levels in animal models of

type 1 and type 2 diabetes [336, 338]. In addition, increased cytosolic PTP1B activity was reported in particulate fractions of skeletal muscle and liver of type 1 diabetic rats [342], suggesting that inhibition of PTP1B could normalize glycemia in diabetic animals. Moreover, inhibition of PTP1B, by means of gene disruption and PTP1B antisense admistration, which leads to enhancement of IR phosphorylation *in vivo*, pointed out PTP1B as a potential target for drug development in diabetes [161, 162, 256, 258].

Taken together, the evidence of increased insulin sensitivity prompted us to look whether PTP1B deficiency could compensate for the reduction or the absence of insulin in type 1 diabetic mice, possibly by promoting IR signaling. We rendered WT and PTP1B null mice diabetic with two injections of STZ. This treatment selectively destroyed pancreatic beta cells and thus mimics insulinopenia of type 1 diabetes [335]. Following the first STZ injection, glucose levels were significantly increased in WT compared to PTP1B null mice. Following the second injection, glycemia increased in both WT and PTP1B null mice, but PTP1B deficient mice maintained lower blood glucose up to 8 days ($p < 0.05$) (Figure 1A). Glycemia was monitored up to three months and, interestingly, PTP1B deficient mice apparently maintained lower blood glucose than WT mice, but not significantly different ($p < 0.057$) (Figure 1B).

In order to verify if the lower blood glucose concentration observed in the STZ-PTP1B null mice was due to the secretion of insulin from residual pancreatic beta cells, we stained islets with anti-insulin antibody. Insulin-producing beta cells are rarely seen in the islets of WT and PTP1B null diabetic mice (Figure 2B), whereas insulin-producing beta cells were clearly detected in normal control islets of WT and PTP1B null mice (Figure 2A). In addition, insulin levels were similar in STZ-treated WT (16.6 ± 3.9) and PTP1B null mice (15.2 ± 4.9) μU (data not shown), and comparable to the ones previously observed in WT and PTP1B null mice [161]. These results suggest that the decreased glycemia observed in PTP1B null STZ mice was not due to

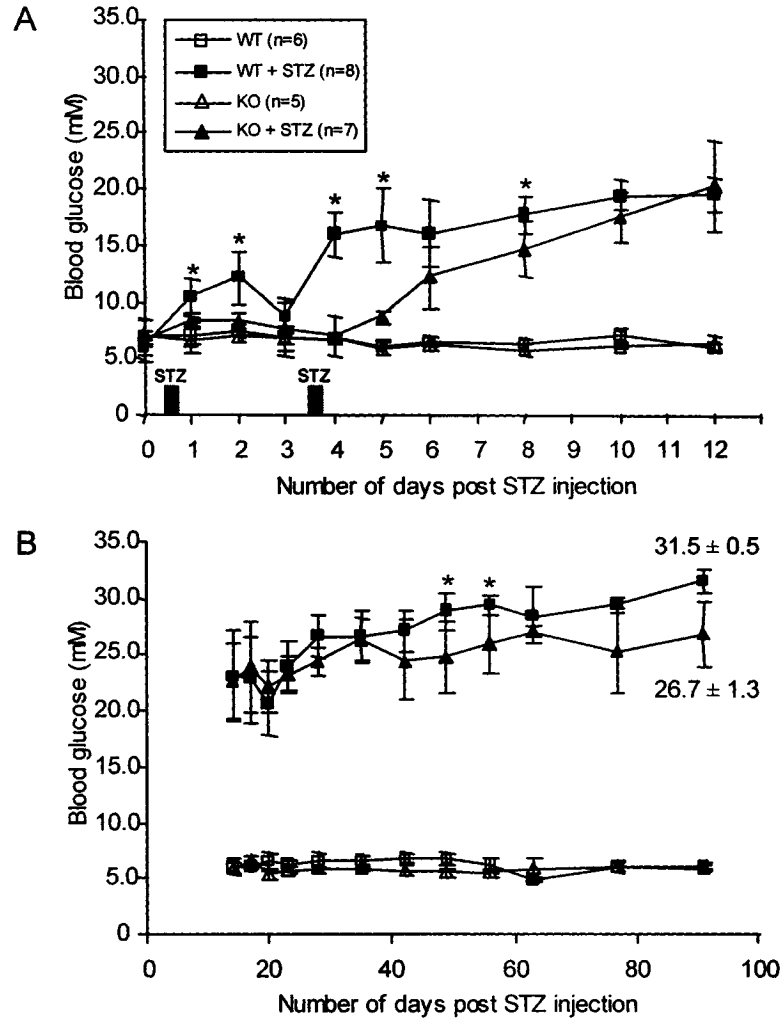


Figure 1. Blood glucose concentrations in streptozotocin-treated WT and PTP1B KO mice. A) Hyperglycemia was induced by two injections of STZ. Blood glucose and body weight were monitored daily for six days, and then every two days until day 12. Glycemia is significantly different between WT+STZ and KO+STZ until day 8. No body weight change was observed following both injections of STZ, but at the end point of the study, WT and PTP1B KO gained 24-27% of their initial weight, and STZ-diabetic WT and PTP1B KO mice gained 6-10%. B) Starting day 20, blood glucose was monitored every week up to three months. At the end point, glycemia was not significantly different between WT+STZ and KO+STZ ($p=0.057$). All the STZ-diabetic mice included in this study were alive up to 40 days following STZ injection and did not required insulin injection to survive. The average mortality rate of STZ treated mice was 11% after 90 days. WT, open squares (n=6); WT + STZ, solid squares (n=8); KO, open triangles (n=5); KO + STZ, solid triangles (n=7). Values are mean ± SEM, * $p < 0.05$ (at least).

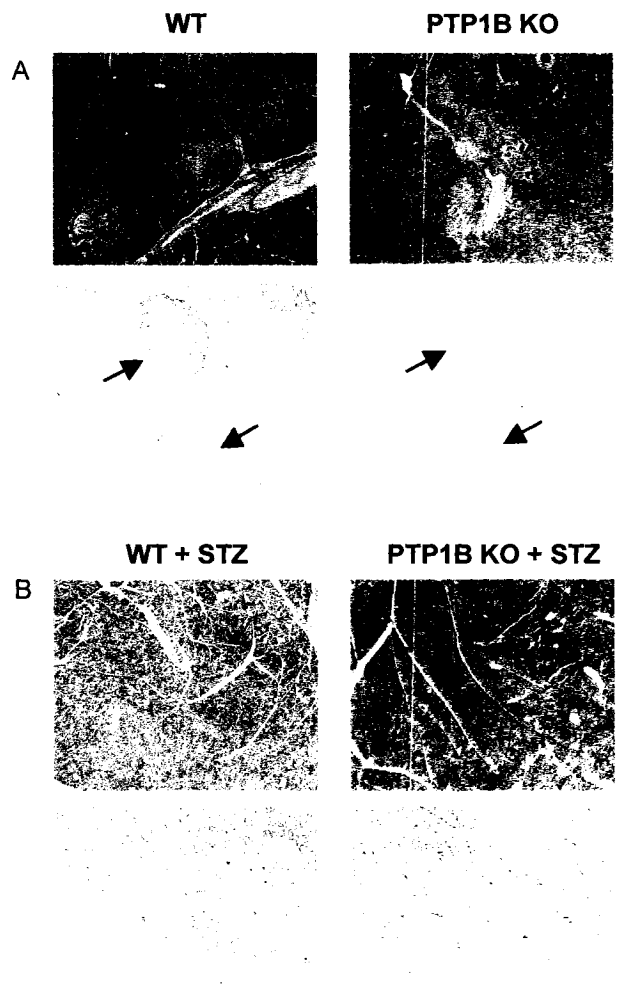


Figure 2. Insulin staining of pancreatic islets in WT and PTP1B KO mice three months following streptozotocin injection. a, and b, Serial pancreatic islet sections of control (A) or STZ-treated mice (B) were stained with hematoxylin and eosin (top panel) or with anti-insulin antibody (bottom panel). Magnification: X10-20.

endogenous pancreatic insulin, but to the absence of PTP1B, and that STZ destroyed beta cells to the same extent in both WT and PTP1B null mice.

In humans, neuropathy is one of the most common complications of diabetes. Diabetic neuropathy is characterized by sensory loss, pain, paresthesiae, and reduced conduction velocity (CV) [331]. CV is considered to be one of the most reliable parameter to evaluate the severity of diabetes in animals. Therefore, we monitored CV of sensory and motor fibers of sciatic nerves in control and STZ-treated WT and PTP1B KO mice. As previously shown, CV is decreased in STZ-treated animals compared to controls [425]. However, no difference was observed between the WT and PTP1B KO STZ treated mice over a three-month period (data not shown). We concluded that PTP1B deficiency does not protect against decreased CV caused by type 1 diabetes.

In accordance with our findings, Kushner et al. recently showed that PTP1B regulates beta cell homeostasis [343]. They observed that the absence of the PTP1B gene improved glucose tolerance and delayed the onset of diabetes in insulin receptor substrate 2 (IRS2) KO diabetic mice. Together with our data, this suggests that the specific inhibition of PTP1B, which enhances IR signaling, can partially compensate for insulin deficiency.

In summary, we have shown that the absence of PTP1B does not prevent induction of type 1 diabetes by STZ, but delays the onset of hyperglycemia and improves blood glucose levels in the long-term. Since many studies demonstrated that the control of glycemia reduces the risk of developing major complications of diabetes [331], our results strongly suggest that a PTP1B inhibitor, in combination with insulin, could be administered to reduce the need for insulin injection. For instance, the use PTP1B inhibitors on genetic type 1 diabetes models such as the non-obese diabetic (NOD) mice, in which diabetes occurs spontaneously with a total dependence on exogenous insulin for survival [426], will further demonstrate that inhibition of PTP1B

can normalize glycemia in combination with insulin injection. In addition, there is accumulating evidence for a role of ER stress in decreased beta cell mass, through apoptosis, in type 1 and 2 diabetes [427]. Interestingly, our laboratory has recently shown that PTP1B null fibroblasts are resistant to tunicamycin and azetidine-2 carboxylic acid-induced ER stress, as they show increased resistance to apoptosis compared to WT fibroblasts [428]. Ongoing work will determine if PTP1B is involved in beta cell homeostasis by investigating whether PTP1B null beta cells are more resistant to ER stress.

3.5 Acknowledgements

N.D. is the recipient of a Canadian Institutes of Health Research doctoral research award. M.L.T. is a Canadian Institutes of Health Research Scientist. This work was supported by a Canadian Institutes of Health Research (MOP-62887) operating grant to M.L.T.

4 Chapter 4: The role of Protein Tyrosine Phosphatase 1B in Ras signaling

4.1 Abstract

Protein tyrosine phosphatase (PTP) 1B has been implicated as a negative regulator of multiple signaling pathways downstream of receptor tyrosine kinases. Inhibition of this enzyme was initially thought to potentially lead to increased oncogenic signaling and tumorigenesis. Surprisingly, we show that platelet-derived growth factor-stimulated extracellular-regulated kinase signaling in PTP1B-deficient cells is not significantly hyperactivated. Moreover, these cells exhibit decreased Ras activity and reduced proliferation by way of previously uncharacterized pathways. On immortalization, PTP1B-deficient fibroblasts display increased expression of Ras GTPase-activating protein (p120RasGAP). Furthermore, we demonstrate that p62Dok is a putative substrate of PTP1B and that tyrosine phosphorylation of p62Dok is indeed increased in PTP1B-deficient cells. Consistent with the decreased Ras activity in cells lacking PTP1B, introduction of constitutively activated Ras restored extracellular-regulated kinase signaling and their proliferative potential to those of WT cells. These results indicate that loss of PTP1B can lead to decreased Ras signaling, despite enhanced signaling of other pathways. This finding may in part explain the absence of increased tumor incidence in PTP1B-deficient mice. Thus, PTP1B can positively regulate Ras activity by acting on pathways distal to those of receptor tyrosine kinases.

4.2 Introduction

Protein tyrosine phosphatase (PTP) 1B is the prototype for the superfamily of protein tyrosine phosphatases (PTPs), and has been implicated in multiple signaling pathways [10]. Of particular interest, gene-targeting studies in mice have established PTP1B as a critical physiological regulator of metabolism by attenuating insulin, leptin and growth hormone signaling [161-164, 429]. PTP1B function seems to be dispensable for embryonic development. However, PTP1B-deficient mice exhibit resistance to diabetes and obesity, the two major metabolic diseases in industrialized societies. Not surprisingly, PTP1B is a highly regarded target of the pharmaceutical industry in the treatment of these disorders [430].

Since PTP1B is a negative regulator of multiple receptor tyrosine kinases (RTKs) [10], the concern is that PTP1B inhibition may lead to increased oncogenic signaling. Indeed, PTP1B-deficient fibroblasts display increased insulin-like growth factor-I (IGF-I) receptor, epidermal growth factor receptor, and platelet-derived growth factor receptor (PDGFR) tyrosine phosphorylation [300, 302]. Regardless of this potentially enhanced oncogenic signaling, PTP1B-deficient mice do not overtly undergo tumorigenesis. One possibility is that PTP1B may not regulate RTK signaling in all cell types, or that functional redundancy may exist. Alternatively, loss of PTP1B may affect the progression of a tumorigenic event, but not its rate of initiation. Finally, it is also possible that PTP1B may be involved in the activation of oncogenic pathways downstream of RTKs.

We decided to pursue the third alternative based on our previous studies with PTP1B in the IGF-I receptor pathway. Paradoxically, IGF-I stimulated extracellular-regulated kinase (Erk) phosphorylation in PTP1B-deficient fibroblasts is significantly diminished. This could, in part, be explained by previous results suggesting that PTP1B is involved in the activation of Src [209, 431]. Indeed, we demonstrated that adhesion

mediated Erk and Src activation in PTP1B-deficient fibroblasts are both impaired [305]. However, most of our studies were performed with cells immortalized with the SV40 Large T antigen (TAg). Importantly, TAg has been shown to abrogate the requirements of Src kinases during PDGF-induced mitogenesis [432], and PDGF-induced Erk activation in TAg-immortalized fibroblasts lacking Src kinases is relatively unchanged [433]. This suggested to us that there were additional mechanisms in PTP1B-deficient fibroblasts that were responsible for the diminished Erk activity.

In this study, we show that, although PTP1B-deficient cells exhibit increased PDGFR and AKT phosphorylation, Erk activation does not occur to the same extent. We show that loss of PTP1B results in diminished Ras activity and that this occurs through increased p120RasGAP (Ras GTPase-activating protein) expression and p62Dok (downstream of tyrosine kinase) phosphorylation. Taken together, these results propose how PTP1B can act as a positive regulator of Ras signaling downstream of RTKs and may in part explain why PTP1B knockout mice do not present an increased incidence of tumors.

4.3 Methods

Antibodies

Rabbit polyclonal antibodies against PTP1B have been described [161]. Additional antibodies were purchased from Cell Signaling Technology (Beverly, MA) (pan and phospho anti-AKT, anti-Erk), Upstate Biotechnology (Lake Placid, NY) (anti-phosphotyrosine 4G10), Santa Cruz Biotechnology (anti-PDGFRbeta, anti-Dok1), Transduction Laboratories (Lexington, KY) (anti-p190RhoGAP, anti-p120RasGAP, anti-H-Ras, and anti-Shc), and Biosource International (Camarillo, CA) (anti-Src).

Cell culture and cell lines

All cell lines were maintained in DMEM (Invitrogen) supplemented with 10% FBS (BioSource International) and antibiotics (5 mg/ml penicillin/streptomycin, Invitrogen). All spontaneously immortalized fibroblast cell lines derived from PTP1B or T cell PTP (TCPTP) knockout embryos have been described [302, 403]. The SV40 TAg cells were rescued as described [302, 403].

Generation of the V12Ras clones

PTP1B WT or knockout spontaneously immortalized fibroblasts were transfected with a linearized V12Ras vector and a hygromycin selection vector (pMC1-HygR-pA) at a 10:1 ratio, respectively. Transfected cells were selected in DMEM containing 50-75 μg/ml hygromycin, and colonies were picked. Screening of positive clones was done by Western blotting by using anti-H-Ras antibody. In each case, at least 10 clones were isolated and further characterized.

Preparation of cell lysate and immunoblotting

Cells were washed in ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Nonidet P-40, 0.25% Na deoxycholate, 1mM Na_3VO_4, 50 mM NaF,) supplemented with Complete EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals). Cell lysates were rotated end-over-end at 4°C for 10 min and cleared by centrifugation at 14,000 x g for 10 min at 4°C. The protein concentration was measured by the Bradford method (Bio-Rad). Protein samples were resolved by 8% SDS/PAGE and subjected to immunoblotting with the indicated antibodies.

Immunoprecipitation

Cell lysates were incubated with anti-p62Dok antibodies and 20 µl of protein G-Agarose (Invitrogen) at 4°C for 2 h. Precipitates were washed in lysis buffer, resuspended in SDS sample buffer, and resolved by 8% SDS-PAGE for immunoblot analysis.

Semi-quantitative RT-PCR

Total RNA was isolated from cells using TRIzol (Invitrogen), and first-strand cDNA synthesis was obtained from 1 µg of RNA with random hexamers using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA was then used as a template for PCR with two sets of synthesized primers. Aliquots of 1 µl of the RT reaction were amplified by using 1 unit of Ampli*Taq* Gold (Applied Biosystems) under the following PCR conditions: 5 min at 95°C, then 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C for 30 cycles, 2 min at 72°C) in a 25-µl reaction mixture, using 100pmol each of the sense and antisense primers: 5'RasGAP, 5'-GGGTGTTTACAGAAATCAGTTC-3'; 3'RasGAP, 5'-CTCATTGCTGAGTGT-TCTCAG-3'. In parallel, a GAPDH PCR was performed to control for the RNA input in the RT-PCR: 5"GAPDH, 5'-AACGACCCCTTCATTGAC-3'; 3'GAPDH, 5'-TCCACGACATACTCAGCAC-3'. Reaction products were separated by 1% agarose gel electrophoresis and detected with ethidium bromide staining.

Subtrate-trapping experiments

Details of the PTP1B WT and D181A constructs have been previously described [145]. NIH3T3 c-Src Y527F cells were transfected by Lipofectamine (Invitrogen) according to manufacturer's instructions. Forty-eight hours post-transfection, cells were lysed in buffer containing 50mM Hepes (pH 7.5), 150mM NaCl, 1.5mM $MgCl_2$, 1mM EGTA, 1% Triton X-100 and 10% glycerol (supplemented with Complete EDTA-free protease

inhibitors). Crude lysates were then cleared by centrifugation at 14,000 x g. GST-tagged proteins were precipitated using 25µl of Glutathione Sepharose beads (Pharmacia), washed extensively in lysis buffer, and then resuspended in SDS sample buffer. Aliquots were resolved by SDS/PAGE and analyzed by immunoblotting with the indicated antibodies.

Soft agar assay

PTP1B WT and knockout cells stably expressing V12Ras were assessed for anchorage-independent growth by colony formation in soft agar. NIH3T3 c-Src Y527F cells were used as positive control. The cells were plated at $3x10^3$ cells per well in a six-well plate in triplicate, by using 0.35% low melting point agarose and grown in DMEM with 20% FBS. Media were changed every 3 days. Colony number was determined by scoring for those with a size >0.1mm in size. Representative colonies were photographed in phase contrast from plates at day 10 of the assay (10X magnification). Values are reported as average of three experiments ± S.E.

Ras-GTP pull-down assay

Cells were lysed in MLB buffer (25 mM Hepes pH 7.5, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 nM NaF, 1 mM Na_3VO_4, 10 mM $MgCl_2$, 1 mM EDTA) supplemented with Complete EDTA-free protease inhibitor mixture. The level of Ras-GTP was determined by precipitation with a GST fusion protein of the Ras-binding domain on Raf1, which recognizes only active, GTP-bound Ras. Pull-downs were resolved by SDS/PAGE and immunoblotted with an anti-Ras antibody to detect precipitated Ras-GTP.

4.4 Results

4.4.1 Differential regulation of downstream pathways of the PDGFR by PTP1B

To investigate the mechanisms by which loss of PTP1B can attenuate Erk activation, we used the PDGF signaling system in TAg immortalized cells (TAg cells), for which Src function is dispensable [432, 433]. Previous studies with PTP1B-deficient TAg cells showed that PDGF-stimulated Erk and AKT activation were not dramatically altered [300, 302]. We used PDGF at a high concentration (50ng/ml), allowing us to suspect that subtle changes by PTP1B may not be detectable in this case. Hence, in our studies, we used PDGF at much lower levels (10-20ng/ml).

As expected, stimulation of TAg cells with low levels of PDGF resulted in increased cellular tyrosine phosphorylation and AKT activation, which was enhanced in PTP1B-deficient cells (Figure 1). In contrast, PDGF-induced Erk phosphorylation was decreased in PTP1B knockout cells. Thus, PTP1B seems to differentially regulate signaling pathways diverging from the PDGFR.

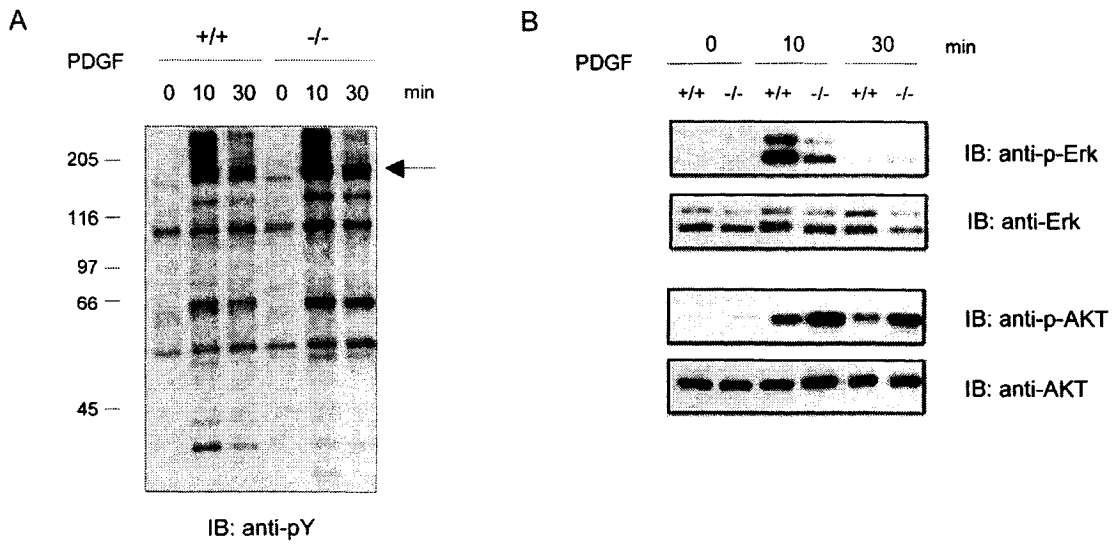


Figure 1. Differential regulation of PDGF signaling pathways by PTP1B. A, Fibroblasts immortalized with SV40 TAg (+/+ PTP1B and -/- PTP1B) were serum-starved and stimulated with 10 ng/ml PDGF for indicated times or left unstimlated. The lysates were analyzed by Western blotting and the membrane was probed with anti-phosphotyrosine antibodies. B, The samples were analyzed for Erk and AKT proteins and phosphorylation levels by using phosphospecific antibodies.

4.4.2 PTP1B-deficient fibroblasts display decreased growth and Ras activity

Consistent with our data that IGF-I [302] and PDGF- induced activation of Erk is impaired in PTP1B-deficient cells, we also observed that these cells display decreased monolayer growth compared to their WT counterparts (Figure 2A). Similarly, although both WT and knockout cells were able to proliferate in 1% serum, PTP1B-deficient cells clearly present a diminished capacity to do so (Figure 2B). Moreover, PTP1B-deficient TAg cells exhibit a consistent and reproducible elevation in PDGFR levels

(Figure 2C). This is unlikely due to defective receptor internalization and degradation as stimulation with PDGF caused a rapid decrease in receptor levels in both PTP1B WT and knockout cells.

It has been established in fibroblasts that expression of the PDGFR is inversely proportional to Ras activity [349]. Furthermore, Ras activity is necessary for full transformation by TAg [434]. Using a Ras-GTP pull down assay, we indeed show that PTP1B-deficient TAg cells display diminished Ras-GTP levels (Figure 2D). In addition, introduction of dominant active V12Ras in both PTP1B WT and knockout cells was able to suppress PDGFR levels (data not shown).

4.4.3 Immortalization increases p120RasGAP expression in PTP1B-deficient fibroblasts

To gain insight into the potential decrease in Ras activity in PTP1B-deficient cells, we first profiled several proteins upstream of the Erk signaling pathway. We found no significant alterations in the expression levels of the adapter proteins Shc or Grb2, or the kinases Src and Erk (data not shown). Importantly, however, we observed that the levels of p120RasGAP are elevated in PTP1B-deficient TAg cells (Figure 3A). Yet, the levels of the related protein p190RhoGAP were not altered. The importance of this finding is underlined by the fact that p120RasGAP can attenuate Ras activity by promoting the intrinsic GTPase activity of Ras [435].

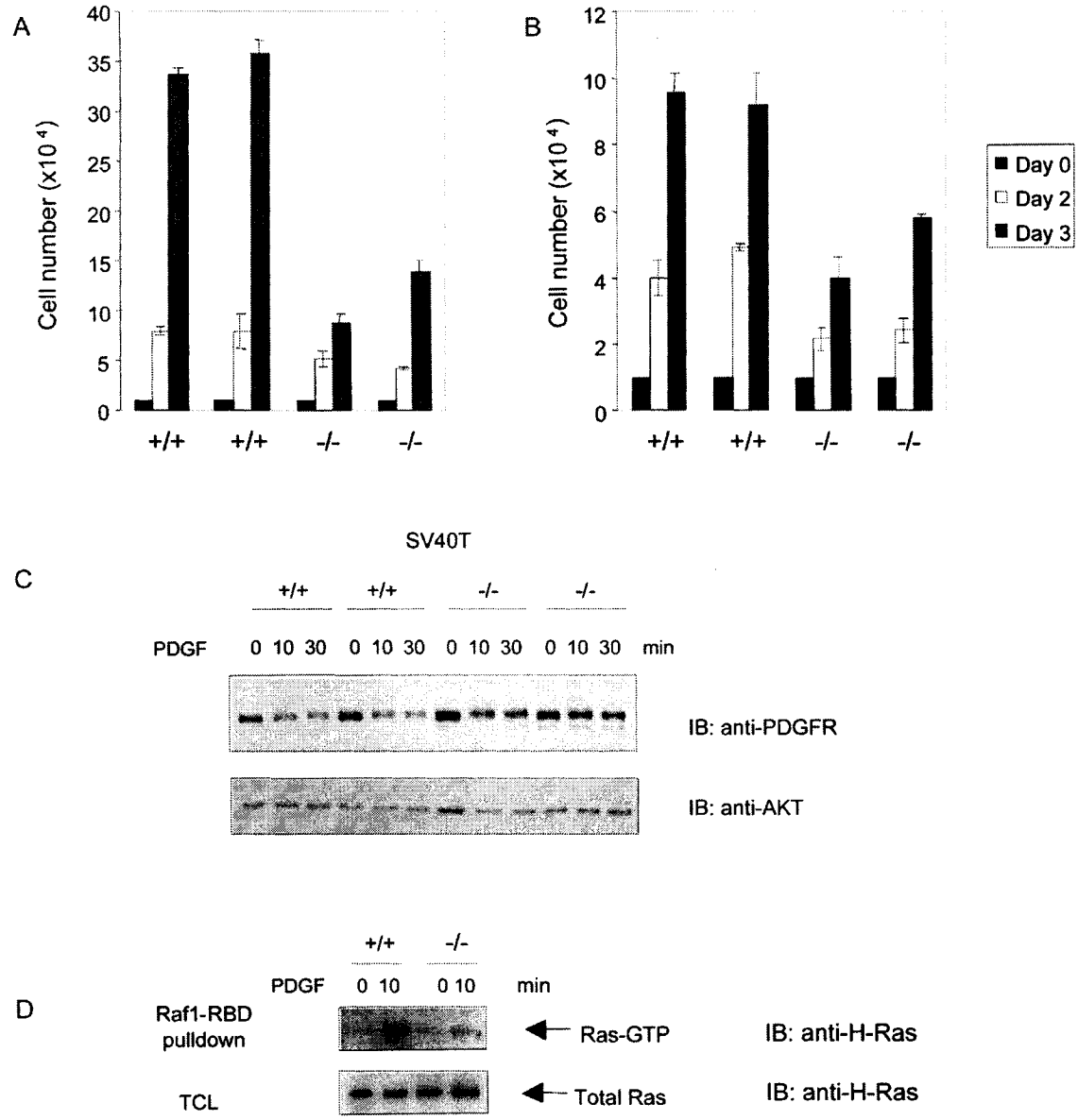


Figure 2. Decreased cell growth and Ras activity of SV40 TAg transformed fibroblasts lacking PTP1B. A and B, Cells were seeded in a 24-well plate at a density of $1x10^4$ cells per well in 10% or 1% FBS. At the indicated time points, the cells were trypsinized and the total cell number per well was determined with a hemacytometer. Values are reported as average of triplicate ± S.E. C, PDGFR expression is increased in cells lacking PTP1B. Serum-starved cells were stimulated with PDGF for indicated times or left unstimlated. The lysates were analyzed by Western blotting for PDGFR expression. Equal loading was done by reprobing the membrane with antibodies against AKT / protein kinase B. D, Serum-starved cells were stimulated with 20ng/ml PDGF for 10 min or left unstimlated. Lysates were incubated with immobilized GST-Raf1-RBD (Raf-RBD) to precipitate active (GTP-bound) Ras. Ras-GTP was detected using anti-H-Ras antibodies.

To exclude the possibility that this phenomenon was due to TAg, we also analyzed p120RasGAP levels in spontaneously immortalized cells, as well as primary fibroblasts. Similar to TAg cells, spontaneously immortalized PTP1B knockout cells also possess increased p120RasGAP levels compared to WT controls (Figure 3B). Interestingly, however, this effect was not seen in primary cells, suggesting that an event during immortalization is required for this process (data not shown). To further exclude the possibility of clonal variation effects, re-expression of myc-tagged PTP1B into knockout cells was able to restore p120RasGAP expression (Figure 3C). Finally, to show that the increase in p120RasGAP expression was specific for PTP1B knockout cells, we used TCPTP knockout cells as a control. Although TCPTP knockout cells also display decreased cell proliferation [403], p120RasGAP levels are not altered (Figure 3D).

To determine whether there were increased levels of p120RasGAP mRNA in PTP1B-deficient fibroblasts, we performed semiquantitative RT-PCR analysis by using the GAPDH gene as a control. As shown in Figure 3E, this is indeed the case in two independent PTP1B-deficient TAg cell lines compared to two WT controls. In Figure 3F, re-expression of PTP1B into PTP1B-deficient spontaneously immortalized cells restores RasGAP mRNA levels. Although the mechanism by which this event occurs is unclear, our results suggest that PTP1B expression is required to suppress expression of the p120RasGAP during immortalization of fibroblasts.

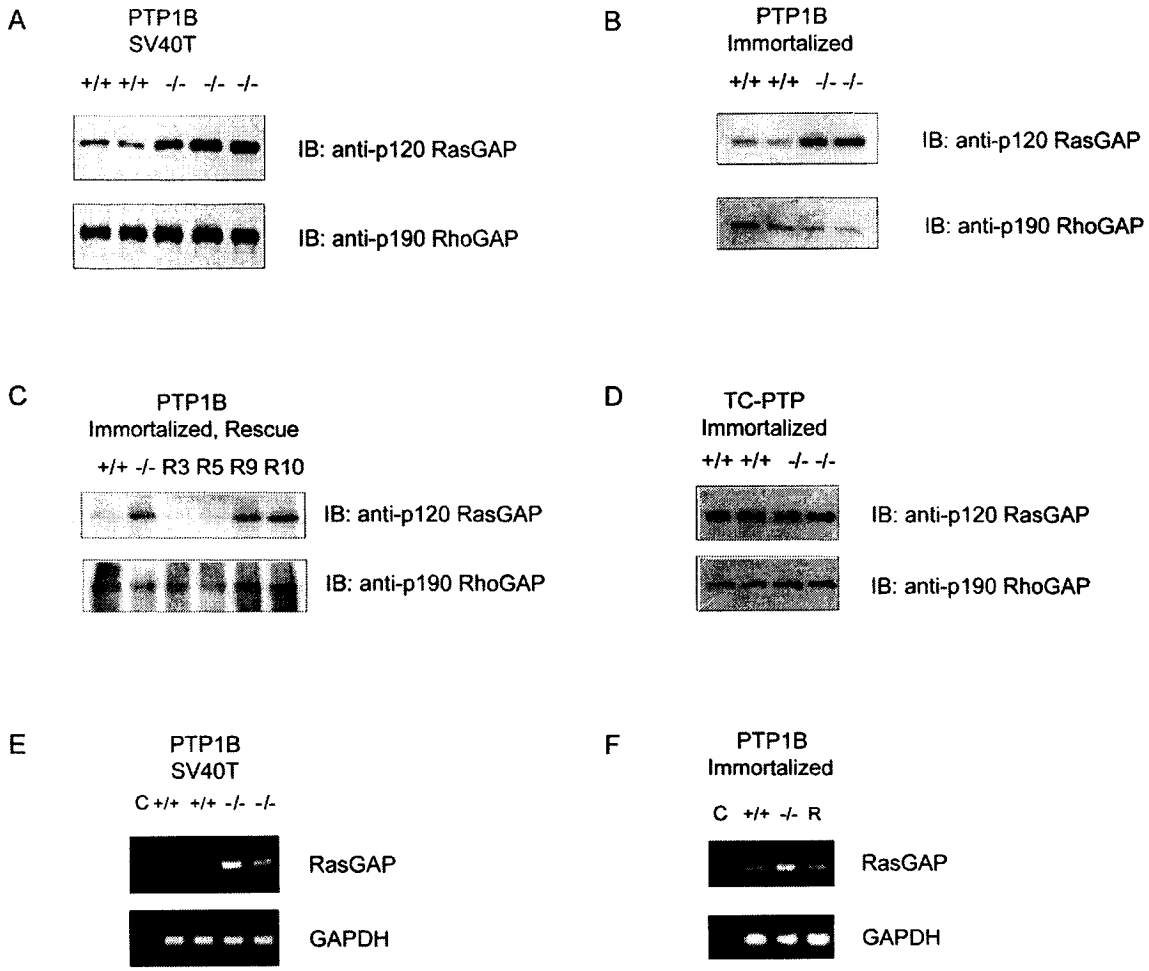


Figure 3. PTP1B deficient cells display increased p120RasGAP expression. A, Increased expression of p120RasGAP but not p190RhoGAP in TAg immortalized PTP1B-deficient fibroblasts. B Increased expression of RasGAP is also seen in spontaneously immortalized PTP1B-deficient fibroblasts. C, Stable re-expression of PTP1B into PTP1B -/- cells decreases RasGAP levels. R3, R5 are -/- cells that expressed PTP1B, and R9, R10 are -/- cells mock transfected. D, Expression level of RasGAP is not affected in TCPTP -/- immortalized fibroblasts. E and F, Increased p120RasGAP mRNA in PTP1B deficient fibroblasts assessed by RT-PCR. GAPDH was used as a loading control, and lane C (no DNA) was used as a negative control.

4.4.4 p62Dok is a putative substrate for PTP1B

Previous studies demonstrated that p120RasGAP is tyrosine phosphorylated in cells transformed by protein tyrosine kinases, including Src [356]. Furthermore, tyrosine phosphorylation of p120RasGAP allows it to bind to other proteins to contribute to its ability to inhibit Ras / mitogen-activated protein kinase (MAPK) pathway [435]. To investigate whether p120RasGAP could be a potential substrate of PTP1B, we used a D181A mutant of PTP1B that was previously shown to possess diminished catalytic activity but retain binding ability, thus producing a "substrate trapping mutant" [167].

We co-expressed GST alone or GST-tagged PTP1B (WT or D181A) into NIH 3T3 cells stably expressing an activated Src mutant (Src Y527F cells) and examined whether we could detect tyrosine-phosphorylated proteins in a complex with PTP1B proteins. Pull-downs of lysates were performed with glutathione beads and then analyzed by immunoblotting with a panel of antibodies. From Figure 4A, it is clear that neither GST alone nor GST-PTP1B (WT) could appreciably precipitate tyrosine-phosphorylated proteins. In contrast, GST-PTP1B (D181A) was found to precipitate two such proteins of ≈60 and ≈130 kDa (pp60 and pp130). We were able to confirm the identity of pp130 as p130Cas, previously shown to be a potential substrate of PTP1B [217]. In contrast, probing with p120RasGAP antibodies failed to demonstrate binding to PTP1B, suggesting that p120RasGAP is not a substrate of PTP1B (data not shown).

We next explored the identity of pp60. Using a candidate approach, we revealed that pp60 is the adaptor protein p62Dok. As controls, two other tyrosine-phosphorylated proteins ≈60 kDa, Shc and Src, were not found to be precipitated by the PTP1B D181A mutant. To further confirm that p62Dok is a substrate of PTP1B, we analyzed the amounts of tyrosine-phosphorylated p62Dok in the PTP1B WT and knockout cells (Figure 4B). As expected, p62Dok phosphorylation was increased in

PTP1B-deficient TAg cells, and re-expression of myc-tagged PTP1B into these cells decreased p62Dok phosphorylation (Figure 4C).

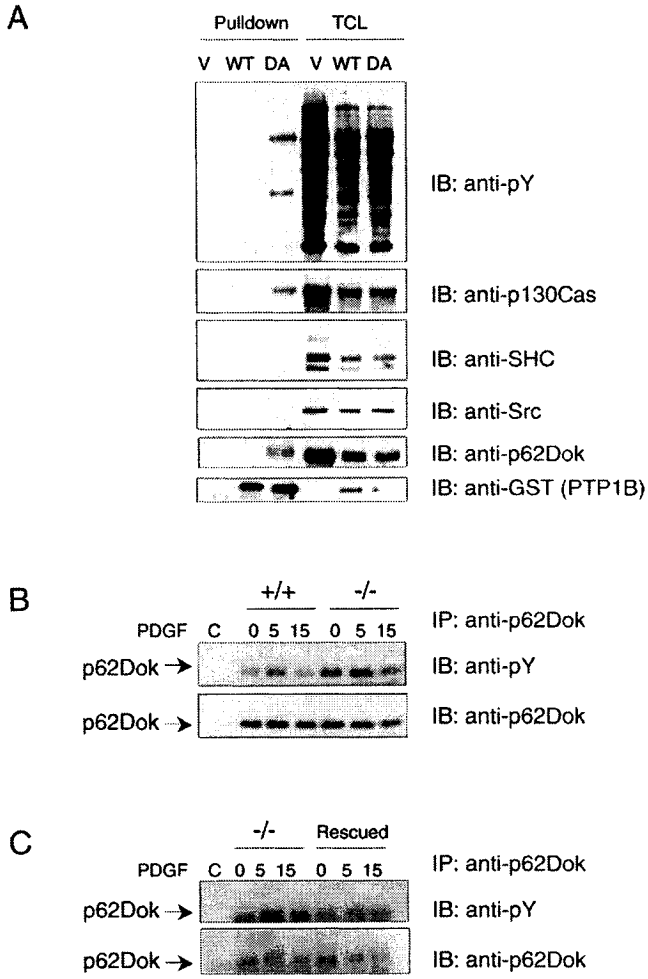


Figure 4. p62Dok is a putative substrate of PTP1B. A, NIH3T3 c-Src Y527F cells were transfected with GST-vector (V), GST-PTP1B WT (WT), GST-PTP1B D181A (DA). Cell lysates were subjected to pull-down with glutathione beads and then analyzed by immunoblotting with the indicated antibodies. TCL; total cell lysate. B and C, Increased p62Dok phosphorylation in fibroblasts lacking PTP1B. Serum-starved cells were stimulated with 20ng/ml PDGF for 5 and 15 min or left unstimlated. p62Dok phosphorylation was analyzed by immunoprecipitating p62Dok, probing with anti-phosphotyrosine antibodies, and then reprobing with anti-Dok1 antibodies. Lane C is antibody and beads alone as a control. Rescued, PTP1B -/- cells that re-expressed PTP1B.

Previous gene targeting studies have shown that p62Dok is a negative regulator of Erk signaling [358, 359]. Furthermore, tyrosine phosphorylation of p62Dok can contribute to its inhibitory effect on Ras [436]. Thus, this provides another mechanism by which PTP1B may regulate Ras activity.

4.4.5 V12Ras rescues the proliferative potential of PTP1B knockout cells

Our results demonstrate that, in immortalized PTP1B-deficient fibroblasts, both p120RasGAP expression and p62Dok phosphorylation are increased. These events can lead to the suppression of the Erk pathway, and lie upstream of Ras activation. Thus, to further confirm our findings, we tested the effect of stably introducing a dominant active Ras mutant (V12Ras) in our PTP1B WT and knockout cells. This mutant is known to inhibit its intrinsic GTPase activity, thus stabilizing the active GTP bound form of Ras [437]. Furthermore, this renders V12Ras independent from the modulation by upstream signals, such as RTKs and GAPs.

For our experiments, we chose three clones each for PTP1B WT and knockout cells. In this group, clones M (mock) do not express V12Ras, which we treated as our negative controls. Expression of V12Ras in the other clones (A +/+, B +/+, A -/-, B -/-), leads to an increase in Ras-GTP levels (Figure 5A). Furthermore, both PTP1B WT and knockout cells were efficiently transformed by V12Ras, as judged by cell morphology and growth in soft agar (Figure 5B, C and D). In fact, the PTP1B-deficient clones even displayed enhanced soft agar growth, probably correlating with the slightly higher levels of Ras-GTP in these cells (Figure 5A). Retroviral expression of PTP1B in these clones was able to suppress approximately half of the number of colonies (Figure 5E), likely through its effects in down-regulating AKT activity [302]. Thus, V12Ras is able to transform fibroblasts, even in the absence of PTP1B.

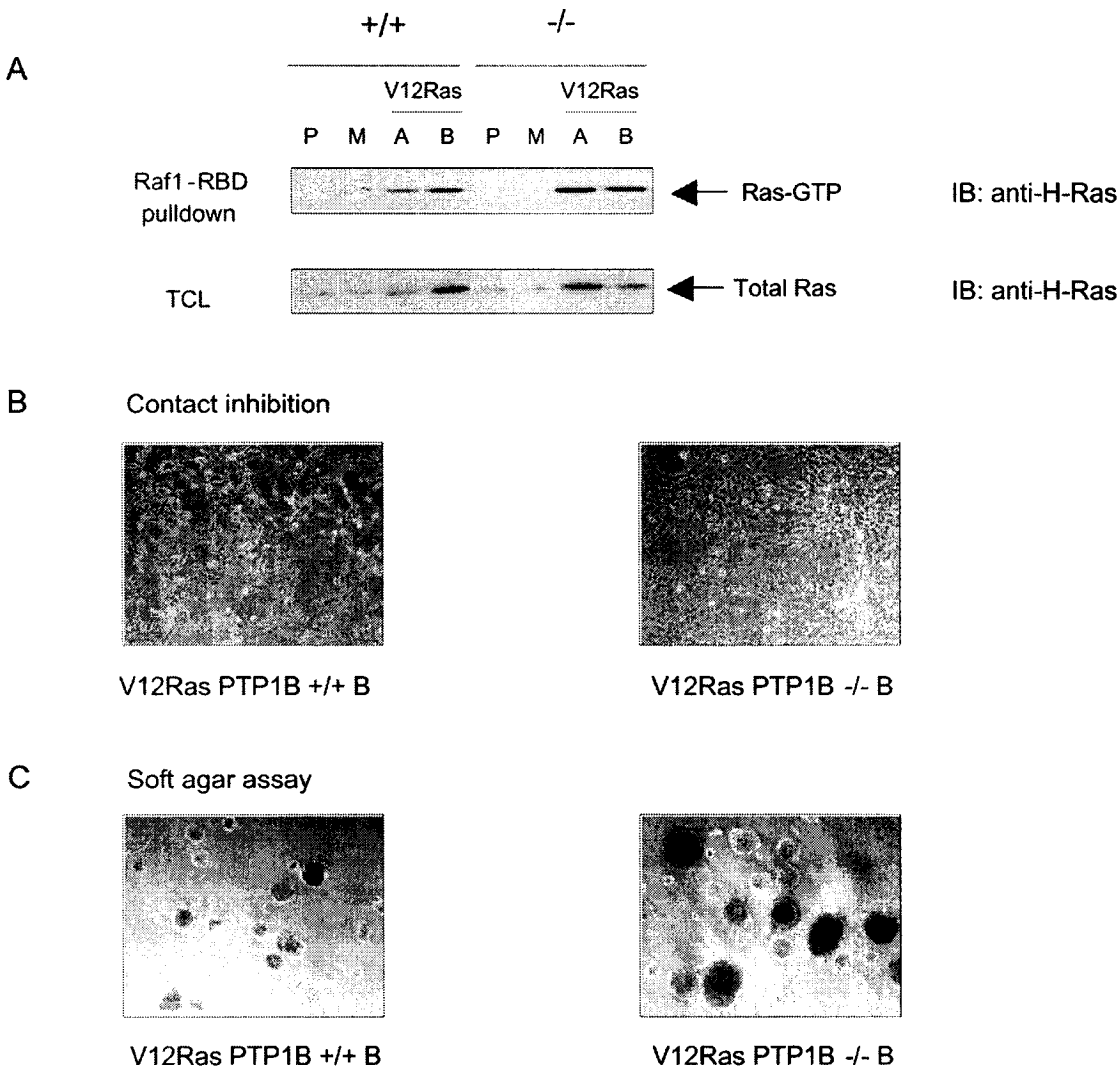
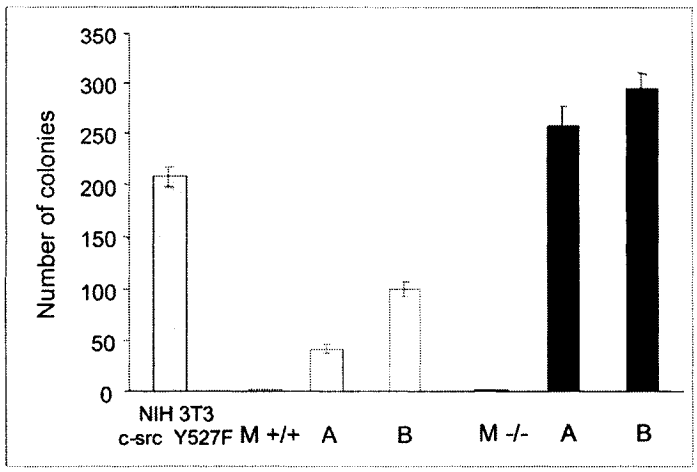


Figure 5. Transformation of PTP1B-deficient fibroblasts by activated Ras. A, Expression of constitutively activated Ras leads to Ras activation. Cells were plated at $1x10^5$ per 10-cm dish and cultured in supplemented DMEM until confluent. The lysates were incubated with immobilized GST-Raf1-RBD (Raf-RBD) to precipitate active (GTP-bound) Ras. Ras-GTP was detected with anti-H-Ras antibodies. B, Stable cell lines expressing V12Ras were grown near confluence. Representative clones were photographed in phase contrast. C, Anchorage-independent growth of PTP1B-deficient fibroblasts. Stable cell lines were grown in soft agar for 10 days. Colonies were counted under a microscope, and pictures of representative clones were taken in phase contrast.

D



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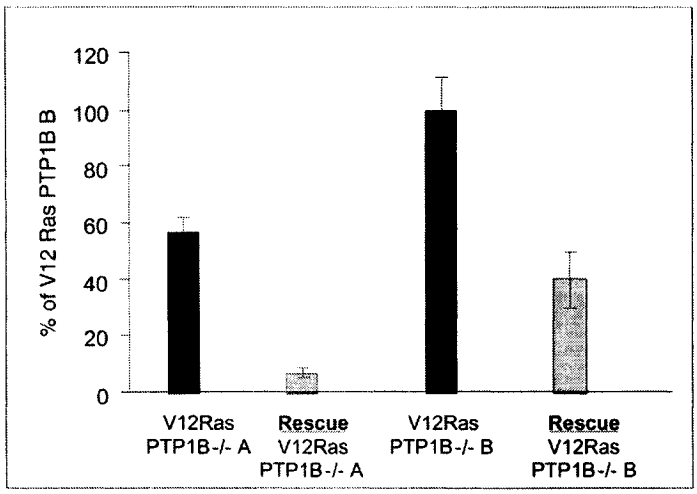


Figure 5. Transformation of PTP1B-deficient fibroblasts by activated Ras. Cont'd.
D, Summary of the results obtained in C. Values are reported as average of triplicate ± S.E. of three independent experiments. NIH3T3 c-src Y527F were used as positive control. P, parental cell line; M, mock stable cell line (negative control); +/+V12Ras A and +/+V12Ras B, wild-type clones expressing V12Ras; -/-V12Ras A and -/-V12Ras B, PTP1B knockout clones expressing V12Ras. E, Re-expression of PTP1B in V12Ras PTP1B -/- fibroblasts decrease colonies' formation in soft agar. Both V12Ras PTP1B -/- cell lines were infected with a retroviral vector encoding PTP1B and grown in soft agar for 10 days. Colonies were counted as described in D.

4.5 Discussion

Overexpression and/or activating mutations of at least 30 protein tyrosine kinases have been linked to malignant transformation and cancer [2]. In contrast, much less is known about the role of PTPs in human diseases. PTP1B is the prototypical PTP, and biochemical studies have implicated this enzyme in the dephosphorylation of several RTKs [10]. We previously demonstrated that PTP1B-deficient fibroblasts display enhanced IGF-I-mediated receptor phosphorylation and AKT activation [302]. Paradoxically, IGF-I-stimulated Erk activation was significantly impaired. Similarly, we also showed that PTP1B-deficient fibroblasts exhibit impaired adhesion-mediated Erk activation [305]. In the present study, we provide evidence to explain how loss of PTP1B can lead to attenuation of Erk activation by way of impaired Ras signaling.

Stimulation of our TAg immortalized cells with PDGF resulted in enhanced tyrosine phosphorylation of cellular proteins in PTP1B knockout cells (Figure 1A). Similarly, AKT phosphorylation was also increased (Figure 1B), but PDGF-stimulated Erk activation was decreased in PTP1B knockout cells. In contrast, Haj et al. reported only minor differences in Erk and AKT activation using independently established PTP1B knockout cell lines [300]. One possible reason for this discrepancy is that those studies used high levels of PDGF (50ng/ml), which may not allow for detecting subtle differences. Indeed, this situation has been shown for ShcA knockout cells [438]. Thus, in our studies, we used lower amounts of PDGF (10ng/ml). When we increased the levels of PDGF to 25ng/ml, the differences we observed were diminished (data not shown).

Analysis of cell growth demonstrated that PTP1B is required for efficient proliferation of immortalized TAg fibroblasts (Figure 2). Furthermore, we also noticed that PTP1B knockout cells had increased levels of the PDGFR (Figure 3A), which suggested that Ras activity was lower in these cells [349]. Consistent with this notion,

Ras activity has been demonstrated to be essential for transformation by TAg in several model systems [434, 439, 440].

Profiling of key proteins involved in the Ras / MAPK pathway revealed an increased expression of p120RasGAP that was due to up-regulated transcription / message stability (Figure 4). The significance of this finding is underscored by the ability of p120RasGAP to negatively regulate Ras activity by promoting the intrinsic GTPase activity of Ras [435]. In addition, p62Dok, a binding partner of p120RasGAP, was found to be a potential substrate of PTP1B (Figure 5). Previously, an unidentified phosphorylated 60-kDa protein was shown to be a potential substrate of PTP1B during epidermal growth factor signaling in COS cells [167]. It is likely that this protein is also p62Dok. Importantly, p62Dok is a negative regulator of MAPK signaling [358, 359], and tyrosine phosphorylation of p62Dok can contribute to its inhibitory effect on Ras [436]. Collectively, this finding suggested that loss of PTP1B in TAg cells leads to impaired Ras signaling.

In cells with high levels of Src activity, p62Dok has been shown to bind Csk, a negative regulator of Src kinases [441]. The binding is thought to recruit Csk to cytoskeletal compartments to attenuate Src activity. Thus, it is intriguing to speculate that increased p62Dok phosphorylation in PTP1B knockout cells may contribute to decreased Src activity that was previously observed during fibronectin signaling [305]. More studies will be required to verify this hypothesis.

If the impaired Ras signaling in PTP1B-deficient cells was due to p120RasGAP and p62Dok, then V12Ras should be able to transform these cells similar to that of WT controls. Indeed this is the case, and V12Ras-transformed PTP1B-deficient clones actually grow slightly better in soft agar (Figure 5C). One possibility is that these clones possess Ras-GTP levels slightly higher than their WT counterparts (Figure 5A). Alternatively, PTP1B-deficient cells also display enhanced AKT activity, which may

promote a survival advantage. Nevertheless, our results show that V12Ras can transform cells in the absence of PTP1B.

Taken together, a model can be put forward to explain the impaired Erk activation seen in TAg immortalized PTP1B-deficient fibroblasts (Figure 6). Loss of PTP1B leads to increased RTK phosphorylation and enhanced signaling of most downstream pathways. However, loss of PTP1B can also lead to cellular alterations that attenuate Erk signaling downstream of RTKs. For example, loss of PTP1B can lead to decreased Src activation by way of increased phosphorylation of the inhibitory site [305]. In addition, loss of PTP1B leads to increased expression of p120RasGAP by way of an unidentified mechanism. Finally, p62Dok, a potential substrate of PTP1B, is hyperphosphorylated in PTP1B-deficient fibroblasts. All these events can contribute to attenuate Ras activity and thus Erk signaling. Consistent with this model, introduction of V12Ras into PTP1B-deficient cells can bypass these inhibitory events on Ras signaling and action.

In addition to the plasma membrane, a recent study demonstrated that Ras activation and signaling can occur at the endoplasmic recticulum (ER) [442]. PTP1B predominantly localizes at the ER where it has been suggested to act on internalized PDGFRs and epidermal growth factor receptors [218, 301]. It will be interesting to determine if loss of PTP1B results in impaired Ras signaling globally, or in specific subcellular compartments. Targeted expression of PTP1B within knockout cells may provide further insight into this issue.

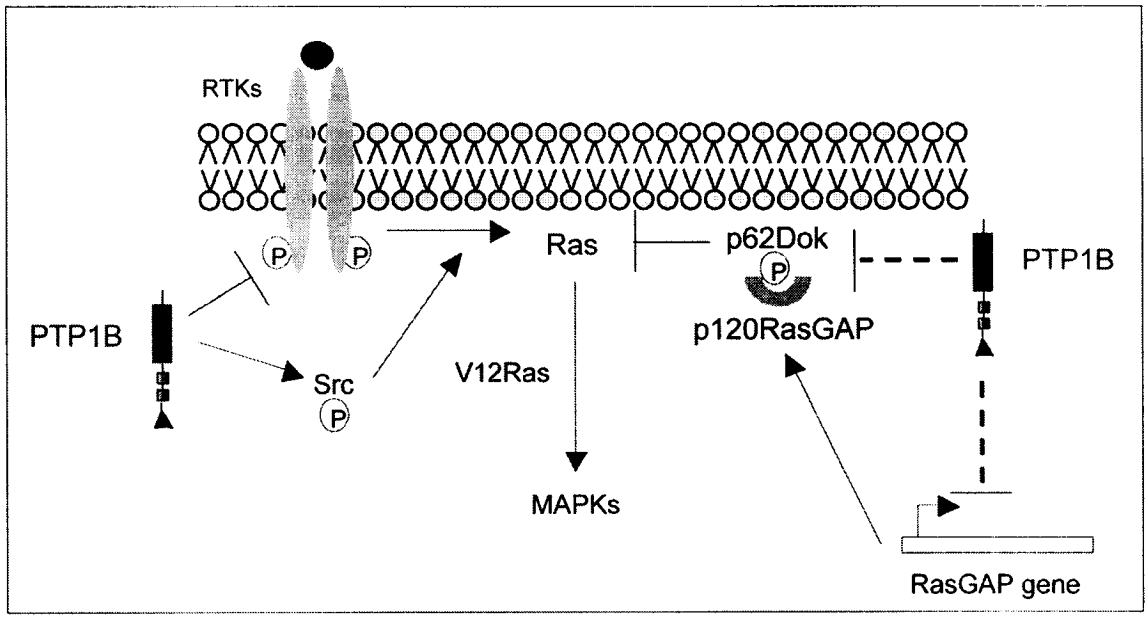


Figure 6. Model of impaired Ras signaling in PTP1B-deficient fibroblasts. Loss of PTP1B leads to increased RTK phosphorylation and enhanced signaling of most downstream pathways. However, the absence of PTP1B can also lead to cellular alterations that attenuate MAPK signaling downstream of RTKs. For example, PTP1B deficiency can lead to decreased Src activation by way of increased phosphorylation of inhibitory site. In addition, loss of PTP1B leads to increased expression of p120RasGAP by way of an unidentified mechanism. Finally, p62Dok, a potential substrate of PTP1B, is hyperphosphorylated in PTP1B-deficient fibroblasts. All these events can contribute to attenuate Ras activity and thus MAPK signaling. Consistent with this model, introduction of activated Ras into PTP1B null cells can bypass these inhibitory events on Ras signaling and action.

If loss of PTP1B leads to impaired Ras signaling, then what is the role of PTP1B during tumorigenesis? Approximately 30% of human cancers harbor activating mutations in the Ras gene [347]. Because most of these mutations render Ras resistant to the actions of p120RasGAP, Src, and p62Dok, it is unlikely that loss of PTP1B would affect the Ras activity in this subset of cancer. However, loss of PTP1B does lead to increased IGF-I-induced AKT / protein kinase B activity [302], and, in this case, inhibition of PTP1B could offer an increased survival advantage to the transformed cells.

Breast cancer provides an interesting aspect in that rarely are Ras mutations found [443]. In fact most breast cancer cases are associated with increased expression of Src and members of the epidermal growth factor receptor family. Importantly, PTP1B has been identified as one of the major phosphatases that activate Src in breast cancer cells [209]. Furthermore, increased expression of PTP1B has also been demonstrated in transformed human breast cells [204] and ovarian carcinomas [205]. This result raises the intriguing possibility that PTP1B may positively contribute to the progression of these cancers by way of activation of Src. It will be interesting to determine the effects of introducing the PTP1B null background into transgenic models of breast cancer [444].

In conclusion, we have identified mechanisms by which PTP1B deficiency can actually lead to impaired Ras signaling and proliferation. Our results suggest that decreasing Ras activity through inhibition of PTP1B could even provide a means to treat a subset of cancers.

4.6 Acknowledgements

We would like to thank Drs. Morag Park, Pankaj Tailor, Christophe Blanchetot, and Feng Gu for helpful discussions. N.D. and A.C. are recipients of a Canadian Institutes of Health Research doctoral research award and studentship, respectively. M.L.T. is a Canadian Institutes of Health Research Scientist. This work was supported by a Cancer Research Society (CRS) and Canadian Institutes of Health Research (MOP-62887) operating grants to M.L.T.

5 Chapter 5: Genetic ablation of PTP1B accelerates tumorigenesis and modifies the tumor spectrum of p53 null mice: potential role for PTP1B in B cell development

5.1 Abstract

Protein tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed enzyme that is involved in multiple signaling pathways by exerting a negative regulation on various protein tyrosine kinases, such as EGFR, PDGFR, IR, IGF-1R, and Src. Of particular interest, gene-targeting studies in mice have established PTP1B as a critical physiological regulator of metabolism by attenuating insulin, leptin and growth hormone signaling. Indeed, PTP1B null mice exhibit resistance to diet-induced diabetes and obesity. Although PTP1B is involved in signaling pathways that contribute to oncogenesis, PTP1B null mice do not develop spontaneous tumors. Since the majority of human cancers harbor mutations in p53, we generated p53/PTP1B double null mice to study the role of PTP1B in tumorigenesis. In the absence of p53, PTP1B heterozygous and null mice display decreased survival rates with the presence of an earlier tumor burden. Compared to p53 null PTP1B wild-type and p53 null PTP1B heterozygous mice, double null mice showed an increased susceptibility towards the development of lymphomas, and specifically towards B-cell lymphomas, suggesting a role for PTP1B in hematopoiesis. Flow cytometry analysis of hematopoietic tissues of PTP1B null mice revealed an increase in the absolute number of B cells in bone marrow due to an accumulation of immature cells. Colony-forming assays in the presence of IL-7 confirmed the increase in precursor B cells in PTP1B null mice. Additionally, B cells are resistant to apoptosis and accumulate in lymph nodes and blood of PTP1B null mice. All together, these results suggest that PTP1B is an important determinant of the latency and type of tumors in a p53 deficient background, and that it regulates B cell differentiation.

5.2 Introduction

The protein tyrosine phosphatases (PTPs) form a superfamily of at least 100 members [12]. Together with the protein tyrosine kinases (PTKs), they modulate the cellular level of tyrosine phosphorylation, and regulate many cellular events such as differentiation, cell growth, motility, and proliferation [13]. Therefore, deregulation of the PTP activity can lead to aberrant signaling that can contribute to the development of various diseases in human, such as cancer, diabetes, inflammation, and autoimmunity [170, 171]. Numerous biochemical and genetic studies suggest a role for the PTPs in transformation and cancer, either acting as oncogenes or tumor suppressors [172-174].

Protein tyrosine phosphatase 1B (PTP1B) is the prototype for the superfamily of PTPs, and has been the most extensively studied within the group. It was the first mammalian PTP identified and purified to homogeneity [210]. This enzyme is widely expressed and localizes predominantly to the endoplasmic reticulum (ER), through a small hydrophobic stretch [218]. Biochemical and substrate trapping studies have implicated PTP1B in the attenuation of various PTK signaling pathways, including the epidermal growth factor (EGF), platelet derived growth factor (PDGF), insulin, and insulin growth factor-1 (IGF-1) receptors [10, 146, 161, 162, 167, 230, 231, 300-302], as well as kinases such as Src, p210Bcr-Abl, JAK2 and TYK2, as well as the transcription factor STAT5 [163, 164, 208, 209, 295, 303, 305, 306, 309, 429], thus associating PTP1B with oncogenic, metabolic, and cytokine signalling [445].

As such, the generation of PTP1B deficient mice has shown that while this enzyme is dispensable for embryonic development, it is important for metabolic control [161, 162]. Despite their potentially enhanced oncogenic signaling through growth factor receptors, PTP1B deficient mice do not spontaneously develop any tumors. Interestingly though, both increased and decreased levels of PTP1B have been

observed in different human tumors [204-209], but strong genetic evidence for a role of PTP1B in cancer is still lacking. Therefore, we introduced a PTP1B deficiency in p53 null mice to study the role of PTP1B in tumorigenesis.

The p53 gene encodes a transcription factor that regulates the expression of cell cycle controlling genes [364, 369, 370], thus regulating normal cell proliferation and apoptosis [365, 371-373]. Indeed, p53 is the most frequently disrupted gene in cancer; almost 50% of human cancers contain a p53 mutation, including tumors of the colon, breast, lung, and brain [378, 379]. Germ line mutations in the p53 gene have been observed in people affected by the Li-Fraumeni syndrome [383]. These individuals are at risk for the development of a number of tumors, including soft-tissue sarcomas, osteosarcomas, brain tumors, breast cancers, and leukemias [384-386]. Inactivation of the p53 gene in mice by gene targeting has resulted in the generation of developmentally viable mice but susceptible to the development of early spontaneous tumors [387-391]. The most frequent tumor in p53 null mice is thymic lymphoma [387-389, 393, 394].

Here, we report that in the absence of p53, PTP1B heterozygous and null mice display decreased survival rates with the presence of an earlier tumor burden. Mice lacking PTP1B in a p53 null background were more susceptible to develop lymphomas. Moreover, compared to p53 -/- PTP1B +/+ and p53 -/- PTP1B +/- littermates, p53/PTP1B double null mice developed a higher percentage of B-lymphomas, suggesting that PTP1B regulates hematopoietic function. Indeed, PTP1B null mice showed an increase in the absolute number of B cells in bone marrow, where immature B cells (IgM^-IgD^-) accumulated. Colony-forming assays in the presence of IL-7 confirmed the increase in pre-B cells progenitors in PTP1B null mice. Additionally, peripheral B cells showed increased resistance to apoptosis and accumulated in lymph nodes and blood of PTP1B null mice. All together, these results indicate that PTP1B is an important determinant of the latency and type of tumors in a p53 deficient

background. Our findings demonstrate that PTP1B regulates B cell development and acts as a tumor suppressor gene in this specific context. To our knowledge, this is the first study to report the role of PTP1B in hematopoiesis and cancer *in vivo*.

5.3 Results

5.3.1 The absence of PTP1B decreases survival of p53 null mice

To explore the role of PTP1B in tumorigenesis, we generated p53 and PTP1B double knockout mice (p53/PTP1B DKO) by breeding the two lines, and we monitored tumor formation and survival of these mice (Figure 1). By 20 weeks of age, 60% of the p53 -/- PTP1B +/+ mice had tumors, compared to 72% of the p53 -/- PTP1B +/-, and 89% of the p53 -/- PTP1B -/-, clearly showing that the absence of PTP1B potentiates tumor development of p53 -/- mice (Figure 1). Out of a study of 20 p53 -/- mice, 75% developed obvious neoplasms by 25 weeks of age, in accordance with the data previously reported by Donehower et al. [387]. Some tumors appeared as early as 12 weeks, and tumor occurrence increased rapidly between 20 and 25 weeks of age. Thus, the mean time to tumor appearance was 21 weeks. Of the 43 p53 -/- PTP1B +/- mice analyzed in the study, 72% did not survive past 20 weeks, and the mean time of tumor occurrence was 18.5 weeks. Finally, the 54 p53 -/- PTP1B -/- mice had a mean time of tumor appearance of 16.5 weeks, and tumor occurrence increased very rapidly between 14 and 18 weeks of age. 89% did not survive past 20 weeks of age. By 25 weeks of age, all p53 -/- PTP1B -/- mice developed tumors and had to be sacrified. Hence, the rate of tumor development was clearly accelerated in p53 null mice lacking one or two alleles of PTP1B, suggesting that PTP1B is an important determinant of tumor onset in p53 null background.

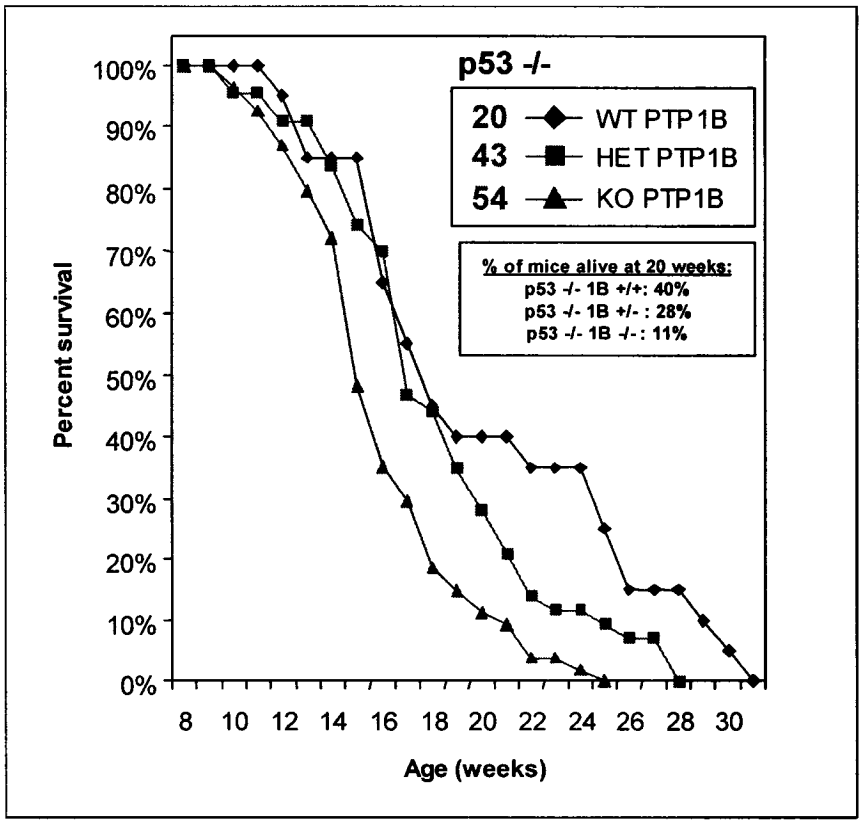


Figure 1. The absence of the PTP1B gene decreases survival of p53 null mice, and accelerates the rate of tumor development. p53/PTP1B double null mice were obtained by breeding the two lines. All mice were on a C57Bl/6XBalb/C mixed background. Tumor formation was followed and mice were sacrified upon tumor appearance, according to animal ethical guidelines. Mice were born with the expected Mendelian ratio (data not shown). n = number of mice studied.

5.3.2 Increased incidence of lymphomas in p53/PTP1B double null mice

We then characterized the type of tumors encountered in p53 -/- PTP1B +/- and p53 -/- PTP1B -/- mice. Hematopoietic tissues such as thymus, lymph nodes, bone marrow, and lymphocytes isolated from blood were analyzed by flow cytometry using T cell (anti-TCRb, CD4, CD8 antibodies) and B cell (anti-CD19 or B220, IgD antibodies) specific markers. At the time of necropsy, two major types of lymphomas were observed. The first type, thymic lymphoma, was invariably of T-cell origin. The second type, peripheral type, was of B-cell origin, and often arose from mesenteric lymph nodes and spleen, as well as in all of the major peripheral lymph node groups. Splenomegaly frequently accompanied the peripheral lymphoma type, and in some cases, the enlarged spleen filled much of the abdominal cavity. Most of the thymic lymphomas were $CD4^{+}CD8^{+}$ (Figure 2A), indicating that they arose from the transformation of relatively immature thymocytes [446]. We also observed some cases where the cells were $CD4^{-}CD8^{+}$ (Figure 2A), suggesting a more differentiated stage. The peripheral lymphomas of B-cell origin were all positive for the B cell markers CD19 or B220, and B cell accumulation was observed in the lymph nodes (axillary and mesenteric) (Figure 2B), as well as in the bone marrow and blood (Figure 2C). In addition, the majority of the B cell content in the lymph nodes stained negative for IgD (data not shown), suggesting an accumulation of immature B cells in the periphery. The mice that exhibited soft or solid tumors without the presence of lymphomas were classified as "other tumors".

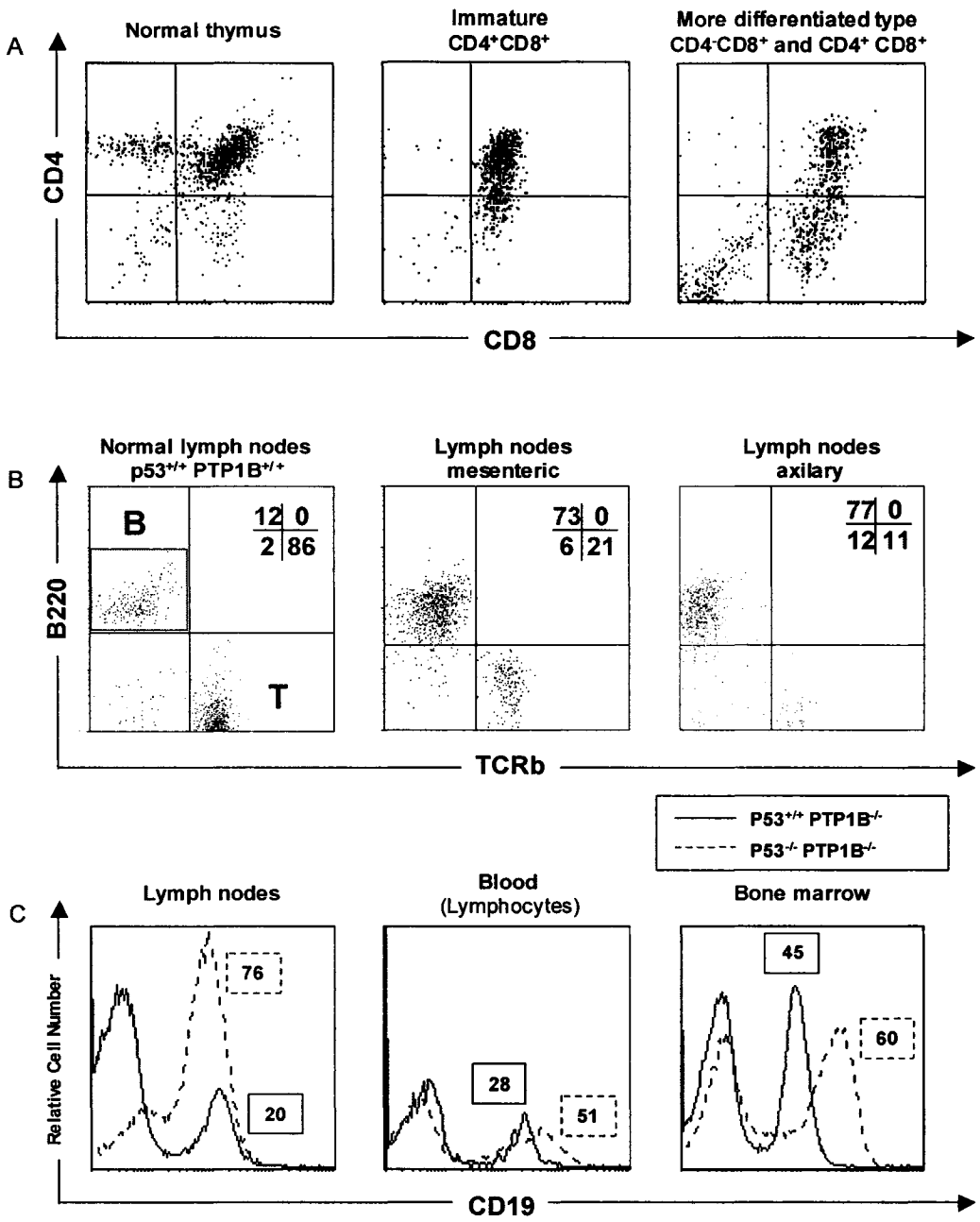
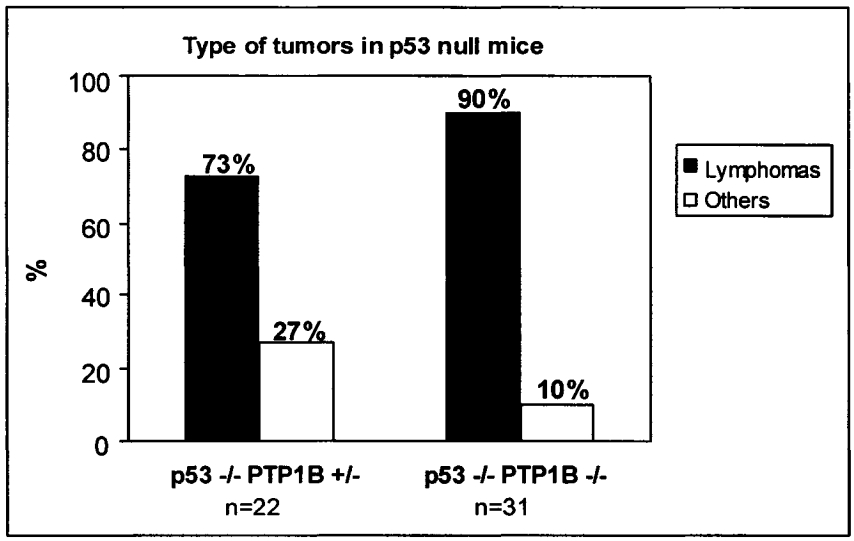


Figure 2. Modification of the tumor spectrum of p53 null mice lacking PTP1B.
A) Characterization of thymic lymphomas. CD4/CD8 profiles of normal p53+/+ PTP1B +/+ and tumorigenic thymus obtained from p53 -/- PTP1B +/- mice.
B, C) Characterization of peripheral lymphomas of B origin derived from p53/PTP1B double null mice. B cells were stained using anti-B220 or anti-CD19 antibodies. Control lymph nodes were obtained from p53+/+ PTP1B +/+ in B), and control hematopoietic organs from p53+/+ PTP1B -/- in C).

5.3.3 Higher proportion of B lymphomas in p53/PTP1B double null mice

Donehower et al. and others previously reported that 70% of the p53 null mice developed lymphomas, 70-75% of which were of T-cell origin, and contained primarily immature ($CD4^+CD8^+$) T-cells, whereas the remaining, which originated in the spleen and peripheral lymph nodes, were characterized as B-cell lymphomas [387-389, 393, 394]. Similarly, we found that 73% of p53 -/- PTP1B +/- mice developed lymphomas, and the 27% remaining proportion of mice exhibited solid tumors (Figure 2D). We observed that 77% the lymphomas of the p53 -/- PTP1B +/- mice were of thymic origin, and the remaining 23% were B-lymphomas (Figure 2E). Thus, p53 -/- PTP1B +/- mice develop lymphomas to the same extent as p53 null mice, but they arise earlier in absence of one allele of the PTP1B gene, at 18 weeks on average. Interestingly, 90% of the double null p53/PTP1B mice developed lymphomas (Figure 2D), where 50% originated from T-cell and 50% from B-cell (Figure 2E). The T lymphomas occurred on average at 17.4 weeks, whereas the B lymphomas occurred at 15.7 weeks. These results suggest that the absence of the PTP1B gene in a p53 deficient background results in an increased proportion of lymphomas, specifically of B-cell origin, and earlier appearance of the tumor burden.

D



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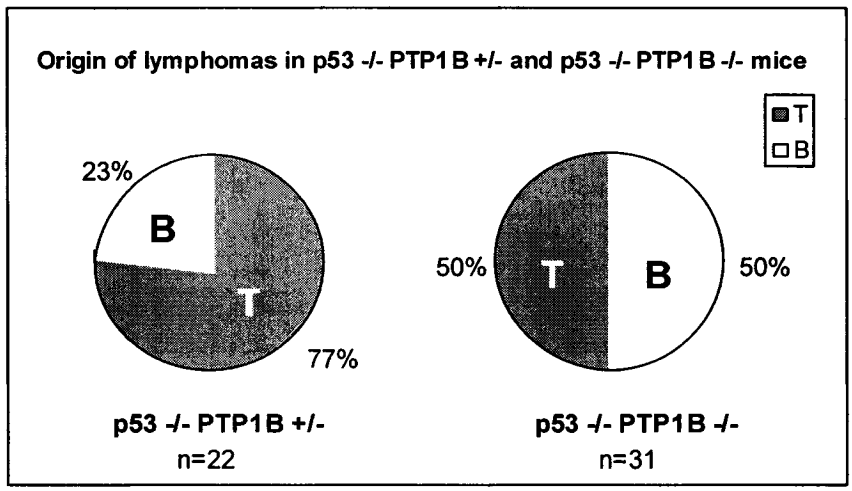

Figure 2. Modification of the tumor spectrum of p53 null mice lacking PTP1B. Cont'd
D) Tumorigenesis in p53/PTP1B mice: tumor classification of p53 -/- PTP1B +/- and p53 -/- PTP1B -/- mice. Lymphomas were characterized by flow cytometry or immunohistochemistry. The remaining tumors were classified as "others", and correspond to all non-lymphoma tumors (soft and solid tumors independently of the site of origin). n = number of mice studied.
E) Proportion of T vs B lymphomas in p53 -/- PTP1B +/- and p53 -/- PTP1B -/- mice.

5.3.4 PTP1B is expressed in hematopoietic tissues

Following the observation that a higher proportion of p53/PTP1B double null succumbed to lymphomas, and especially of B-cell origin, we next examined whether PTP1B null mice presented hematopoietic abnormalities, since immune defects could contribute to the accelerated rate of tumor development and to the shift in T to B lymphoma ratio. Given that Donehower et al. reported that p53 null mice presented normal numbers of B and T cells in thymus and spleen, as well as normal levels of IgA, IgM, and IgG subclasses, and they were are able to mount a normal humoral immune response to an antigen test [387], we focused our analysis on the hematopoietic function of PTP1B null mice.

PTP1B is a ubiquitously expressed enzyme but its pattern of expression in hematopoietic tissues has not been confirmed yet. Therefore, tissues and cells were isolated from adult PTP1B WT and KO mice, and expression was verified by immunoblotting using an antibody against PTP1B. As shown on Figure 3, PTP1B is expressed in stromal cells derived from the bone marrow of 7-day-old WT pups, as well as in adult bone marrow, lymph nodes, spleen, thymus, and splenic B cells. These results demonstrate that PTP1B is expressed in all hematopoietic tissues.

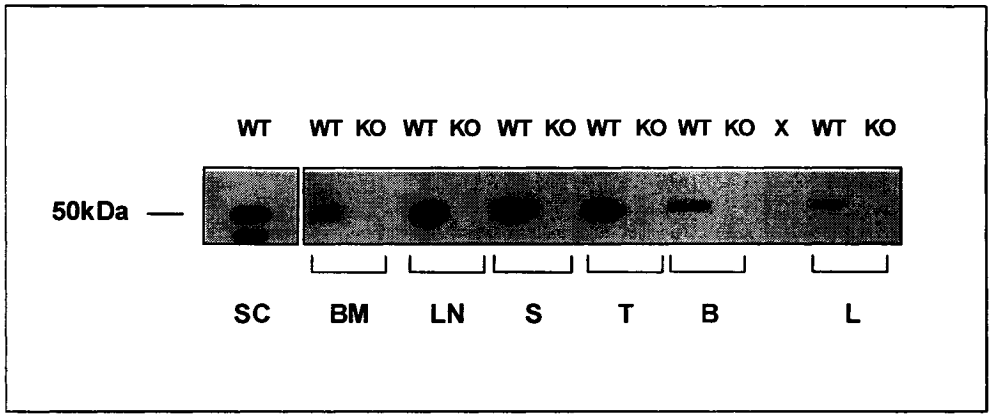

Figure 3. PTP1B is expressed in hematopoietic tissues and cells. Western blot analysis of stromal cells, bone marrow, lymph nodes, spleen, thymus, splenic B cells isolated from spleen, and liver shows that PTP1B is expressed in hematopoietic tissues and cells. Tissues were lysed in M-RIPA and lysates were resolved by SDS-PAGE and immunoblotting using anti-PTP1B antibody. SC: stromal cells, 20μg; BM: bone marrow, 60μg; LN: lymph nodes, 10μg; S: spleen, 10μg; T: thymus, 10μg; B: spleenic B cells, 10μg; L: liver, 20μg.

5.3.5 Accumulation of immature B cells in bone marrow of PTP1B null mice

The observation that p53/PTP1B double null mice exhibited a higher proportion of B lymphomas prompted us to verify the number and developmental stage of B cells in the bone marrow of PTP1B null mice. Bone marrow cells were isolated and stained with anti-CD19 or B220 antibodies, and analyzed by flow cytometry. As shown in Figure 4A, PTP1B null mice displayed a significant 18% increase in the proportion of B lineage cells ($p < 0.02$). To examine whether the absence of PTP1B altered B cell differentiation, we stained the bone marrow cells with antibodies against B220, IgM, and IgD, developmental markers of B cells. A significant 15% increase was observed in the immature B cell fraction (R3; IgM^-IgD^-) ($p < 0.004$) (Figure 4B). Thus, the absence of PTP1B causes an accumulation IgM^-IgD^- immature B cells in the bone marrow.

As depicted on Figure 4B, the mature population of B cells (R5; IgM^+IgD^{++}) is decreased PTP1B null bone marrow. To confirm this observation, we stained the cell suspension with anti-CD19 and anti-IgD antibodies. Two distinct IgD^+ B cell populations

are observed in WT mice (Figure 4C). In contrast, these populations are decreased by 32% and 57% ($p < 0.004$) respectively in the bone marrow of PTP1B null mice, averaging 45% in total.

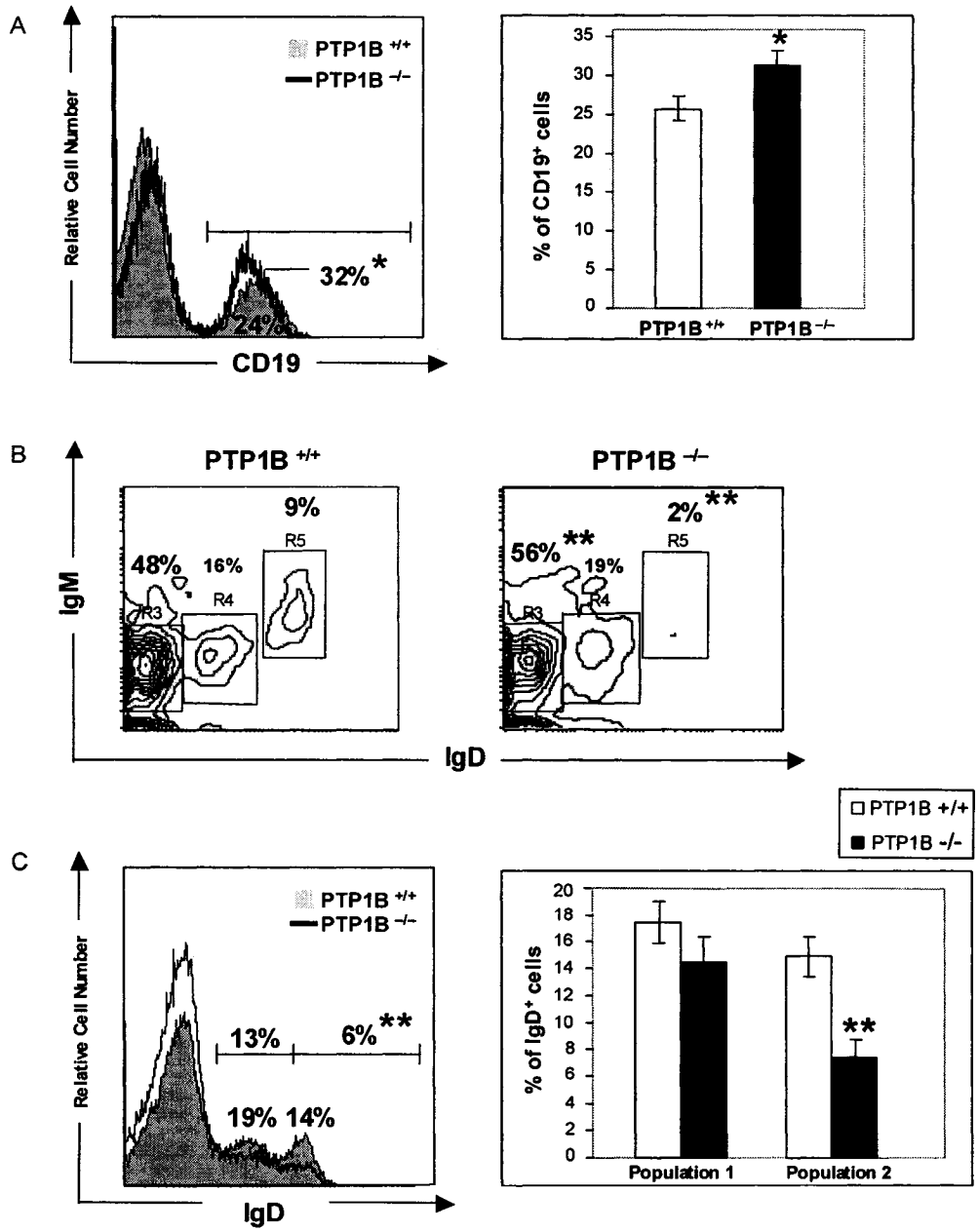

Figure 4. Accumulation of immature B cells in bone marrow of PTP1B null mice.
A, B, C) Flow cytometry analysis of antibody-stained bone marrow cell suspensions isolated from PTP1B +/+ and -/- mice. Representative of five independent experiments, with at total of n = 12-14 mice per group. * $p < 0.02$; ** $p < 0.0004$.

5.3.6 Increased pre-B colony formation in bone marrow of PTP1B null mice

To confirm the increase in progenitor B cells in PTP1B null bone marrow, colony-forming assays were performed using bone marrow cells harvested from WT, heterozygous, and PTP1B null 6-7 weeks mice. The cells were grown in 1% methylcellulose supplemented with 10ng/ml interleukin-7 (IL-7), which induces the growth of pro-B and pre-B colonies (StemCell Technologies). Nearly twice as many colonies were counted in PTP1B +/- as in +/+ bone marrow cultures ($p < 0.003$). Moreover, cells isolated from PTP1B null bone marrow formed three times more colonies than +/+ cells ($p < 0.003$) (Figure 5A), showing that the defect is in part cell autonomous. Flow cytometry analysis of B220, CD43, and CD25 antibody-stained colonies confirmed their identity as pre-B cells, $CD43^+CD25^+$ and $CD43^+CD25^-$ (Figure 5B). In all cases, over 99% live cells were B220 positive (data not shown).

To determine whether the increased B cell numbers in PTP1B null bone marrow are due to higher rates of cell proliferation or to an increased resistance to apoptosis, Annexin V staining, a marker of apoptosis, was performed on freshly isolated bone marrow cells. No difference was observed in the percentage of Annexin V positive cells in WT (average 11%) and PTP1B null (average 9%) $CD19^+$ cells (Figure 5C), suggesting that the increase of B cell numbers in PTP1B null mice bone marrow results from an increased number of progenitors of B cells and not from an alteration in the resistance of B cells to apoptosis. Therefore, it is possible that the increased number of B cell precursors observed in colony assays in PTP1B null bone marrow accounts for the increased incidence of B lymphomas in p53/PTP1B double null mice. For instance, Wnt5a heterozygous mice are susceptible to the development of B lymphomas with age, and Wnt5a has been reported as a negative regulator of B cell proliferation and it inhibits the response of B cells to IL-7 [447].

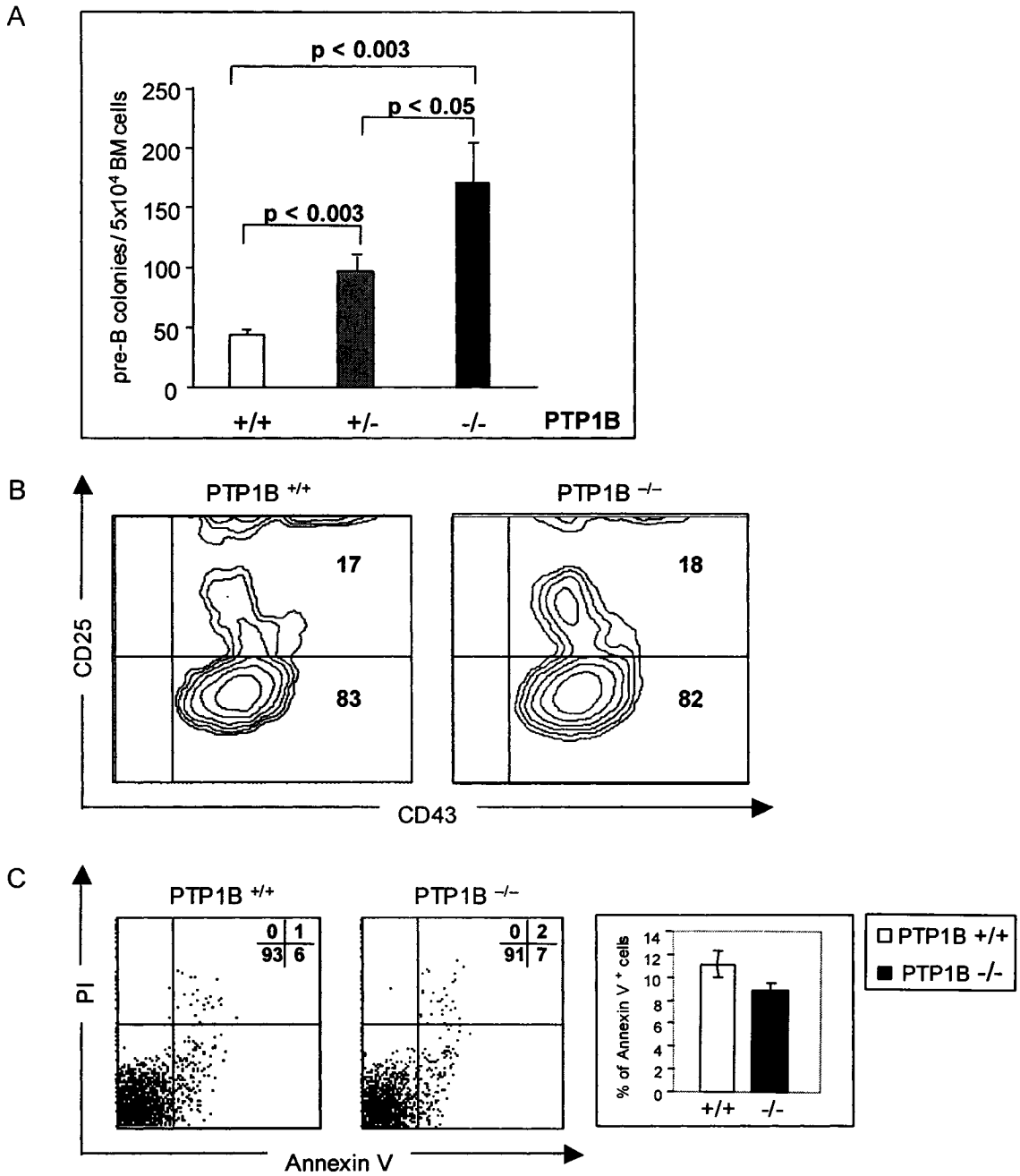

Figure 5. Increased pre-B colony formation in bone marrow of PTP1B null mice
A) Methylcellulose colony-forming assay for B cells in IL-7. Representative of three independent experiments, with a total of n =3-4 mice (6-7 weeks old) per group. The results are given as the number of colonies formed per $5x10^4$ cells. The error bars represent SEM.
B) Representative flow cytometry analysis of pre-B colonies gated on $B220^+$ cells and then analyzed for CD25 and CD43 expression. In both cases, over 99% live cells were B220+.
C) Apoptosis assay using Annexin V staining of freshly isolated WT and PTP1B null bone marrow cells. Apoptotic index was determined by counting the number of Annexin V positive cells. Representative of three independent experiments, with a total of n = 8-9 mice per group.

5.3.7 Increased proportion of B cells in peripheral lymph nodes of PTP1B null mice

We next determined if B cells accumulated in peripheral lymphoid organs such as lymph nodes, blood, and spleen of PTP1B null mice. To verify B/T cell ratio, cell suspensions of lymph nodes and spleen, or peripheral blood lymphocytes from adult mice (7-14 weeks) were stained with anti-CD19 and anti-TCRb antibodies, and analyzed by flow cytometry. In PTP1B null mice, a 40% increase in the proportion of B lineage cells was observed in the lymph nodes and blood (Figure 6A and B). However, PTP1B null mice displayed a normal B/T cell ratio in the spleen (Figure 6C). Additionally, we determined whether the maturity of B cells was modified in these organs by staining the cell suspensions with anti-CD19 and anti-IgD antibodies. These cells appeared to express IgD in equivalent proportions in the lymph nodes, blood, and spleen (Figure 6D).

To verify if resistance to apoptosis caused the accumulation of B cells in the lymph nodes of PTP1B null mice, we stained cells harvested from lymph nodes with Annexin V and propidium iodide, and found that B cells lacking PTP1B exhibited a 50% reduction in apoptosis (on average WT 20% vs KO 10%; $p < 0.002$) (Figure 6E). Therefore, B cells accumulate in lymph nodes of PTP1B null mice and are more resistant to apoptosis. In contrast, the apoptotic index of B cells is unchanged in the spleen of PTP1B null mice (21%) compared to WT controls (19%) (Figure 6F).

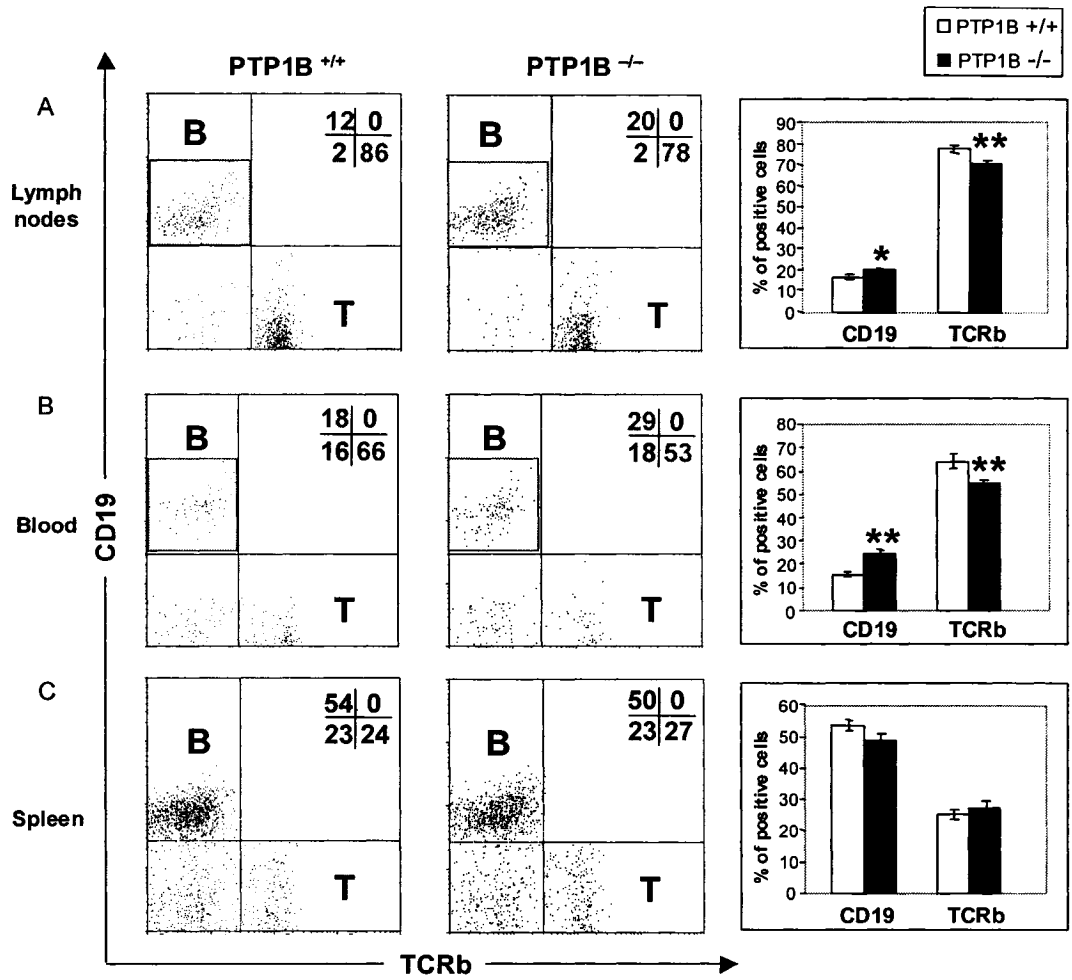

Figure 6. Abnormal B/T cells ratio in lymph nodes and blood, but not in spleen of PTP1B null mice.
A - C) Flow cytometry analysis of antibody-stained lymph nodes, lymphocytes isolated from blood, and spleen cell suspensions isolated from WT and PTP1B null mice. Representative of at least three independent experiments, with a total of n = 8-11 mice per group. * $p < 0.02$; ** $p < 0.007$.

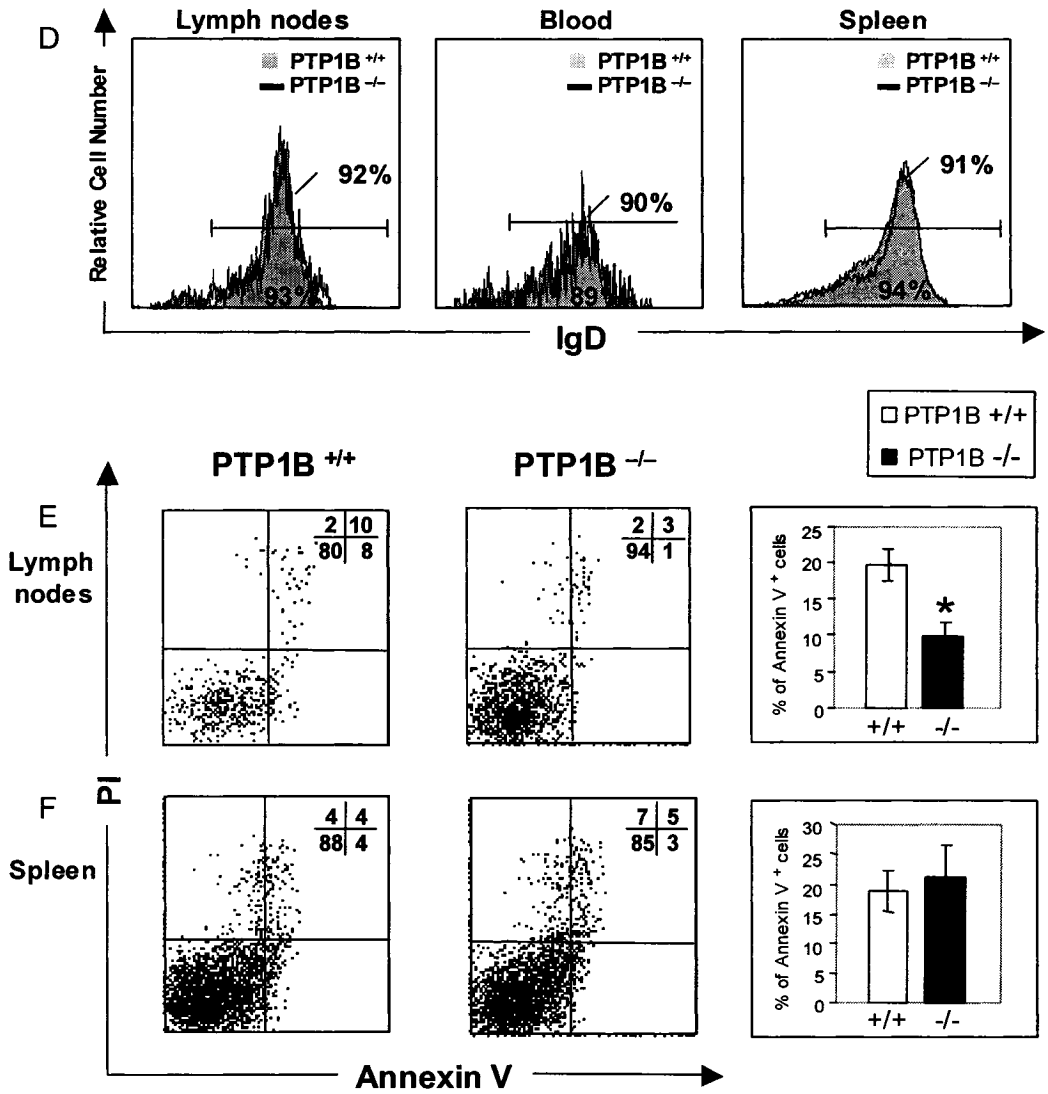

Figure 6. Abnormal B/T cells ratio in lymph nodes and blood, but not in spleen of PTP1B null mice. Cont'd

D) Flow cytometry analysis of antibody-stained lymph nodes, lymphocytes isolated from blood, and spleen cell suspensions isolated from WT and PTP1B null mice. Representative of at least three independent experiments, with a total of n = 8-18 mice per group.

E, F) Annexin V staining of lymph nodes and spleen cell suspensions. Apoptotic index was determined by counting the number of Annexin V positive cells. Representative of three independent experiments, with a total of n = 9-11 mice per group. * $p < 0.002$.

5.3.8 PTP1B null mice present lymphadenopathy and splenomegaly

Although it was never reported before, we observed that adult PTP1B null mice presented enlarged lymph nodes (lymphadenopathy). Cell count from lymph nodes of 6-7 weeks male mice revealed an increase of 50% in the number of cells in PTP1B null mice (Figure 7A), confirming that the increase in the percentage of B cells was truly due to accumulation of these cells. Moreover, the size of B cells was greater at 7 weeks in PTP1B null mice (data not shown), and it increased with age. Hence, 15-week-old PTP1B null mice had 50% larger B cells than WT mice, as shown on the forward side scatter (FSC) plot gated on $CD19^+$ cells (Figure 7B).

5.4 Discussion

Previous studies have implicated PTP1B as a positive as well as a negative regulator of oncogenesis, and PTP1B expression is either upregulated or downregulated in a variety of human cancers [70, 203-209]. Furthermore, PTP1B is involved in the negative regulation of cytokine signaling, through dephosphorylation of the kinases JAK2 and TYK2 [163, 164, 295, 429], as well as STAT5 [309]. Disregulation or aberrant regulation of these kinases is involved in diseases such as lymphomas and leukemias [448-451]. Importantly, this function suggests that PTP1B could be a negative regulator of these diseases. An obvious possibility is that p53/PTP1B double null mice succumb to increased lymphomagenesis, consistent with the proposed function of PTP1B as a negative regulator of cytokine signaling. As yet, no alteration in the expression or phosphorylation of JAK2, TYK2, STAT3, and STAT5 were observed in hematopoietic tissues of these mice (data not shown). Whether specific signaling pathways are modified in absence of PTP1B in a p53 null background in vivo remains to be determined.

Using p53 deficient mice, we now demonstrate that PTP1B negatively regulates tumorigenesis and B cell development. We have generated p53/PTP1B double null mice by breeding the two lines and observed that mice lacking both genes exhibited accelerated development and increased incidence of B lymphomas. Bone marrow of PTP1B null mice contained an increased proportion of immature $IgM^{-}IgD^{-}$ B cells, and B cells accumulated the in lymph nodes, correlating with a decrease in apoptosis. Accordingly, we observed a higher number of pre-B colonies in colony-forming assay of bone marrow cells in the absence of PTP1B.

Daily, there are about $2x10^{7}$ BCR^{+} (IgM^{+}) B cells that develop in the murine bone marrow. Approximately 10% exit to the periphery, and 1-3% of these cells progress to the mature B cell pool [452]. When B cells emerge from bone marrow into the periphery, they are still functionally immature, expressing high levels of IgM but little IgD. Most of these immature cells will not survive to become fully mature B cells bearing low levels of IgM and high levels of IgD [452]. $IgM^{+}IgD^{+}$ B cells in the bone marrow are thought to be B cells that have completed their maturation process in the periphery and return to the bone marrow by recirculating within the lymph and the blood, entering the lymphoid follicles of spleen and lymph nodes. These abilities are very important for the mounting of an efficient humoral immune response [453, 454]. Therefore, the decrease in recirculating mature B cells observed in the PTP1B null bone marrow may have consequences on their immune response to bacterial or viral agents (Figure 4B and C).

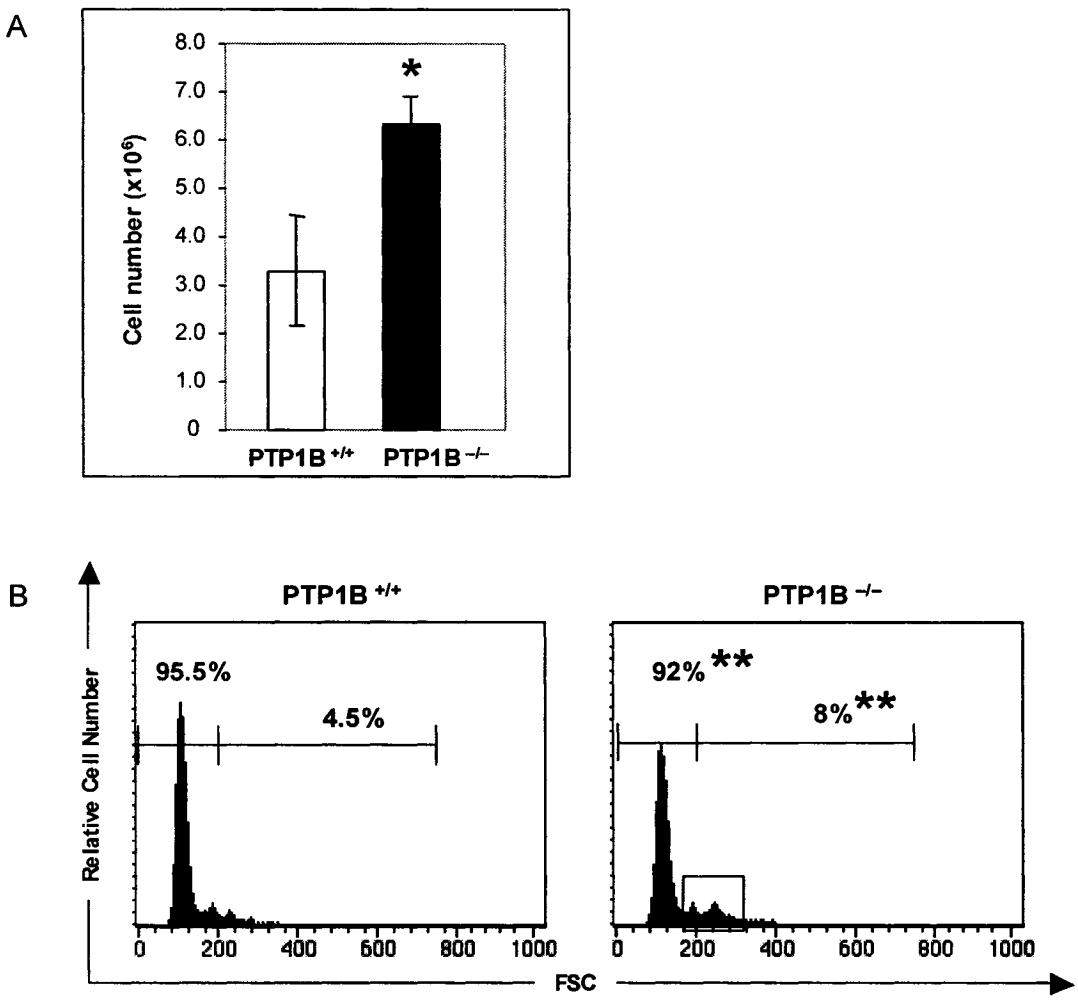

Figure 7. PTP1B null mice present lymphadenopathy.
A) Cell count of the four axillary lymph nodes from 6-7-week-old WT and PTP1B null mice (n=3-4 per group). Representative of three independent experiments. Error bars represent SEM. * $p < 0.03$.
B) Cell size analysis of $CD19^+$ cells in WT and PTP1B null mice. The foward side scatter (FSC) shows increased proportion of larger $CD19^+$ cells in 15-week-old PTP1B null mice. Representative of two independent experiments, with a total of n = 8 mice per group. ** $p < 0.05$.

An increasing amount of evidence suggests that disruption of various components of the B cell receptor (BCR) signaling pathways leads to a block in different stages of transitional B-cell development [452]. Of interest, CD45 null mice have been shown to accumulate $IgM^{high}IgD^{low}$ late transitional B cells [150, 151]. Similarly, bone marrow B-cell development in PTP1B null mice is normal up to the immature stage ($IgM^{low}IgD^{low}$), during which B cells accumulate. This suggests that PTP1B could be involved in BCR signaling or that BCR expression could be modified in the absence of PTP1B. IL-7 is another important player in B cell differentiation, as shown by gene-targeted mice deficient in IL-7 or IL-7R, where the loss of IL-7 / IL-7R interaction led to a severe impairment of development beyond the pro-B cell stage [455, 456] Since this cytokine receptor signals through the JAK1 and JAK3 kinases, it is unlikely that PTP1B regulates signaling downstream of this pathway. Alternatively, the levels of IL-7 and/or IL-7R could be modulated in the absence of PTP1B.

It was shown earlier that disruption of apoptosis is the only consequence of p53 loss required for the generation of aggressive murine lymphomas [457]. Furthermore, Lu et al. reported that the absence of p53 reduced pro-B cell apoptosis [458]. In this regard, Buckley et al. previously observed that loss of PTP1B does lead to resistance to apoptosis [302]. Thus, inhibition of PTP1B could provide a pro-survival signal to transformed cells, suggesting that PTP1B contributes to the maintenance of apoptosis in specific cell types. It is specifically the case in the lymph nodes of PTP1B null mice, where B cells accumulated and are resistant to apoptosis (Figure 6E), which could also explains the lymphadenopathy (Figure 7). Additionally, the consequences of removal of PTP1B on IL-7 induced cell proliferation and apoptosis is under investigation at the moment. This should give further insight on the signaling pathways involved in this process, and how does PTP1B regulate B cell proliferation/apoptosis. Therefore, it stands to reason that the removal of PTP1B in a p53 deficient background would further decrease apoptosis as observed in the lymph nodes, and, together with the

higher number of B cell progenitors in bone marrow of PTP1B null mice, these events could trigger the development of B lymphomas.

Hence, a model can be put together to explain our results. As depicted in Figure 8, we propose that PTP1B exerts a negative regulation at the immature to transitional stage by controlling the production of immature (IgM^-IgD^-) cells in the bone marrow. It is possible that the proportion of B cells that does not recirculate to the bone marrow accumulates in blood and lymph nodes of PTP1B null mice.

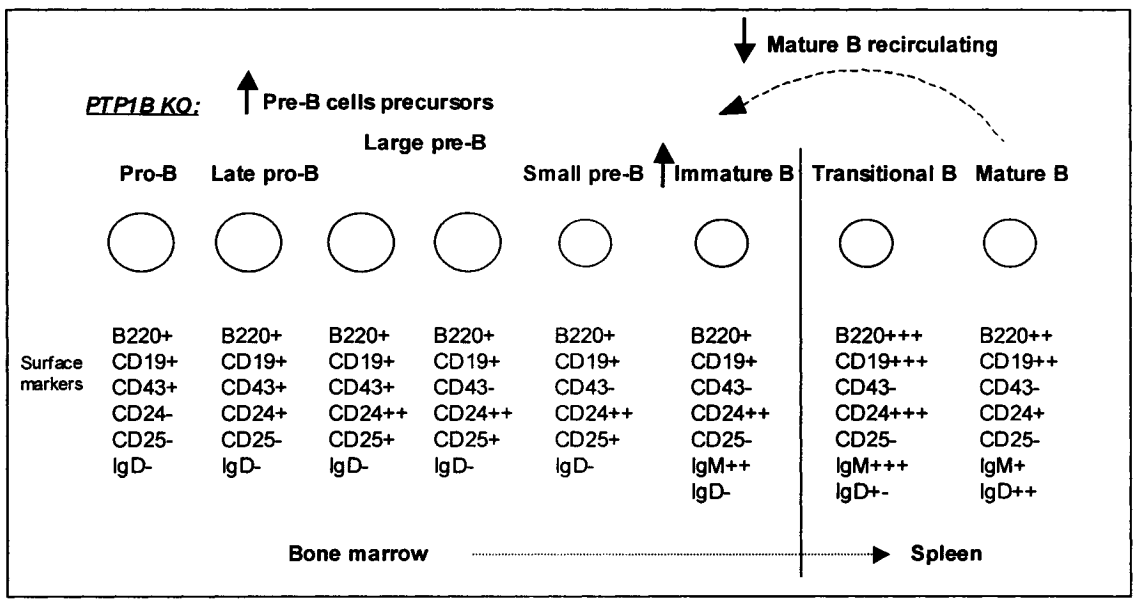

Figure 8. Proposed model of B cell development in PTP1B null mice.
Developmental stages of the various B cell types. Our results suggest that PTP1B exerts a negative regulation at the immature to transitional stage by controlling the production of immature (IgM^-IgD^-) cells in the bone marrow. Accumulation of mature B cells (IgM^+IgD^{++}) is observed in lymph nodes of PTP1B null mice. However, a decreased number of these mature B cells recirculates to the bone marrow. Adapted from Benschop and Cambier 1999 [459].

In summary, our studies reveal that PTP1B is an important determinant of the latency and tissue distribution of tumors in a p53 deficient background. Additionally, our results reveal a novel and important role of PTP1B in immune and oncogenic function. To our knowledge, this is the first demonstration of a role for PTP1B in B cell development and hematopoiesis, as well as in cancer *in vivo*. Further studies on the role of PTP1B in lymphoid cell development, proliferation, and differentiation are currently ongoing.

5.5 Experimental procedures

Reagents and antibodies

Antibodies were purchased from BD Pharmigen (anti-CD4, CD8, TCRb, CD19, B220, CD43, CD25, IgM, IgD, Fcgamma block), Biosource (anti-Annexin V), Upstate Biotechnology (anti-PTP1B), and reagents for colony-forming assay from StemCell Technologies (1% methylcellulose, and recombinant human IL-7). Tissue culture media and components were from InVitrogen (IMDM, fetal bovine serum (FBS), L-glutamine, Beta-Mercaptoethanol).

Mice

Generation of PTP1B mutant mice and genotyping was described previously [161]. Mice with a heterozygous-targeted p53 allele were obtained from Taconic. To eliminate the effects due to genetic background, we used multiple breeding pairs to generate p53-/- mice that were wild-type, heterozygous, or homozygous null for PTP1B. All animal procedures were approved by the McGill University Research and Ethic Animal Committee, and experiments were carried out under the Canadian Council on Animal Care (CCAC) ethical regulations.

Tumorigenesis studies

p53 null mice either PTP1B +/+, +/-, or -/- on C57Bl/6 X Balb/C genetic background were monitored for spontaneous tumor formation. Mice showing tumors or signs of possible lymphomas (weight lost, enlarged lymph nodes or abdomen, wasting and ruffled fur in the absence of overt tumors) were sacrified and subjected to necropsy. The thymus, spleen, and peripheral lymph nodes of each animal were examined for signs of enlargement. The tissues were either placed in OCT for further sectioning, or cell suspensions were prepared and stained with T or B cells markers, and analyzed using a FACS Callibur (Becton Dickinson). Sections were prepared and stained with hematoxylin/eosin or B220, TCRb, and examined microscopically.

Genomic Multiplex PCR for p53 alleles

Technical advice was provided by Taconic. Forward PCR primers used were: 5' GTG GGA GGG ACA AAA GTT CGA GGC C 3' (intron 4; wild-type and null) Reverse primers used were 5' ATG GGA GGC TGC CAG TCC TAA CCC 3' (wild-type), 5' TTT ACG GAG CCC TGG CGC TCG ATG T 3' (null) and 5' CTA GTT TAC ACA CAG TCA GGA TGG 3' (control). PCR was performed in a buffer (Roche) containing 1.5 mM $MgCl_2$, 100 pmol of each primer, 0.2 mM dNTPs, and 0.4 units of Ampli*Taq* Gold DNA polymerase (Roche). Reaction conditions were as follows: denaturation at 94°C for 50 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min for 35 cycles. Products were analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. A 200 bp band from the 5' primer in intron 4 plus a 3' primer in the polII promoter in the targeting construct is diagnostic for the presence of the null allele and a wild-type allele correspond to a 600 bp band from the same 5' primer in intron 4 but with a 3' primer that is at the exon5/intron 6 junction which is present in the normal p53 gene. PTP1B mice were genotyped as previously described [161].

Preparation of cell lysate and immunoblotting

Cells were washed in ice-cold PBS and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% Nonidet P-40, 0.25% Na deoxycholate, 1mM Na_3VO_4, 50 mM NaF,) supplemented with Complete^{TM} EDTA-free protease inhibitor cocktail (Roche Molecular Biochemicals). Cell lysates were rotated end-over-end at 4°C for 10 min and cleared by centrifugation at 14000 x g for 10 min at 4°C. The protein concentration was measured by the Bradford method (Biorad). Protein samples were resolved by 8% SDS-PAGE and subjected to immunoblotting with the indicated antibodies.

Methylcellulose colony-forming assay

$5x10^4$ bone marrow cells isolated from 6-7 weeks old PTP1B WT and KO mice were plated in duplicate in 1% methylcellulose, IMDM 30% FBS, 2mM L-glutamine, 10^{-4}M Beta-ME, and 10 ng/ml recombinant human IL-7. Colonies were counted under a microscope after 7-8 days.

Flow cytometry

Single-cell suspension of total spleen, thymus, bone marrow, lymph nodes, and lymphocytes isolated from blood were prepared from 6-7 or 14-15 weeks old male mice, in PBS containing 2% FBS, and filtered through a 70-100uM cell strainer. Equal volume of cell suspension was used and incubated with Fcgamma block, as well as with the indicated antibodies, and analyzed using a FACScan or Callibur flow cytometer (Becton Dickinson). The analysis was performed using Cell Quest software (Becton Dickinson).

Lymphocyte isolation from whole blood

Lymphocytes were isolated using "Lympholyte" (Cedarlane) according to the manufacturer's instructions, and resuspended in PBS containing 2% FBS.

B cell isolation from spleen

B cells from PTP1B WT and KO mice were obtained by isolating $CD19^+$ cells using EASY Sep magnetic beads system (StemCell Technologies), according to the manufacturer's protocol.

Annexin V staining

Single-cell suspensions of lymph nodes, spleen, and bone marrow were prepared and apoptosis was determined by Annexin V staining following the manufacturer's instructions (Biosource). Cells were analyzed using a FACscan or Callibur flow cytometer (Becton-Dickinson).

Statistical analysis

Values are reported as the average of at least three independent experiments ± SEM. Statistical analysis were performed using a two-tailed, unpaired, Student's *t*-test.

5.6 Acknowledgements

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6 Chapter 6: General Discussion

The phosphorylation of proteins at tyrosine residues is an important regulatory component in a variety of biological processes including cell growth and differentiation, metabolism, and transformation. Indeed, deregulation of tyrosine phosphorylation of cellular proteins can lead to deleterious effects, including diseased states in humans. For example, overexpression or activating mutations of at least thirty PTKs have been linked to malignant transformation and cancer [2]. Additionally, other diseases also result from aberrant PTKs signaling, such as diabetes mellitus, autoimmune disease, and infectious diseases (see Table 5 for references).

Although the protein tyrosine phosphatases (PTPs) are best known as downregulators of PTKs signaling, their functions and regulation are recently beginning to be elucidated. Accordingly, the PTPs have been shown to regulate many cellular events such as differentiation, cell growth, motility, and proliferation [14]. Moreover, biochemical and genetic studies are suggesting an emerging role for the PTPs in transformation and cancer, either acting as oncogenic or tumor suppressor PTPs [160].

PTP1B is the prototype for the superfamily of PTPs, and has been the most extensively studied within the group. Biochemical studies have implicated PTP1B in multiple signaling pathways, through the dephosphorylation of a variety of growth factor receptors, including the EGFR, PDGFR, IR, and IGF-1R [10, 146, 161, 162, 167, 230, 231, 300-302], as well as kinases such as Src, p210Bcr-Abl, JAK2, TYK2, and STAT5 [163, 164, 208, 209, 295, 303, 305, 306, 309, 429], associating PTP1B with oncogenic, metabolic, and cytokine signaling. Similarly, overexpression of PTP1B in cells can attenuate signaling from these receptors.

As such, generation of PTP1B deficient mice has shown that this enzyme is dispensable for embryonic development. PTP1B deficient mice are resistant to both diet-induced diabetes and obesity [161, 162], which established PTP1B as an

important regulator of metabolism. However, the negative regulation exerted by PTP1B on several growth factor receptors, including IGF-1R [302], raises the possibility that PTP1B deficiency can potentially lead to increased oncogenic signaling through heightened signaling activity of those receptor tyrosine kinases. In spite of this, PTP1B deficient mice do not overtly develop any tumors.

Therefore, my doctoral research had focused on understanding the role of PTP1B in metabolism and tumorigenesis by using the target-disruption the PTP1B gene in mouse as a model. First, in the metabolic angle, these studies underlined the role of PTP1B in the growth hormone signaling pathway and type 1 diabetes. Then, in the cancer point of view, my results revealed a role for PTP1B in the regulation of Ras signaling pathway and p53 dependent tumorigenesis.

6.1 PTP1B as a major regulator of metabolism: additional evidence

Genetic and biochemical evidence that PTP1B is a critical regulator of metabolism have emerged in recent years. PTP1B null mice are hypersensitive to the action of insulin and are resistant to diet-induced obesity [161, 162]. Moreover, mice lacking both leptin and PTP1B are leaner than their leptin deficient counterpart, and are more sensitive to the anorexic and weight-reducing effects of exogenous leptin [163, 164]. Hence, PTP1B exerts these actions by dephosphorylating tyrosine residues involved in recruiting and activating signaling molecules [14, 221].

6.1.1 PTP1B attenuates growth hormone action *in vivo* through the dephosphorylation of JAK2 and attenuation of STAT3 and STAT5 activation: physiological consequences

Growth hormone (GH) promotes growth by acting directly on tissues such as liver and bone, and by stimulating the synthesis of IGF-1, a growth factor required for

complete postnatal development [314, 315, 397]. Most of the actions of GH are mediated by IGF-1, which is mainly secreted by the liver [314]. IGF-1 is an anabolic hormone that acts on growth and storage. In addition, GH is a major metabolic hormone, with effects on glucose, protein and lipid metabolism [313, 397].

The GH receptor (GHR) is a member of the superfamily of cytokine receptors and lack intrinsic kinase activity. Therefore, it relies on the tyrosine kinase JAK2 to get activated and to transmit signal to downstream effectors of the cascade. Given the central role of JAK2 in initiating GH signaling, identification of phosphatases involved in its deactivation is important.

In this aspect, molecular modeling studies predicted that JAK2 was a candidate substrate for PTP1B. In addition to the IR recognition, structural studies on the PTP1B enzyme by Dr. David Barford's laboratory suggested that PTP1B has a 70-fold greater affinity for tandem-pY than for mono-pY containing peptides [238]. PTP1B might preferentially recognize substrates containing two adjacent phosphorylated tyrosine residues (X-pY-pY-X) [238], and specificity of this interaction is increased when an acidic and a basic residue flank the tandem pY residues (E/D-pY-pY-R/K) [295], a motif that is exactly conserved in the activation loop of JAK2.

Afterward, our laboratory also contributed to demonstrate that both JAK2 and TYK2 kinases (that contain such tandem phosphotyrosines) were hyperphosphorylated in PTP1B-deficient fibroblasts upon interferon stimulation [295]. Physiological relevance was further demonstrated by showing that PTP1B negatively regulates JAK2 phosphorylation, which attenuates leptin signaling *in vivo* [163, 164].

Accordingly, we have shown that PTP1B interacts with JAK2 in a GH-dependent manner, and dephosphorylates tyrosine residues present in the activation loop of JAK2. This leads to further enhanced activation of STAT3 and STAT5. Additionally, some PTPs were shown to be involved in JAK2 dephosphorylation in GH-treated cells, including SHP-1, a PTP expressed predominantly in tissues and cells of

hematopoietic origin [325], and the widely expressed SHP-2 [326, 401]. TCPTP and CD45 were also shown to dephosphorylate multiple JAK family members [30, 145], but a role for CD45 in GH signaling would be limited by its near exclusive expression in hematopoietic tissues [30]. Although PTP1B is not the unique PTP that downregulates JAK2 activity, its widespread expression suggests that it could modulate the action of GH in many target tissues. The possibility that these PTPs regulate in a tissue-specific manner various aspects of JAK signaling remains to be determined.

PTP1B plays a role in GH action and signaling *in vivo* as determined by evaluating the effects of starvation on hepatic GH-induced response. Of importance, liver is a major GH target tissue in postnatal animals [314, 412]. In rodents, fasting induces a GH-resistance state, which is characterized by reduced ability of GH to induce tyrosine phosphorylation of JAK2, GHR, and STAT5, and is responsible for low circulating IGF-1 levels [413]. However, we found out that GH-induced JAK2, STAT3, and STAT5 phosphorylation were as efficient in fasted as in fed liver in PTP1B null mice (Chapter 2, Figure 6). This points out that during fasting, PTP1B null livers display significantly higher activation of these molecules than WT liver, indicating that the development of GH resistance normally associated with fasting is impaired [413, 417]. This failure resulted in a functional consequence, which is the increased induction of SOSC2, a negative feedback responsive gene. Accordingly, induction of SOCS2 appears to be particularly important in the attenuation of GH action in liver and in the whole animal [418-420]. Whether the absence of PTP1B would rescue chronic anabolic actions of GH during states normally associated with hepatic GH resistance such as inflammation, burn injury and aging [421-423] remains to be determined.

Interestingly, fasting reduces PTP1B expression in liver (Chapter 2, Figure 6), whereas levels of the closely related tyrosine phosphatase TCPTP remained unchanged. This reduction in PTP1B may be the consequence of decreased needs for negative regulation when plasma concentrations of metabolic hormones such as GH

and insulin are lower. In this context, it has been recently shown that feeding hamsters with high-fructose diet leads to increased hepatic expression of PTP1B [254]. Based on these findings, PTP1B seems to be unique among PTPs in its ability to respond to changes in metabolic stress. Further studies are needed to determine whether this regulation extends to other important metabolic tissues, and to identify the basis for altered PTP1B expression. A potential candidate in the regulation of PTP1B expression is the transcription factor Y box-binding protein-1 (YB-1). Within the PTP1B promoter, there is an enhancer sequence that serves as a binding site for YB-1 [262]. It was shown that overexpression of YB-1 in Rat 1 fibroblasts and in HepG2 cells increases the level of PTP1B protein. Inversely, depletion of YB-1 by specific antisense construct led to a 70% decrease in PTP1B expression, and resulted in increase insulin sensitivity in Rat 1 fibroblasts. Moreover, enhanced signaling through the cytokine receptor gp130, which signals through JAK2, a substrate of PTP1B, was also observed [262].

6.1.2 PTP1B: a potential survival gene?

It is interesting to note that PTP1B controls signaling pathways downstream of three major regulators of metabolism, namely insulin, leptin, and growth hormone. As yet, the PTP1B null mice live "happily ever after". Then, what is the physiological utility of PTP1B? One potential justification could be that this enzyme is part of a core of signaling molecules that are responsible to sense and respond to basic metabolic survival needs. A proposed model explores the following: in a stress period, an increase in expression and/or activation of PTP1B would be protective and contribute to survival. For example, it stands to reason that in a period of food deprivation, PTP1B activity is extremely useful; by attenuating the action of IR, glucose uptake would be reduced and thus, would prevent hypoglycemia. Similarly, during starvation conditions, attenuation of leptin action by PTP1B would reinforce hunger and the need to seek

food. Finally, as mentioned above, during starvation, mammals develop a decreased sensitivity to GH action in the liver [413]. Consequently, decreased GH action will reduce IGF-1 secretion, a situation that will favor catabolism instead of anabolism. To this extent, catabolism will promote fat burning instead of storage and would permit a longer survival. As such, the body needs catabolism to survive during a starvation period, likely to maintain available all the nutrients to peripheral tissues. Here again, the presence of PTP1B that increases GH resistance might allow mammals to survive longer in a fasted period. Therefore, in an evolutionary perspective, PTP1B, through its action on food intake, glucose uptake, lipid metabolism, body metabolic rate, as well as modulation of its expression under various metabolic stress conditions, appears to have a crucial role in maintaining body weight and homeostasis during periods of food deprivation or other metabolic and environmental stresses (Figure 1).

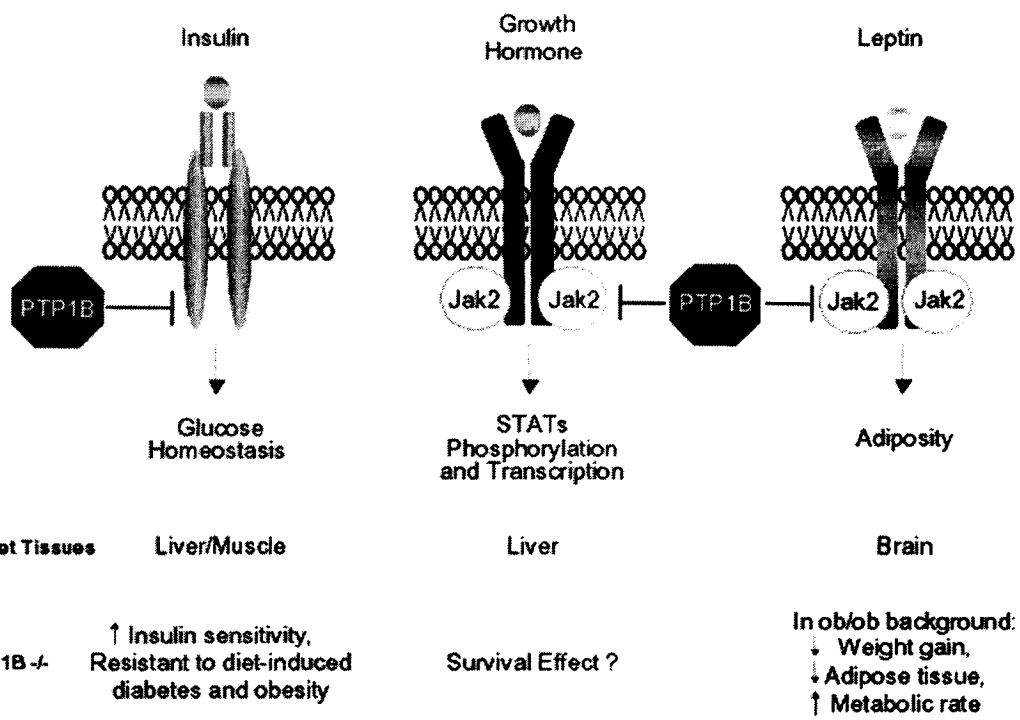

Figure 1. PTP1B controls metabolic responses. PTP1B dephosphorylates the insulin receptor and JAK2, thus negatively regulating insulin, leptin, and growth hormone actions.

6.1.3 The absence of PTP1B normalizes glycemia in a model of type 1 diabetes

Type 1 diabetes, which accounts for 5-10% of total diabetes mellitus cases, is characterized by high levels of blood glucose resulting from absolute lack of insulin due to beta cell destruction of the pancreas. As a generalized metabolic disorder, the disease is associated with long-term complications that affect almost every major system in the body, such as diabetic cardiomyopathy, retinopathy, neuropathy, nephropathy, and vascular diseases [331]. Currently, the treatment of type 1 diabetes consists of maintaining glycemia as close to the normal range as possible, which is achieved by multiple daily insulin injections. In addition, there are several experimental promising approaches in the treatment of this disease, such as islet transplantation, but still, some major problems are encountered with this method [334]. Consequently, development of better insulinomimetics remains one of the objectives of studies for better type I diabetes therapy, with properties that would be superior to insulin itself in the control of diabetes.

Previous studies showed that vanadium compounds, which possess insulinomimetic activity by inhibiting the activity of PTPs, normalized blood glucose levels in animal models of type 1 and type 2 diabetes [336, 338]. In addition, increased cytosolic PTP1B activity was reported in particulate fractions of skeletal muscle and liver of type 1 diabetic rats [342]. These results suggest that the downregulation of PTP1B could normalize glycemia in diabetic animals. Moreover, inhibition of PTP1B, by means of gene disruption and PTP1B antisense admistration, which leads to enhancement of IR phosphorylation *in vivo*, pointed out PTP1B as a potential drug target in diabetes [161, 162, 256, 258]. Taken together, the evidence of increased insulin sensitivity prompted us to look at the effect of removal of the PTP1B gene on glycemia of a mouse model of type 1 diabetes induced by destruction of beta cells by streptozotocin (STZ), which mimics insulinopenia of type 1 diabetes [335].

Indeed, injection of STZ rendered both WT and PTP1B null mice hyperglycemic. However, my results demonstrate significantly lower blood glucose levels in absence of PTP1B (Chapter 3, Figure 1A), which is maintained up to 8 days following STZ injection. Then, lower glycemia is maintained in STZ-treated PTP1B null mice up to three months, the end point of the study (Chapter 3, Figure 1B). Therefore, to verify if the lower blood glucose concentration observed in the STZ-PTP1B null mice was due to the secretion of insulin from residual pancreatic beta cells, we stained islets with anti-insulin antibody and we measured insulin concentrations. As expected, insulin-producing beta cells are destroyed by STZ treatment, both in WT and PTP1B null mice (Chapter 3, Figure 2B), and insulin levels were similar; STZ-treated WT (16.6 ± 3.9) and PTP1B null mice (15.2 ± 4.9) μU of insulin. Thus, STZ destroyed beta cells to the same extent in both WT and PTP1B null mice. These results are consistent with the fact that the lower glycemia observed in type 1 diabetic PTP1B null mice is not due to higher insulin secretion from beta cells but to increased insulin sensitivity. However, as suggested by electromyography measurements of motor and sensory fibers conduction velocity in the sciatic nerve, decreased glycemia in STZ-treated PTP1B null mice does not protect against development of reduced conduction velocity, a common complication of diabetes.

In accordance with our data, Kushner et al. recently showed that PTP1B regulates beta cell homeostasis [343]. They observed that the absence of the PTP1B gene improved glucose tolerance and delayed the onset of diabetes in insulin receptor substrate 2 (IRS2) KO diabetic mice. Together with our data, this suggests that the specific inhibition of PTP1B, which enhances IR signaling, can partially compensate for insulin deficiency.

We have shown that the absence of PTP1B does not prevent induction of type 1 diabetes by STZ, but delays the onset of hyperglycemia and improves blood glucose levels in the long-term. Since the major problem with actual therapy for type 1 diabetic

patients is the need of insulin injection to maintain blood glucose homeostasis, the aim remains to find a more convenient way to treat this disease. Our results strongly suggest that a PTP1B inhibitor, in combination with insulin, could be administered to reduce the need for insulin injection. The use PTP1B inhibitors on genetic type 1 diabetes models such as the non-obese diabetic (NOD) mice, in which diabetes occurs spontaneously with a total dependence on exogenous insulin for survival [426], will further demonstrate that inhibition of PTP1B can normalize glycemia in combination with insulin injection.

6.2 PTP1B is involved in the regulation of oncogenic signaling and tumorigenesis

As mentioned previously, PTP1B deficient mice are resistant to both diabetes and obesity, which established PTP1B as an important regulator of metabolism. However, the negative regulation exerted by PTP1B on several growth factor receptors, including IGF-1R [302], raises the possibility that PTP1B deficiency can potentially lead to increased oncogenic signaling through heightened signaling activity of those receptor tyrosine kinases. In spite of this, PTP1B deficient mice do not overtly develop any tumors. In this aspect, a mouse model of cancer combined with PTP1B deficiency is still lacking.

6.2.1 PTP1B regulates signaling downstream of RTKs through novel pathways

To clarify the potential consequences of removing or inhibiting PTP1B on cellular signaling pathways implicated in oncogenesis, our laboratory isolated mouse embryonic fibroblasts derived from PTP1B wild-type and knockout mice. Immortalization of these cells was achieved either spontaneously or by introduction of

the SV40 large T antigen (TAg). This provided a useful model to study signaling by various growth factors, adhesion molecules, and transformation.

As reported by Cheng et al., we unexpectedly found that PTP1B deficient fibroblasts display decreased fibronectin mediated cell spreading, and ERK phosphorylation [305]. In these cells, phosphorylation on Src Y527, the inhibitory tyrosine residue of Src, was increased. This suggested to us that PTP1B lies upstream of Src activation during fibronectin signaling, and that PTP1B could positively regulate Src activity. Additionally, immortalization of wild-type fibroblasts with SV40TAg increased PTP1B expression [305]. In collaboration with Dr. O'Connor (National University of Ireland, Cork, Ireland), our laboratory showed that PTP1B null fibroblasts display increased IGF-1R and AKT phosphorylation. Interestingly here again, similar effects of reduced ERK phosphorylation were observed when PTP1B deficient fibroblasts were stimulated with IGF-1 [302].

As follows, several biochemical studies demonstrated that PTP1B is involved in the attenuation of various PTKs. In addition, PTP1B can attenuate cellular transformation by oncogenic PTKs such as p210Bcr-Abl [208, 303] and p185v-Erb2 [460]. Thus, a common theme in transformation by all these kinases is their requirement for the activation of the small GTPase Ras [437]. In quiescent cells, Ras exists in an inactive GDP bound state. Upon stimulation with growth factors, Ras switches to an active GTP bound state, which allows it to bind to and activate its effector proteins. The most notable example is the Raf kinase, which subsequently activates in cascade the kinases MEK1 and ERK1/2, and leads to gene transcription and other cellular responses.

6.2.2 Loss of PTP1B in SV40TAg fibroblasts impairs Ras signaling

Therefore, we undertook the elucidation of this decreased ERK activation in SV40 TAg fibroblasts lacking PTP1B, by using the PDGFR signaling pathway. Once more, we revealed that loss of PTP1B function in immortalized cells leads to impaired Ras signaling. Stimulation of the fibroblasts with low levels of PDGF resulted in increased cellular tyrosine phosphorylation and AKT/PKB activation, which was enhanced in PTP1B null cells. In contrast, PDGF-induced ERK phosphorylation was decreased in these cells (Chapter 4, Figure 1B). Consistent with our data that fibronectin, IGF-1 and PDGF-induced activation of ERK is impaired in PTP1B deficient cells, we observed that these cells display decreased monolayer growth compared to their wild-type counterparts (Chapter 4, Figure 2A and B). Moreover, immortalized cells lacking PTP1B exhibit a consistent and reproducible elevation in PDGFR levels (Chapter 4, Figure 2C). This increase in PDGFR levels correlates with their decreased proliferation capacities. It is established that expression of the PDGFR is inversely proportional to Ras activity [349], and we found that PTP1B deficient fibroblasts have decreased Ras activity upon PDGF stimulation (Chapter 4, Figure 2D).

6.2.3 Involvement of p120RasGAP and p62Dok in the regulation of Ras activity

The decreased Ras activity observed in immortalized fibroblasts lacking PTP1B was most likely caused by increased expression of p120RasGAP, a negative regulator of the GTPase activity of Ras, whose protein and mRNA levels are increased in PTP1B deficient cells (Chapter 4, Figure 3). We found that p62DOK, a negative regulator of ERK signaling, is hyperphosphorylated in PTP1B deficient cells, and is a substrate of PTP1B (Chapter 4, Figure 4). Tyrosine phosphorylation of p62DOK contributes to its inhibitory effect on Ras [358, 359]. Indeed, introduction of a constitutively active form of Ras into PTP1B knockout cells rescues their proliferative potential, similar to the one

observed in wild-type cells, and even triggers an increased in anchorage-independent growth compared to wild-type cells (Chapter 4, Figure 5), suggesting that activated Ras can bypass the negative regulation from p62DOK and p120RasGAP. Therefore, we proposed the following model: PTP1B seems to be necessary for Ras signaling in immortalized fibroblasts, and is a positive modulator of this pathway, as it also positively modulates Src signaling (Figure 2).

One interesting aspect of these studies is that, in contrast to immortalized cells, PTP1B deficient primary fibroblasts do not exhibit decreased ERK activation. The increased dependency on PTP1B in Ras signaling in immortalized cells may reflect alterations in gene expression caused by immortalization. We have observed that spontaneous or TAg immortalization of PTP1B deficient fibroblasts leads to increased p120RasGAP expression at the protein and mRNA level (Chapter 4, Figure 4). However, immortalization of TC-PTP deficient fibroblasts, a PTP 74% identical to PTP1B [238], does not lead to the same modification. This suggests that PTP1B could indirectly regulate the transcription of the RasGAP gene. Noteworthy, p120RasGAP null fibroblasts also display alterations in cell migration and morphology [461]. Therefore, the increased p120RasGAP expression in PTP1B deficient cells may contribute to the phenotype previously observed by Cheng et al. in adhesion signaling [305].

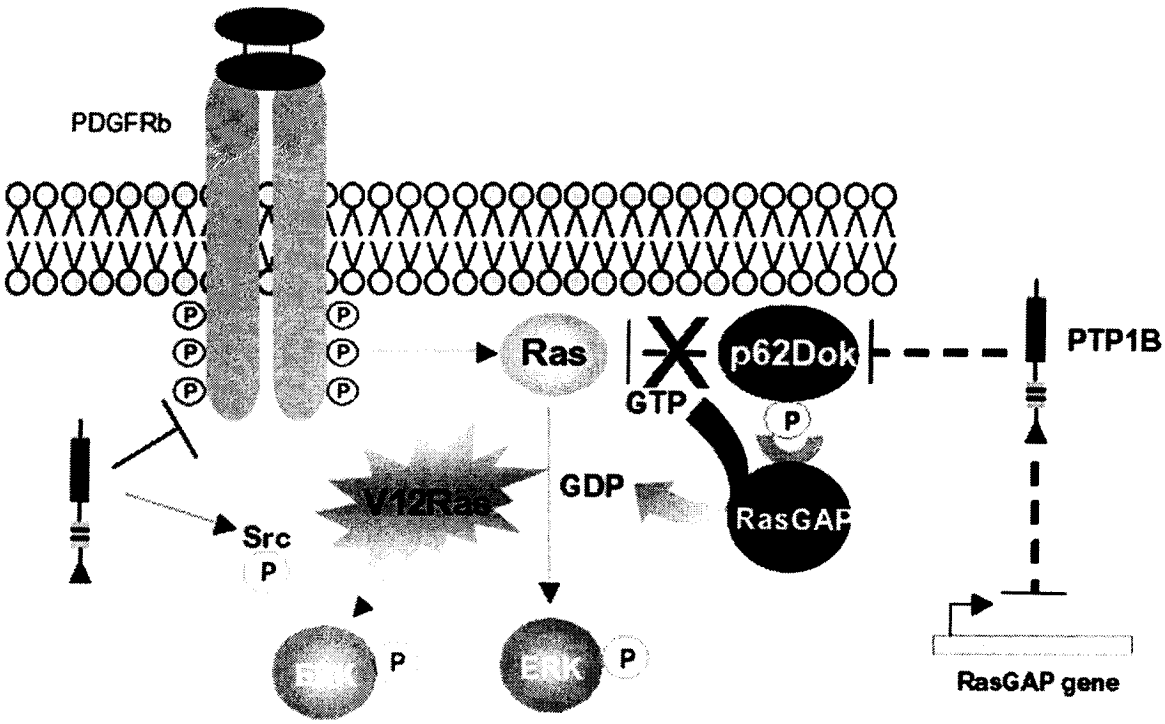

Figure 2. PTP1B regulates signaling downstream of RTKs. Loss of PTP1B leads to increased RTK phosphorylation and enhanced signaling of most downstream pathways. However, the absence of PTP1B can also lead to cellular alterations that attenuate MAPK signaling downstream of RTKs. For example, PTP1B deficiency can lead to decreased Src activation via increased phosphorylation of inhibitory site. In addition, loss of PTP1B leads to increased expression of p120RasGAP, via an unidentified mechanism. Finally, p62Dok, a potential substrate of PTP1B, is hyperphosphorylated in PTP1B deficient fibroblasts. All these events can contribute to attenuate Ras activity and thus MAPK signaling. Consistent with this model, introduction of activated Ras into PTP1B null fibroblasts can bypass these inhibitory events on Ras signaling and action.

6.2.4 PTP1B: tumor suppressor or oncogene?

Intuitively, it is often speculated that inhibition of PTP1B may lead to increased PTKs signaling, and ultimately oncogenesis. However, as described previously, despite potentially enhanced oncogenic signaling, PTP1B deficient mice do not overtly undergo tumorigenesis. One possibility is that PTP1B may not regulate RTKs signaling in all cell types, or that functional redundancy may exist. In addition, PTP1B is involved in the activation of oncogenic pathways downstream of RTKs, such as regulation of Ras activity, as described in Chapter 4. Interestingly, both increased and decreased levels of PTP1B have been observed in different tumors. Yet, strong genetic evidence for a role of PTP1B in cancer is still lacking.

Hence, the question whether PTP1B act as an oncogene or a tumor suppressor remains. Some evidence suggests that it could act as a positive as well as a negative regulator of oncogenesis. First, the fact that PTP1B dephosphorylates various growth factor receptors such as PDGF, EGF and IGF-1 receptors, would suggest that it negatively regulates transformation. In addition, the expression of PTP1B mRNA is decreased in oesophageal cancer lesions [203], which places PTP1B as a tumor suppressor in accordance with its negative effect on cell growth. To this extent, Buckley et al. have previously shown that loss of PTP1B does lead to increased IGF-1 induced AKT activity and resistance to etoposide-induced apoptosis [302], and that inhibition of PTP1B could provide pro-survival signal to transformed cells.

Furthermore, PTP1B is involved in the negative regulation of cytokine signaling, through dephosphorylation of the kinases JAK2, TYK2 [163, 164, 295, 429], and STAT5 [309]. Deregulation or aberrant regulation of theses kinases is involved in diseases such as lymphomas and leukemias [448-451]. Importantly, this function suggests that PTP1B could act as a negative regulator in these diseases.

In contrast, overexpression of PTP1B is characteristic of various cells expressing p210Bcr-Abl, including a cell line derived from a patient with chronic myelogenous leukemia (CML) [208], as well as many types of epithelial carcinomas [207], and also observed in ovarian carcinomas [205] and in human breast carcinoma cells [204, 206]. Accordingly, Wiener et al. have shown that overexpression of ErbB2/HER2 in ovarian cancer cells induces an increase in PTP1B [70], and, consequently, that PTP1B expression is associated with ErbB2/Neu expression in human breast cancer [206]. In fact, most breast cancer cases are associated with increased expression of Src and members of the EGFR family [61, 462-464]. Importantly, PTP1B has been identified as one of the major phosphatases that activate Src in breast cancer cells. PTP1B dephosphorylates an inhibitory tyrosine residue on Src, thus, activating the kinase [209, 305]. Furthermore, increased expression of PTP1B has also been demonstrated in transformed human breast cells [204], as well as in fibroblasts immortalized with SV40TAg [305]. This raises the intriguing possibility that PTP1B may positively contribute to the progression of breast cancer, via activation of Src but also negatively via regulation of EGFR and/or ErbB2/HER, and that inhibition of PTP1B could be a means of treating a subset of cancers. Introducing the PTP1B null background into transgenic models of breast cancer [444] would certainly help to elucidate this question. Predicting whether PTP1B is a good drug target for cancer is particularly difficult, and might depend on the type tissue and/or cancer.

In summary, it is important to consider that the localization and the tissue expression of PTP1B might determine its involvement in cancer, acting either as an oncogene or a tumor suppressor. To address this question appropriately, introduction of PTP1B siRNA in various cancer cell lines would surely help to determine its role in transformation, and might give valuable information as to whether PTP1B has a tissue specific action. In addition, studies correlating expression levels of PTP1B with

neoplastic disease status in humans will be of invaluable help to define its role in tumorigenesis.

6.2.5 Absence of PTP1B decreases survival rate and increases lymphoma incidence of p53 null mice

To study the *in vivo* role of PTP1B in oncogenesis, we have generated p53 PTP1B double null mice by breeding the two lines. The p53 deficiency constitutes a good model to study tumorigenesis since this gene is mutated in the majority of human cancers [378, 379], and germ line mutations in the p53 gene have been observed in patients affected by the Li-Fraumeni syndrome [383]. These individuals are at risk for the development of a number of tumors, including soft-tissue sarcomas, osteosarcomas, brain tumors, breast cancers, and leukemias [384-386]. The p53 null mice are viable but susceptible to the development of early spontaneous tumors. Their phenotype is well characterized, and the most frequent tumor observed in p53 null mice is thymic lymphoma (70%) [387-391].

We have found that in the absence of p53, PTP1B heterozygous and null mice display decreased survival rates with the presence of an earlier tumor burden. By 20 weeks of age, 60% of the p53 -/- PTP1B +/+ mice developed tumors (n=20) (in accordance with the initial gene-targeting study published by Donehower et al. [387]), compared to 72% of the p53 -/- PTP1B +/- mice (n=43), and 89% of the p53 -/- PTP1B -/- (n=54). These results clearly showed that the absence of PTP1B potentiates tumor development of p53 -/- mice (Figure 1, Chapter 5). In addition, p53 -/- PTP1B -/- mice were more susceptible (by 19%) to develop lymphomas compared to p53 -/- PTP+/- and, interestingly, developed twice as more B-cell lymphomas. This prompted us to look at the role of PTP1B in hematopoiesis since immune defects could contribute to the accelerated rate of tumor development and to the shift in T to B lymphoma.

6.2.6 Novel role for PTP1B in hematopoiesis

PTP1B null mice showed an increase in the absolute number of B cells in bone marrow, where immature B cells ($IgM^{-}IgD^{-}$) accumulated. Colony-forming assays in the presence of IL-7 confirmed the increase in pre-B cell precursors in PTP1B null mice, which does not result from increased resistance to apoptosis. We need to further characterize whether IL-7 induced proliferation is modified in the PTP1B null B cells. Preliminary results suggest that PTP1B null bone marrow B cells express higher levels of IL-7Ra, which could have consequences on their proliferative responses. At this point, it is possible that the increased number of B cell precursors observed in the colony assays in PTP1B null bone marrow accounts for increased incidence of B lymphomas in p53/PTP1B double null mice. For instance, Wnt5a heterozygous mice are susceptible to the development of B lymphomas with age, and Wnt5a has been reported as a negative regulator of B cell proliferation and it inhibits the response of B cells to IL-7 [447].

When B cells emerge from bone marrow into the periphery, they are still functionally immature. Most of these immature cells will not survive to become fully mature B cells [452]. The B cells that have completed their maturation process in the periphery and return to the bone marrow are called mature recirculating B cells and are important to mediate an efficient humoral immune response [453, 454]. Therefore, it would be interesting to determine the physiological consequences of the reduced number of mature recirculating B cells in the bone marrow of the PTP1B null mice, which could have consequences on their ability to mount an immnune response to bacterial or viral agents.

The accumulation of immature B cells in the bone marrow of PTP1B null mice is similar to the phenotype reported for the CD45 null mice [151]. Since B and T cells originate from a common lymphoid progenitor (CLP), it is possible that PTP1B null mice also display a defect in T cell development or maturation of thymic cells, such as

the CD45 null mice. Using the method previously described by Kondo et al. [465] and Sitnicka et al. [466], we found no difference in the number of hematopoietic stem cells (HSC) and CLP in PTP1B WT and null mice (data not shown). Therefore, we hypothesized that PTP1B exerts a negative regulation downstream of the CLP to downregulate the production of B cells (Figure 3). Ongoing studies will determine how PTP1B potentially regulates B cell development.

Lu et al. reported that the absence of p53 reduced pro-B cell apoptosis [458]. To this extent, Buckley et al. previously observed that loss of PTP1B led to resistance to apoptosis [302]. Thus, inhibition of PTP1B could provide a pro-survival signal to transformed cells, suggesting that PTP1B contributes to the maintenance of apoptosis in specific cell types. It is specifically the case in the lymph nodes of PTP1B null mice where B cells accumulated and are resistant to apoptosis. Survival assays on the subsets of B cells in the bone marrow will determine whether the absence of PTP1B amplifies the p53 pro-B cell apoptosis phenotype.

Hence, a model can be put together to explain our results. As depicted on Figure 4, we propose that PTP1B exerts a negative regulation at the immature to transitional stage by controlling the production of immature (IgM^-IgD^-) cells in the bone marrow. It is possible that the proportion of B cells that does not recirculates to the bone marrow, for an unknown reason yet, accumulates in blood and lymph nodes of PTP1B null mice.

In summary, our studies revealed that PTP1B is an important determinant of the latency and tissue distribution of tumors in a p53 deficient background, suggesting that it acts as a tumor suppressor in this specific context. Additionally, our results underlined a novel and important role of PTP1B in immune and oncogenic function. To our knowledge, this is the first demonstration of a role for PTP1B in B cell development and hematopoiesis, as well as in cancer *in vivo*. Further studies on the role of PTP1B in lymphoid cell development, proliferation, and differentiation are currently ongoing.

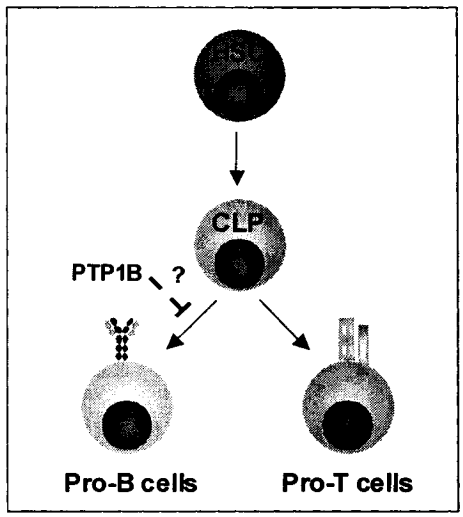

Figure 3. Potential role of PTP1B in hematopoietic lineage differentiation. We hypothesized that PTP1B might negatively regulate differentiation from common lymphoid progenitor based on the higher number of pre-B progenitors were observed in the bone marrow of PTP1B null mice.

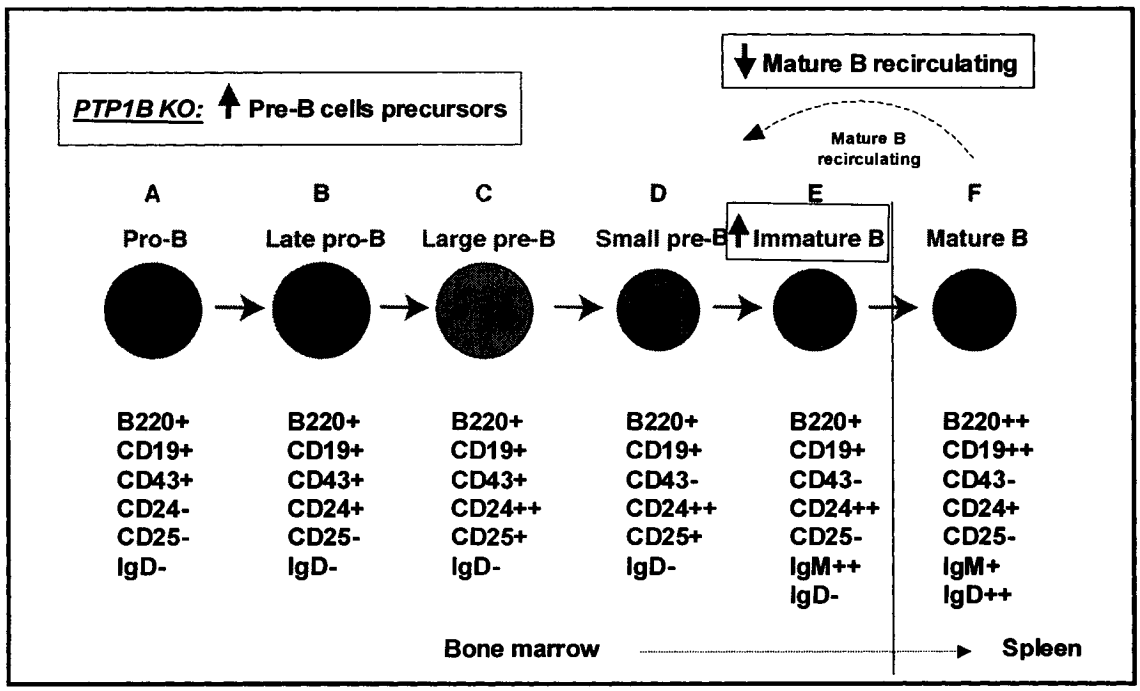

Figure 4. Developmental stages of the various B cell types and potential role of PTP1B in B cell development. Our results suggest that PTP1B exerts a negative regulation at the immature to transitional stage by controlling the production of immature ($IgM^{++}IgD^{-}$) cells in the bone marrow. However, a decreased number of mature B cells recirculates to the bone marrow. Adapted from Benschop and Cambier [459].

6.3 Summary and perspectives: looking for PTP1B within the cell

In the past five years, a considerable amount of evidence has been provided to define the role of PTP1B in metabolism. It is now clear that inhibition of PTP1B is an effective mean of treating type 2 diabetes and obesity. My research has highlighted another important physiological aspect of this enzyme in metabolic signaling by showing that PTP1B regulates the activation of the GH signaling pathway (Chapter 2), and that the absence of PTP1B reduces glycemia of type 1 diabetic mice (Chapter 3). Furthermore, the role of PTP1B in oncogenesis is starting to be elucidated. In that aspect, my results confirmed that PTP1B can modulate signaling downstream of growth factor receptors (Chapter 4). I have also provided *in vivo* evidence that PTP1B acts as a tumor suppressor in a p53 deficient background (Chapter 5). This study also revealed a novel role of PTP1B in B cell development.

Hence, important questions remain: How is the spatial temporal regulation of PTP1B controlled? Where do PTP1B and its substrates interact? Does PTP1B localize differently in all cell types? What is the physiological significance of these interactions? Haj et al. reported that PDGFR and EGFR internalize to sites near the ER for dephosphorylation, where they interact with PTP1B [301]. Furthermore, JAK2 has been shown to partly localize with PTP1B on the ER membrane [410]. Future studies will have to determine what are the signals that regulate PTP1B relocalization if they exist.

Another issue that shoud be addressed is the role of the proline-rich domains of PTP1B. These domains enable PTP1B to bind to a variety of adapter molecules, which could provide a means for PTP1B to participate in multiple signaling pathways. Re-introduction of proline-rich mutants in PTP1B null cells could contribute to define the function of these motifs in cell signaling. The physiological significance of the proline-rich motifs could be further studied by developing knock in mouse models.

In summary, as depicted in Figure 5, my doctoral research underlined important information on the mechanism of action of PTP1B in normal and transformed cells, and should provide novel insight into the development of inhibitors toward specific pathways regulated by PTP1B that are relevant to diabetes, obesity, and cancer.

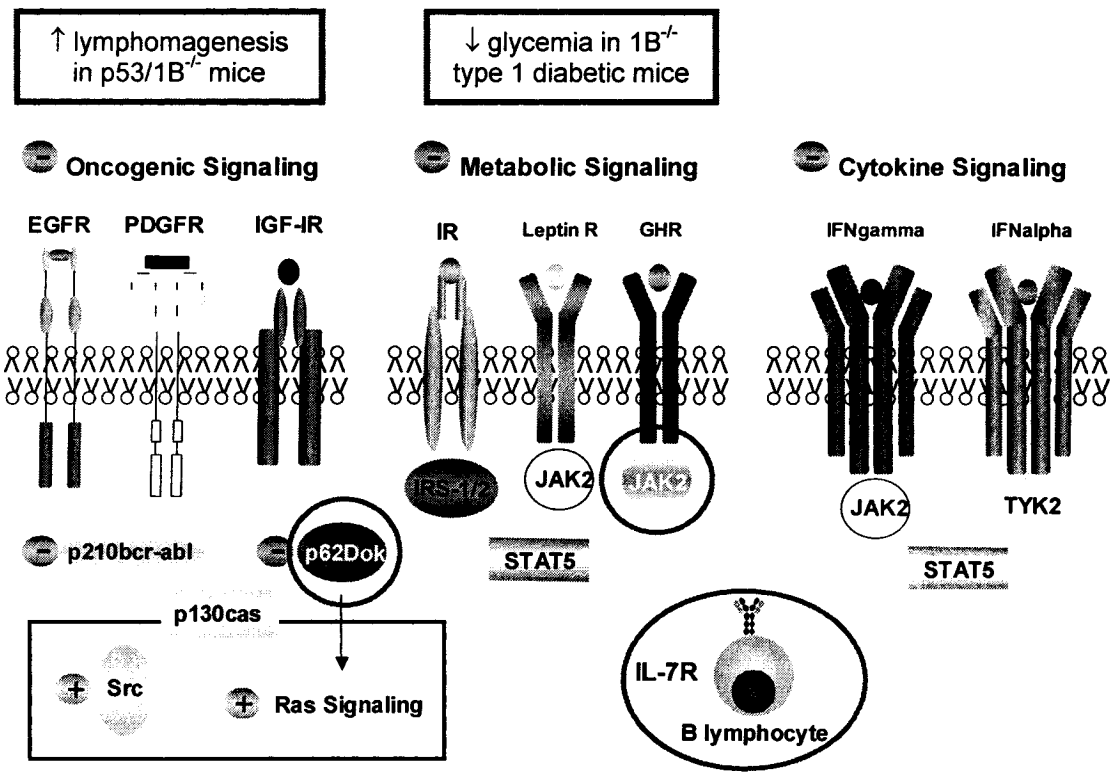

Figure 5. PTP1B is involved in multiple signaling pathways. PTP1B dephosphorylates various receptor tyrosine kinases, as well as cytosolic tyrosine kinases. The modulation of these signaling cascades, either positive or negative, implicates PTP1B in oncogenic, metabolic, cytokine signaling, and hematopoiesis. The novel substrates and/or roles of PTP1B that I have identified are circled or squared.

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8 Appendix

- Research compliance certificates

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

No

Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes

What precautions are being taken to reduce production of infectious droplets and aerosols?

Fumehood used, Biological safety cabinet

List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
McIntyre	715	Nuaire	NU-425-300	26052XX	October 2003