

Molecular Control of Haematopoiesis via the JAK-STAT Signalling Pathway

A dissertation submitted in total fulfilment of the requirements of the
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Abstract

Maintenance of the haematopoietic system is controlled by intercellular signalling molecules known as cytokines. Cytokines function by binding to receptors at the surface of target cells and activating a number of signalling pathways inside the cell that lead to changes in gene transcription and ultimately a cellular response, whether it be differentiation, division, cell death or other. These processes need to be tightly controlled and regulated, and so there are cellular mechanisms for controlling both the duration and intensity of the signal produced by cytokines.

This thesis investigates the structure and function of two regulators of cytokine signalling; PTP1B and LNK. PTP1B is a protein tyrosine phosphatase that interacts with and dephosphorylates the JAK proteins, returning them to their inactive state. Similarly, LNK, a lymphocyte adaptor protein also interacts with JAK2, and negatively regulates a subset of cytokines by an unknown mechanism. The work presented in this thesis details the study of the interactions between PTP1B and LNK with the JAK proteins using structural and biochemical techniques, allowing for the characterisation of the mode of substrate recognition, and substrate preference.

The studies in chapter 3 revealed that the PTP1B phosphatase can directly dephosphorylate JAK kinases, inducing their transition to an inactive state. The interaction between PTP1B and the motif on JAK that is its target was studied in detail. While previous studies of PTP1B substrate recognition have defined a second aryl binding site that allows PTP1B to interact with substrates containing two consecutive phosphotyrosine residues, here it is shown that there is a second mode of binding where this second binding site is not used. Given that PTP1B is the prototypical phosphatase in the category I phosphatases, this may warrant redefining this category. Moreover, the accessibility and position of the JAK activation loop to phosphatases was shown to be critical for dephosphorylation rate, and so it can be postulated that some conformations of the JAK proteins may inhibit their dephosphorylation by phosphatases.

The results presented in chapter 4 study the interaction between the LNK SH2 domain and various phosphorylated targets, including JAK. It was shown that LNK can interact with several sequence motifs from proteins involved in haematopoiesis

but that JAK2 and JAK3 are its favoured targets. Structural studies were performed to uncover the molecular basis for this high-affinity interaction with JAK. In addition, studies of mutations found in the LNK SH2 domain which were identified in patients with myeloproliferative neoplasms revealed how these changes to the SH2 domain reduced the ability of LNK to bind to its targets, which may contribute to disease.

In summary, the information presented in this thesis add to our understanding of the regulation of the JAK-STAT pathway, and how changes to essential protein-protein interactions may be involved in disease.

Declaration

The work presented in this thesis was conducted through The University of Melbourne, at The Walter and Eliza Hall Institute of Medical Research in the laboratory of Associate Professor Jeffrey Babon.

I, Rhiannon Morris, declare that:

I. This thesis comprises only my original work towards the degree of Doctor of Philosophy except where indicated in the Preface

II. Due acknowledgement has been made in the text to all other material used

III. The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies, appendices and footnotes

Signed:



Rhiannon Morris

July 2020

Preface

Contributions of others

In accordance with the regulations governing the degree of Doctor of Philosophy at the University of Melbourne, this thesis comprises my original work except where indicated in the preface:

- I. Jeff Babon, Nicos Nicola and Dina Stockwell carried out the initial screen of the phosphatases in the M1 cells in Chapter 3.
- II. Artem Laktyushin and Nicholas Liau had previously purified the majority of the JAK constructs used in Chapter 3.
- III. Artem Laktyushin. previously purified WT PTP1B used for experiments in Chapter 3.
- IV. James Murphy provided the WT LNK SH2 domain construct used for protein expression in chapter 4.
- V. James Murphy previously generated the PTP1B 2-298 and 2-321 D181A and Q262A substrate trapping mutant constructs used in chapter 3.
- VI. Janet Newman and Bevan Marshall provided initial assistance crystallising proteins through CSIRO.
- VII. Nadia Kershaw assisted with the collection and refinement of structures throughout this thesis.
- VIII. Jeff Babon assisted with the collection and analysis of NMR data in Chapter 3.
- IX. Jeff Babon assisted with several competition assays presented in Chapter 4.
- X. Andrew Jarratt generated and provided the M1-Cas9 cell line
- XI. Craig Hyland and Maria Kauppi provided the M1-Cas9 + MPL cell line.

I assess my overall contribution to the work found in this thesis to be at least 85%

Publications

It should be noted that sections of the introduction presented in this thesis have been published in the review article listed below, and found in Appendix I. This review article was written by myself, Jeff Babon and Nadia Kershaw. Some sections initially written by me appear verbatim in this thesis, while others have been expanded upon for the purposes of this work. Text written primarily by Jeff Babon has been re-written for use in this thesis.

Morris, R., Kershaw, N.J. and Babon, J.J., 2018. The molecular details of cytokine signaling via the JAK/STAT pathway. *Protein Science*, 27(12), pp.1984-2009.

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If I listed all the friends who have supported me along this journey, I would surely run out of words for this thesis so I will hopefully be able to thank you all personally instead – you all know who you are. A massive thank you to the whole structural biology division, it has been a pleasure to come to work every day and be surrounded by such an amazing and talented group of people who I am also lucky to

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List of appendices

Appendix I: Publication (Review article)

Morris, R., Kershaw, N.J. and Babon, J.J., 2018. The molecular details of cytokine signaling via the JAK/STAT pathway. *Protein Science*, 27(12), pp.1984-2009.

Appendix II: Omit maps for five PTP1B structures with phosphopeptides bound.

Appendix III: Raw data for NMR pYpY dephosphorylation assays of IRK and JAK2.

Appendix IV: Fo-Fc map for water modelled into catalytic pocket of PTP1B D/Q plus JAK2 pYpY.

List of abbreviations

Abbreviation	Meaning
2D	Two dimensional
3D	Three dimensional
A₂₈₀	Absorbance at 280 nm
Ab	Antibody
ABL	Tyrosine kinase abelson
AKT	Protein kinase B
ALP	Activation loop peptide
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
APS	Adaptor protein containing SH2 (Also SH2B2)
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region protein
BSA	Bovine serum albumin
C3	Collaborative Crystallisation Centre
CALR	calreticulin
CAPS	N-cyclohexyl-3-aminopropanesulfonic acid
CD	Cluster of differentiation
c-FMS	Colony stimulating factor 1 receptor
CHR	Cytokine binding homology region
CIS	Cytokine inducible Sh2 domain containing protein
c-KIT	Proto-oncogene receptor tyrosine kinase
CLC	Cytokine like factor
CML	Chronic myeloid leukaemia
CNTF	Ciliary neurotrophic factor
CRLF2	Cytokine receptor like factor 2
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CT-1	Cardiotrophin 1

DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3 alpha
Dox	Doxycyclin
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EPO	Erythropoietin
EPOR	Erythropoietin receptor
ER	Endoplasmic reticulum
ET	Essential thrombocythemia
FERM	Four-point-one (4.1), ezrin, radixin, moesin
FL	Full length
FLT3	FMS-like tyrosine kinase 3
FNIII	Fibronectin type III domain
GAS	γ -activated sequences
GRB2	Growth factor receptor bound protein 2
GST	Glutathione S transferase
HSC	Haematopoietic stem cell
IC₅₀	Half maximal inhibitory concentration
IDH1	Isocitrate dehydrogenase 1
IDH2	Isocitrate dehydrogenase 2
IE	Idiopathic erythrocytosis
IKZF1	IKAROS family zinc finger 1
IFN	Interferon
IgE	Immunoglobulin E
IL	Interleukin
IL2RG	Interleukin 2 receptor gamma (gamma common chain)
IL6Ra	Interleukin 6 receptor alpha

IL6ST	Interleukin 6 signal transducer
IPTG	Isopropyl β -D-1-thiogalactopyranoside
IRSE	Interferon-stimulated response elements
IRK	Insulin Receptor Kinase
ITC	Isothermal titration calorimetry
JAK	Janus Kinase
KD	Kinase domain
kDa	Kilodalton
K_D	Equilibrium dissociation constant
K_m	Michaelis constant
KIR	Kinase inhibitory region
LEPR	Leptin receptor
LIF	Leukaemia inhibitory factor
LIFR	Leukaemia inhibitory factor receptor
LNK	Lymphocyte adaptor protein (also SH2B3)
MAM	Meprin, A-5 protein and receptor protein tyrosine phosphatase mu
MAPK	Mitogen-activated protein kinase
MES	2-(N-morpholino)ethanesulfonic acid
Min	Minutes
MPL	Myeloproliferative leukaemia virus
MPN	Myeloproliferative neoplasm
mTOR	Mammalian target of rapamycin
MUNC18	Mammalian uncoordinated-18
MW	Molecular weight
MX2	Macromolecular crystallography 2
NADH	Nicotinamide adenine dinucleotide + hydrogen
NMR	Nuclear magnetic resonance
OSM	Oncostatin-M
OSMR	Oncostatin-M receptor

PAGE	Polyacrylamide gel electrophoresis
PCM1	Pericentriolar material 1 protein
PCR	Polymerase chain reaction
PDB	Protein Databank
PDGFR	Platelet derived growth factor receptor
PEG	Polyethylene glycol
PH	Plekstrin homology
PI3K	Phosphoinositide 3-kinase
pKa	Dissociation constant
PMF	Primary myelobibrosis
PTP	Protein tyrosine phosphatase
PTP1B	Protein tyrosine phosphatase 1B
PTPRT	Protein tyrosine phosphatase receptor type T
PV	Polycythemia vera
PVDF	Polyvinylidene fluoride
pY (pTyr)	phosphotyrosine
Raf	Rapidly accelerated fibrosarcoma kinases
Ras	RAS Guanosine triphosphatases
RMSD	Root mean standard deviation
ROS	Reactive oxygen species
RT	Room temperature
s	Seconds
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SH2	Src homology 2
SH2B	SH2 domain containing adaptor protein 1 (also SH2B1)
SHP1	SH2 domain containing phosphatase 1
SHP2	SH2 domain containing phosphatase 2
SOS	Son of sevenless

SOCS	Suppressor of cytokine signalling
SPR	Surface plasmon resonance
SRSF2	Serine and arginine rich splicing factor 2
STAT	Signal Transducer and Activator of Transcription
TCEP	Tris(2-carboxyethyl)phosphine
TC-PTP	T-cell protein tyrosine phosphatase
TEL	Translocation-Ets-leukaemia virus
TET2	Ten Eleven translocase 2
TEV	Tobacco etch virus
TNF	Tumor necrosis factor
TPO	Thrombopoietin
Tris	Tris(hydroxymethyl)aminomethane
TYK	Tyrosine kinase
U2AF1	U2 auxiliary factor 1
VEGFR2	Vascular endothelial growth factor receptor 2
Vmax	Maximum velocity
WB	Western blot
WT	Wild-type
μ	Micro
°C	Degrees celcius
Å	Angstroms

Chapter 1 Introduction

All Figures in the introduction excluding structures were created with BioRender.com.

As indicated in the preface, sections in the introduction have been published as part of the review found in **Appendix I**.

1.1 Haematopoiesis and cytokine signalling

1.1.1 Haematopoiesis

Every mature blood cell present in the human body arises from a pool of cells termed the haematopoietic stem cells (HSCs). These cells are remarkable in that a single HSC can give rise to the entire haematopoietic system of an organism via a process termed haematopoiesis. These HSCs also have the capacity to self-renew, a process which generates an identical copy of the original cell while remaining in an undifferentiated state. Together, these properties of HSCs ensure that haematopoiesis can be sustained for the lifetime of an organism (Orkin 2000). During haematopoiesis, the HSCs differentiate into progenitor cells, which in turn differentiate into each mature cell type. With each successive cycle of proliferation and differentiation, the cells become more abundant, and more committed to a specific lineage (**Figure 1.1**). Generally, the more mature the cell type, the shorter the life span, with many of the mature blood cells having a lifespan of only a few weeks or months (Franco 2012; Mason et al. 2007). Long-term HSCs, however, reside in the bone-marrow indefinitely (Oguro, Ding, and Morrison 2013), and continuously produce an estimated 10^{11} - 10^{12} new blood cells every day in an average human adult (Brune 2019). Due to this constant turnover, the process of haematopoiesis is highly dynamic, complex, and thus tightly regulated.

The process of cellular proliferation and differentiation is governed by changes in gene transcription and genome organisation within the cell. These changes occur in response to extracellular signals received at the cell surface. In the haematopoietic and immune systems, cytokines play a critical role in regulating haematopoiesis and controlling immune responses (Alexander 2001). Cytokines are a group of small, secreted proteins that act on their target cells by binding to specific receptors found at the cell surface. Their binding to these receptors causes an intracellular signalling cascade that ultimately leads to changes in gene expression, resulting in a wide array of biological responses, including the cellular proliferation and differentiation required for haematopoiesis.

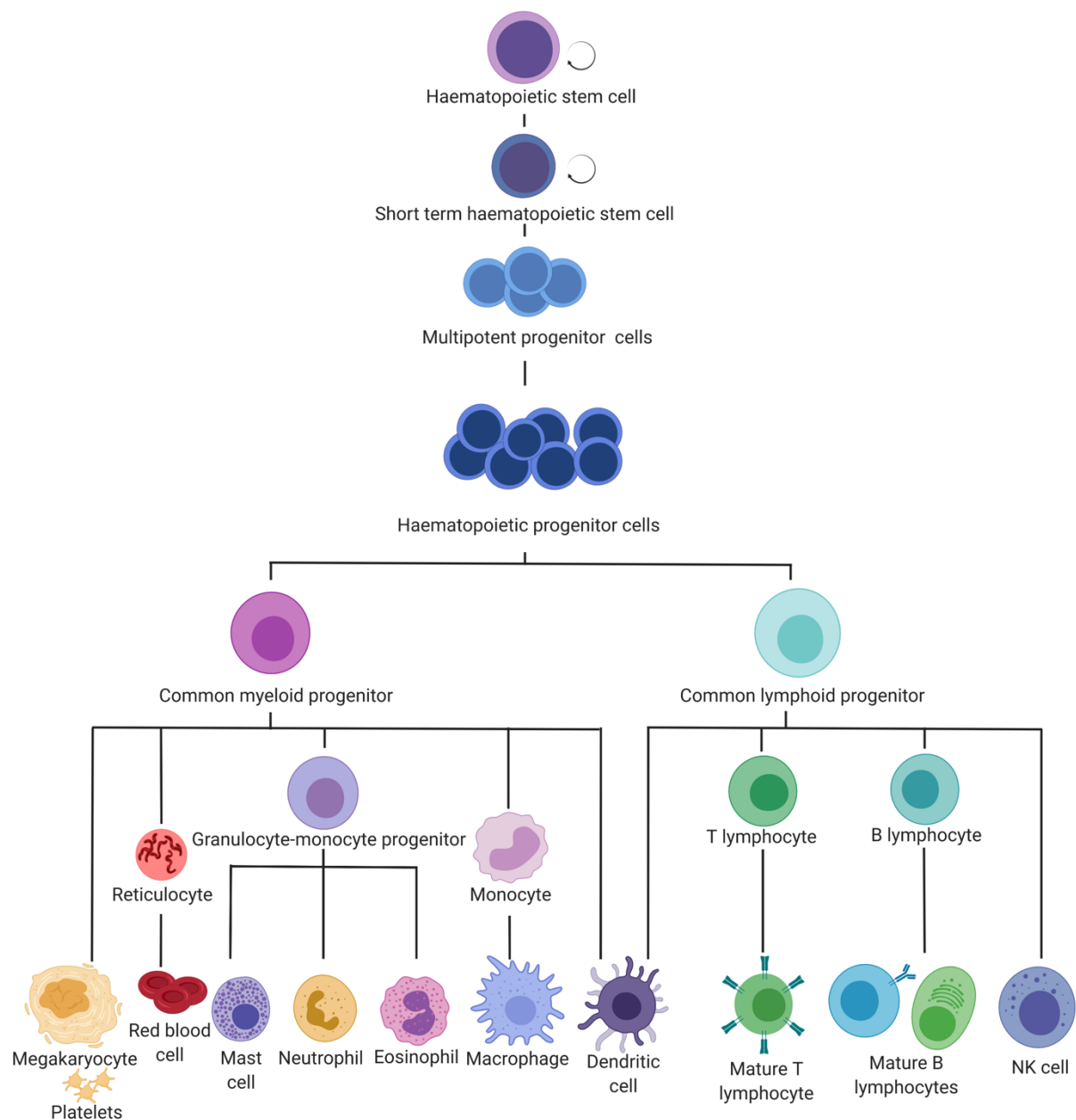


Figure 1.1.1 The major classes of cells in haematopoiesis.

During haematopoiesis, the hematopoietic stem cells that reside in the bone marrow undergo progressive differentiation as they commit to specific lineages that ultimately lead to the production of all the mature blood cells in an organism. A multipotent haematopoietic stem cell receives signals from its environment to differentiate into either myeloid or lymphoid progenitors. From here the cells are able to differentiate into their respective mature blood lineage through a set of differentiation and commitment stages.

1.1.2 Cytokines and their receptors

Cytokines can be separated into five classes based on their biological properties; i) The IL-1 family of cytokines, ii) The TNF- α related cytokines, iii) growth factors, iv) the chemokines, and v) the cytokines which act via the Janus Kinase-Signal transducer and activator of transcription (JAK-STAT) signalling pathway. Each of these groups are structurally distinct from one another, which reflects their ability to activate different signalling pathways in the cell. The cytokines that activate the JAK-STAT signalling pathway include the haematopoietic cytokines, growth factors, immunomodulatory cytokines, and the interferons. This thesis will focus predominantly on this fifth group of cytokines.

In mammals, more than 50 JAK-STAT activating cytokines have been described and while most have distinct biological roles, many have overlapping or redundant cellular outcomes, such as in the case of the IL-6 family. Often cytokines with similar biological effects activate receptors that are closely (both structurally and functionally) related to one another, and so it can be useful to classify them into families. Given the diverse roles and lack of sequence similarities between different cytokines, classification can be difficult, however they can be broadly grouped together on the basis of the receptors they interact with and their biological roles. The haematopoietic cytokines, their families and their biological functions are listed in **Table 1.1**.

Cytokines typically act as monomeric proteins, however in some cases will also form and act as dimers. Cytokine receptors typically comprise two or more individual protein chains that are single pass transmembrane proteins. All cytokines bind to the extracellular portion of cytokine receptors termed the cytokine homology region (CHR), which are highly variable and dependent on the specific receptor chains that make up the receptors, the details of these receptors is reviewed in more detail in Morris et al., 2018. The intracellular portion of cytokine receptors are unstructured regions that act as scaffolds upon which signalling is initiated. These cytoplasmic domains exist to provide sequence specific docking sites for the JAKs, the STATs, and other signalling and regulatory molecules.

Table 1.1 Cytokines, their receptors and biological function.

Cytokine	Receptor chain 1	Receptor chain 2	Receptor chain 3	Biological function	Reference
Class I cytokines					
The IL-2 Family					
IL-2 interleukin-2	IL2RG*	IL2RA	IL2RB	Lymphopoiesis and T cell activation/differentiation	(Gaffen and Liu 2004)
IL-4 (I) interleukin-4	IL2RG*	IL4RA		Induces T _H 2 differentiation	(Paul 1991)
IL-4 (II) interleukin-4	IL2RG*	IL13RA			(Paul 1991)
IL-7 interleukin-7	IL2RG*	IL7RA		Growth factor for B and T cells	(Sudo et al. 1993)
IL-9 interleukin-9	IL2RG*	IL9RA		Pleiotropic, Stimulates T-, B- and NK cells	(Demoulin and Renauld 1998)
IL-15 interleukin-15	IL2RG*	IL15RA	IL2RB	Regulation of T, and NK cell proliferation and activation	(Fehniger and Caligiuri 2001)
IL-21 interleukin-21	IL2RG*	IL21RA		Regulation of T, B- and NK cell proliferation and activation	(Parrish-Novak et al. 2002; Parrish-Novak et al. 2000)
The IL-3 Family					
IL-3 interleukin-3	CSF2RB**	IL3RA		Differentiation of HSCs	(Lopez et al. 1987)
IL-5 interleukin-5	CSF2RB**	IL5RA		Eosinophil activation, B-cell development	(Lopez et al. 1988)
GM-CSF Granulocyte/Macrophage Colony Stimulating Factor	CSFR2B**	CSF2RA		Multi-lineage haematopoietic growth factor	(Francisco-Cruz et al. 2014)
The IL-6 Family					
IL-6 interleukin-6	IL6ST	IL6RA		Pleiotropic functions, haematopoiesis, acute phase response, lymphoid differentiation	(Kishimoto et al. 1995; Kishimoto, Akira, and Taga 1992)
LIF leukaemia inhibitory factor	IL6ST	LIFR		Pleiotropic functions, bone remodelling, CNS functions	(Hilton 1992)
CNTF ciliary neurotrophic growth factor	IL6ST	CNTFR	LIFR	Motor neuron survival and development	(Stahl and Yancopoulos 1997)
CT1 cardiotrophin 1	IL6ST	LIFR		Cardiac myocyte growth factor	(Stahl and Yancopoulos 1997)
CLC cardiotrophin-like cytokine	IL6ST	LIFR		Neurological growth factor	(Kraves and Weitz 2006; Senaldi et al. 2002)
IL-11 Interleukin 11	IL6ST	IL11RA		Pleiotropic functions	(Trepicchio et al. 1999)

OSM Oncostatin M	IL6ST	OSMR	Pleiotropic functions, bone formation	(Stahl and Yancopoulos 1997)
IL-31 interleukin-31	OSMR	IL31RA	Inflammatory	(Zhang et al. 2008)
Homodimeric cytokines				
GCSF Granulocyte Colony Stimulating Factor	GCSFR		Stimulates granulopoiesis, mobilises stem cells	(Lieschke and Burgess 1992)
EPO Erythropoietin	EPOR	EPOR	Stimulates erythropoiesis	(Richmond, Chohan, and Barber 2005)
TPO Thrombopoietin	MPL	MPL	Stimulates thrombopoiesis, regulates HSCs	(Fishley and Alexander 2004)
GH Growth Hormone	GHR	GHR	Growth	(Frank 2001)
PRL Prolactin	PRLR	PRLR	Mammopoesis and lactation	(Boyle et al. 2007)
LEP Leptin	LEPR	LEPR	Appetite control	(Ahima et al. 1996)
Other cytokines				
IL-12 interleukin-12	IL12B1	IL12B2	Differentiation of T- and NK cells and inflammation	(Trinchieri 2003, 1998)
IL-13 interleukin-13	IL4RA	IL-13RA	Allergic inflammation	(McKenzie et al. 1993; Wills-Karp et al. 1998)
IL-23 interleukin-23	IL12RB1	IL23R	Inflammatory	(Volpe et al. 2008)
TSLP Thymic stromal lymphopoietin	CRLF2	IL7RA	Allergic inflammation, stimulates T- and B-cells	(Liu 2006)
Class II cytokines				
Type I interferon				
IFN α interferon alpha	IFNAR1	IFNAR2	Antiviral response, secreted by lymphocytes, fibroblasts and monocytes	(Kotenko and Langer 2004)
IFN β interferon beta	IFNAR1	IFNAR2	Antiviral response, ubiquitously expressed	(Kotenko and Langer 2004)
IFN ϵ interferon epsilon			Antiviral response, expressed in female reproductive tract	(Kotenko and Langer, 2004)
IFN κ interferon kappa	IFNAR1	IFNAR2	Antiviral response, expressed by keratinocytes	(Kotenko and Langer 2004)
IFN ω interferon omega	IFNAR1	IFNAR2	Antiviral response, secreted by leukocytes	(Kotenko and Langer 2004)
Type II interferon				

IFN γ interferon gamma	IFNGR1	IFNGR2	Pro-inflammatory, secreted by T- and NK-cells. Activates macrophages/monocytes.	(Kotenko and Langer 2004)
Type III interferon				
IFN α 1	IL28RA	IL10RB	Antiviral response, similar to type I but acts on fewer cell types.	(Kotenko and Langer 2004)
IFN α 2	IL28RA	IL10RB	Antiviral response, similar to type I but acts on fewer cell types.	(Kotenko and Langer 2004)
IFN α 3	IL28RA	IL10RB	Antiviral response, similar to type I but acts on fewer cell types.	(Kotenko and Langer 2004)
Other interferons				
IL-10 interleukin-10	IL10RA	IL10RB	Anti-inflammatory processes, inhibits macrophage activation	(Renauld 2003)
IL-19 interleukin-19			Inflammatory, acts on dermal cells	(Gallagher 2010)
IL-20 interleukin-20	IL20RA	IL20RB	Inflammatory, acts on dermal cells	(Blumberg et al. 2001)
IL-22 interleukin-22	IL22R1	IL10RB	Pro-inflammatory processes, secreted by TH1 cells and acts on dermal cells	(Conti et al. 2003)
IL-24 interleukin-24	IL20RA	IL20RB	Inflammatory, acts on dermal cells	(Conti et al. 2003)

* also called γ common

** also called β common

1.1.3 Molecular mechanisms of cytokine signalling

Cytokine binding to receptors triggers the activation of a suite of signalling pathways in cells, including the JAK-STAT pathway (Nicola 1994), the mitogen-activated protein kinase (MAPK) pathway (Fahmi et al. 2013), and the Phosphoinositide 3-kinase (PI3K) pathway (Takano et al. 2007).

1.1.3.1 The JAK-STAT signalling pathway

JAK-STAT signalling is elegantly simple in that the cytokine requires only three components to elicit a response: a receptor, a kinase (JAK) and a transcription factor (STAT). Each cytokine binds to a specific receptor on the surface of its target cell. Using classical IL-6 signalling as an example (**Figure 1.2**), IL-6 associates first with the IL6RA receptor prior to forming a hexameric complex (2:2:2) with the signalling receptor, IL6ST (Boulanger et al. 2003; Xu et al. 2010). IL6ST contains an intracellular domain which is constitutively associated with members of the JAK family of tyrosine kinases. The JAKs are inactive prior to cytokine exposure, however binding of a cytokine to its receptor induces their autoactivation (O'shea et al. 1997; Haan et al. 2001). Upon activation, the JAKs are able to phosphorylate nearby tyrosine residues, such as those located on the receptor tails, which then become available for proteins with SH2 domains to bind, including the STAT proteins. STATs are found in the cytoplasm as pre-existing dimers in their inactive state (Braunstein et al. 2003; O'shea et al. 1997) and upon binding to the phosphorylated tyrosine residues on the receptor they are rapidly phosphorylated by the JAK proteins. Following phosphorylation, the STAT proteins re-orient themselves to form active dimers and subsequently translocate to the nucleus where they bind to sequences in DNA and regulate the transcription of target genes (Schindler and Darnell Jr 1995).

1.1.3.2 Activation of MAPK and PI3K signalling

IL-6 is an example of a cytokine that activates multiple signalling pathways in target cells. In the case of the receptors IL6ST and leukaemia inhibitory factor receptor (LIFR), the MAPK pathway is activated when an SH2 domain containing phosphatase, SHP2, binds to phosphotyrosine residues (Cha and Park 2010) and links the GRB2-SOS complex to these receptors. Upon binding to the phosphorylated receptor, SHP2 is also tyrosine

phosphorylated by JAK, which allows for interaction with GRB2. Activation of the MAPK cascade through the oncostatin M receptor (OSMR) occurs through interactions with the adaptor protein SHC at Tyr 317 in the receptor (Hermanns et al. 2000). Similar to SHP2 in the case of IL6ST and LIFR, SHC is then phosphorylated and recruits GRB2. SOS recruitment to the receptor-SHP2 or SHC-GRB2 complex promotes activation of Ras which then drives the Ras-Raf-MAPK cascade.

IL-6 signalling can also lead to the activation of PI3K, an enzyme that phosphorylates phosphatidylinositides which leads to the recruitment and phosphorylation of AKT (Takahashi-Tezuka et al. 1997). Activation of AKT, a serine/threonine kinase subsequently leads to activation of numerous other pathways including the mTOR pathway, which is important for cell survival and cell cycle.

This thesis will focus predominantly on signalling through the JAK-STAT pathway, with the following sections discussing the components of the pathway, its regulation, and involvement in disease.

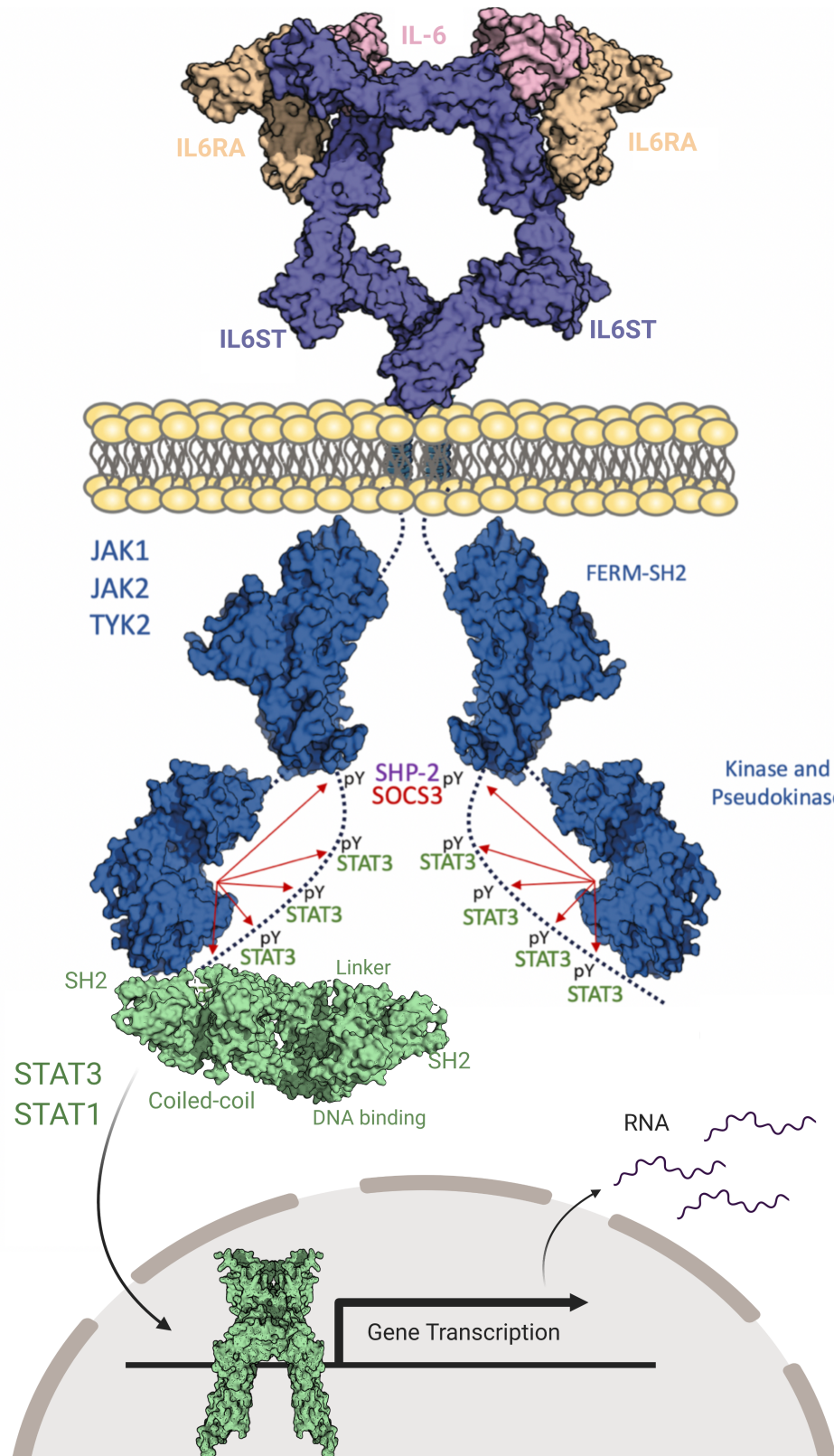


Figure 1.1.2 The IL-6 JAK-STAT signalling pathway.

IL-6 signalling activates JAK1, JAK2 and TYK2, which in turn activate STAT3 and STAT1 to a lesser extent. STATs drive gene expression in the nucleus. SHP2 and SOCS3 can bind to the IL6ST receptor tail, with SOCS3 downregulating signalling, and SHP2 activating the MAPK pathway.

1.1.4 The Janus Kinase (JAK) proteins

Four mammalian JAK proteins have been identified; JAK1, JAK2, JAK3 and TYK2 (Firmbach-Kraft et al. 1990; Harpur et al. 1992; Wilks 1989; Wilks et al. 1991). Each JAK comprises four domains and are ca. 1000 residues in length. The first domain is a 4.1, ezrin, radixin, moesin (FERM) domain, followed by an SH2-like domain. The third is a pseudokinase domain, followed by a C-terminal kinase domain (**Figure 1.3**).

The FERM and SH2 domains of the JAKs are often grouped as one functional unit, as together they are responsible for binding to the Box I and Box II motifs of the cytokine receptor (Wallweber et al. 2014; Zhang, Wlodawer, and Lubkowski 2016; Ferrao et al. 2016). The N-terminal FERM domain comprises three lobes, the second of which is responsible for interactions with the Box I motif of receptors. The SH2-like domain interacts with the Box II motif of the receptor, and together they constitutively tether the JAKs to the receptor.

The pseudokinase domain of the JAK proteins retains the canonical kinase fold (Bandaranayake et al. 2012; Lupardus et al. 2014), however it is catalytically inactive. The pseudokinase domain has been shown to have regulatory functions, modulating the activity of the active tyrosine kinase domain (Saharinen, Takaluoma, and Silvennoinen 2000; Saharinen and Silvennoinen 2002; Ungureanu et al. 2011). The functional significance of the pseudokinase domain was further highlighted upon the discovery of the JAK2 V617F mutation, which causes the protein to be constitutively active. The V617F mutation was identified in patients with myeloproliferative neoplasms (Kralovics et al. 2005; Levine et al. 2007; James et al. 2005) a group of diseases that will be discussed in detail later. The kinase domain is the catalytically active domain of the JAK proteins and is absolutely essential for downstream signalling. The kinase domain adopts the canonical kinase fold, forming two lobes. The N-terminal lobe contains the majority of the conserved residues required for ATP-binding, while the C-terminal lobe contains those required for phosphotransfer and Mg^{2+} -binding, with the ATP-binding site being situated between the two lobes. The region of the JAKs that become phosphorylated upon cytokine binding is termed the activation loop (Feng et al. 1997). When unphosphorylated, the JAKs are inactive, and only upon activation loop phosphorylation

do they become fully active. All four JAK proteins possess an activation loop that contains two tandem tyrosine residues. The first of these appears to be critical for activation of the enzyme, however both are found fully or partially phosphorylated upon activation (Williams et al. 2009; Lucet et al. 2006; Boggon et al. 2005; Chrencik et al. 2010).

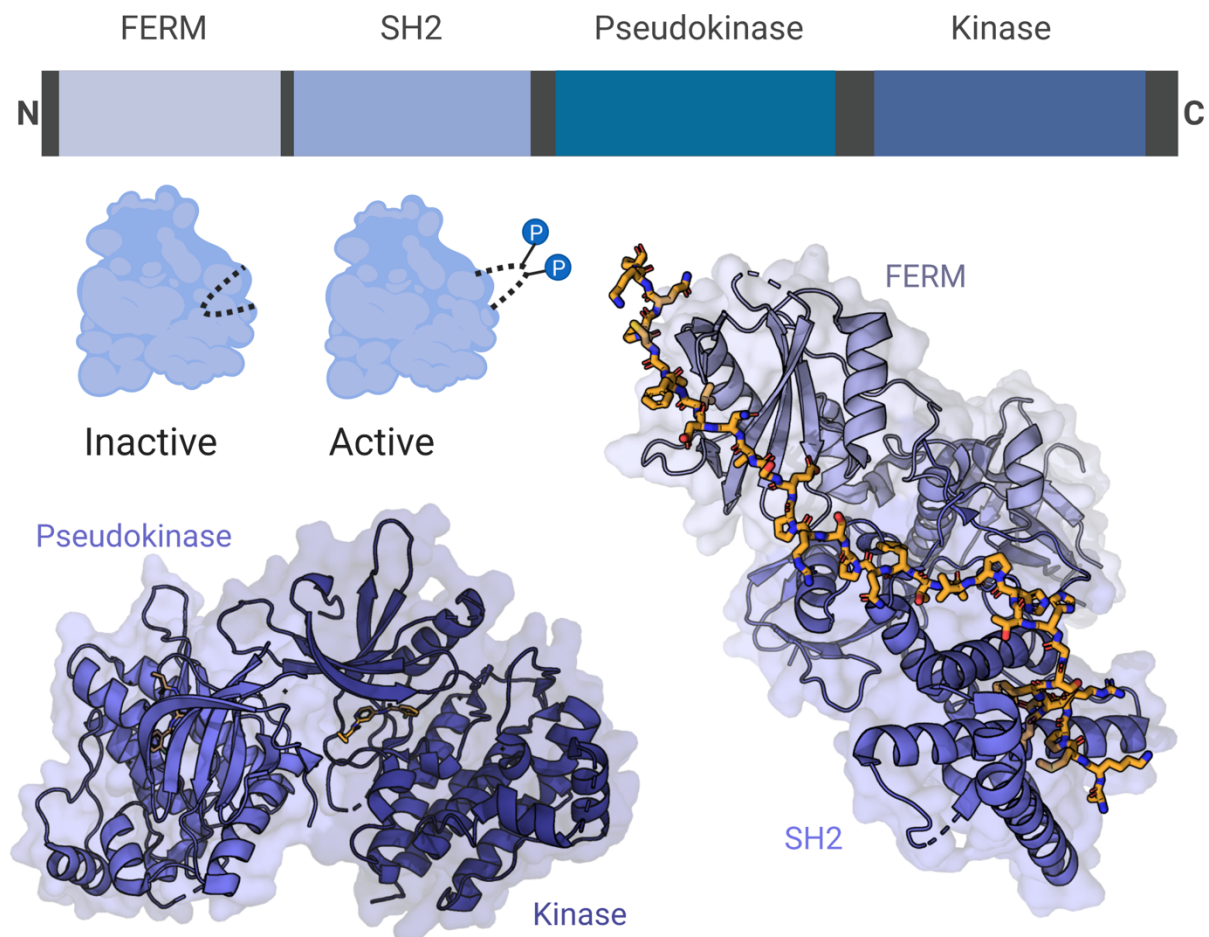


Figure 1.1.3 The Janus Kinase (JAK) proteins.

The four JAK proteins comprise four domains. The FERM and SH2 domains tether the JAKs to the cytokine receptor by binding the Box I and Box II motifs (structure of these domains is shown on the right. PDB ID: 5L04 (Zhang, Wlodawer, and Lubkowski 2016)). The pseudokinase domain has no kinase activity but has regulatory functions, and the kinase domain is the active catalytic domain (structure of these domains is shown on the left. PDB ID: 4OLI (Lupardus et al. 2014)). The kinase domain contains a motif termed the activation loop, which contains two tandem tyrosine residues that are phosphorylated upon cytokine signalling and kinase domain activation.

1.1.5 The Signal Transducer and Activator of Transcription (STAT) proteins

The STATs are a family of proteins named for their dual roles in transducing signals from cytokines and promoting transcription of specific genes in their target cells. Inside cells, the STAT proteins are found in their inactive state and are activated upon cytokine signalling, allowing for the rapid and specific activation of gene transcription in response to cytokine binding (Darnell 1997; Shuai et al. 1992; Darnell Jr, Kerr, and Stark 1994). Seven mammalian STAT proteins have been identified (STATs 1-4, STAT5a, STAT5b and STAT6) (Zhong, Wen, and Darnell 1994; Copeland et al. 1995; Shuai, Stark, et al. 1993) and each contains several conserved features discussed below.

The N-terminal ~100 residues in the STAT proteins are largely conserved between all seven proteins and are thought to provide stability to the overall STAT structure as well as providing a surface for interactions with other proteins (Hossain et al. 2013; Strehlow and Schindler 1998). This is followed by a coiled-coil domain that comprises several α -helices which form a bundle that projects outward from the core domain during DNA binding, providing a surface for other proteins such as transcription factors to bind (Chen et al. 1998). In the case of STAT1 and STAT3, the coiled-coil domain also contains a sequence for nuclear translocation (Ma et al. 2003; Begitt et al. 2000). The DNA binding domain of the STATs was identified in 1995 as the domain involved in the recognition and binding to specific DNA sequences (Horvath, Wen, and Darnell 1995). While all STATs bind the same DNA motif (5'-TTCN₂₋₄GAA-3' where N₂₋₄ denotes a spacer of 2 to 4 of any nucleotide (Decker, Kovarik, and Meinke 1997)), their sequence preferences differ, and target gene specificity is dictated by differences in STAT dimer formation. Prior to activation, the STAT proteins are recruited to specific phosphorylated tyrosine residues on cytokine receptors. The selectivity of STAT activation for any particular cytokine signalling pathway is therefore dependent on these STAT-receptor interactions (Heim et al. 1995; Shuai et al. 1994). Studies of the STAT SH2 domains have demonstrated that the SH2 domains of the STAT proteins recognise specific phosphotyrosine motifs found on cytokine receptor tails, each of which is distinct for each STAT. In short, which STAT is activated by a particular cytokine depends on motifs in the cytokine receptor, which in turn determines which genes are activated.

Subsequent to STATs docking to phosphorylated cytokine receptors, the JAKs rapidly phosphorylate a conserved C-terminal tyrosine in the STATs (Darnell, Kerr, and Stark 1994). Structures of the STATs prior to and following activation by the JAK proteins has provided insights into the role of the SH2 domains in dimerization of the STATs. Further structural studies on various STAT proteins bound to DNA has shown how STATs are able to recognise specific DNA sequences and upregulate gene expression via their DNA-binding domain (Horvath, Wen, and Darnell 1995). Prior to tyrosine phosphorylation, and activation, the STATs are found as inactive dimers in the cytoplasm. Crystal structures of the inactive forms of STAT1, STAT3, and STAT5 show that the DNA binding domain and coiled-coil domains of two STAT monomers interact to form a reciprocal dimer (Ren et al. 2008; Neculai et al. 2005; Mao et al. 2005) **(Figure 1.4)**. Upon phosphorylation of a critical tyrosine residue at the transactivation domain (TAD) of STAT proteins, these STAT dimers undergo a major structural change where the phosphorylated tyrosine of one STAT molecule sits in the SH2 domain of the other, forming an almost parallel (rather than a head-to-tail) dimer. These phosphorylated STAT dimers then translocate to the nucleus and bind γ -activated sequences (GAS) or interferon-stimulated response elements (IRSE) in DNA (Reich et al. 1987).

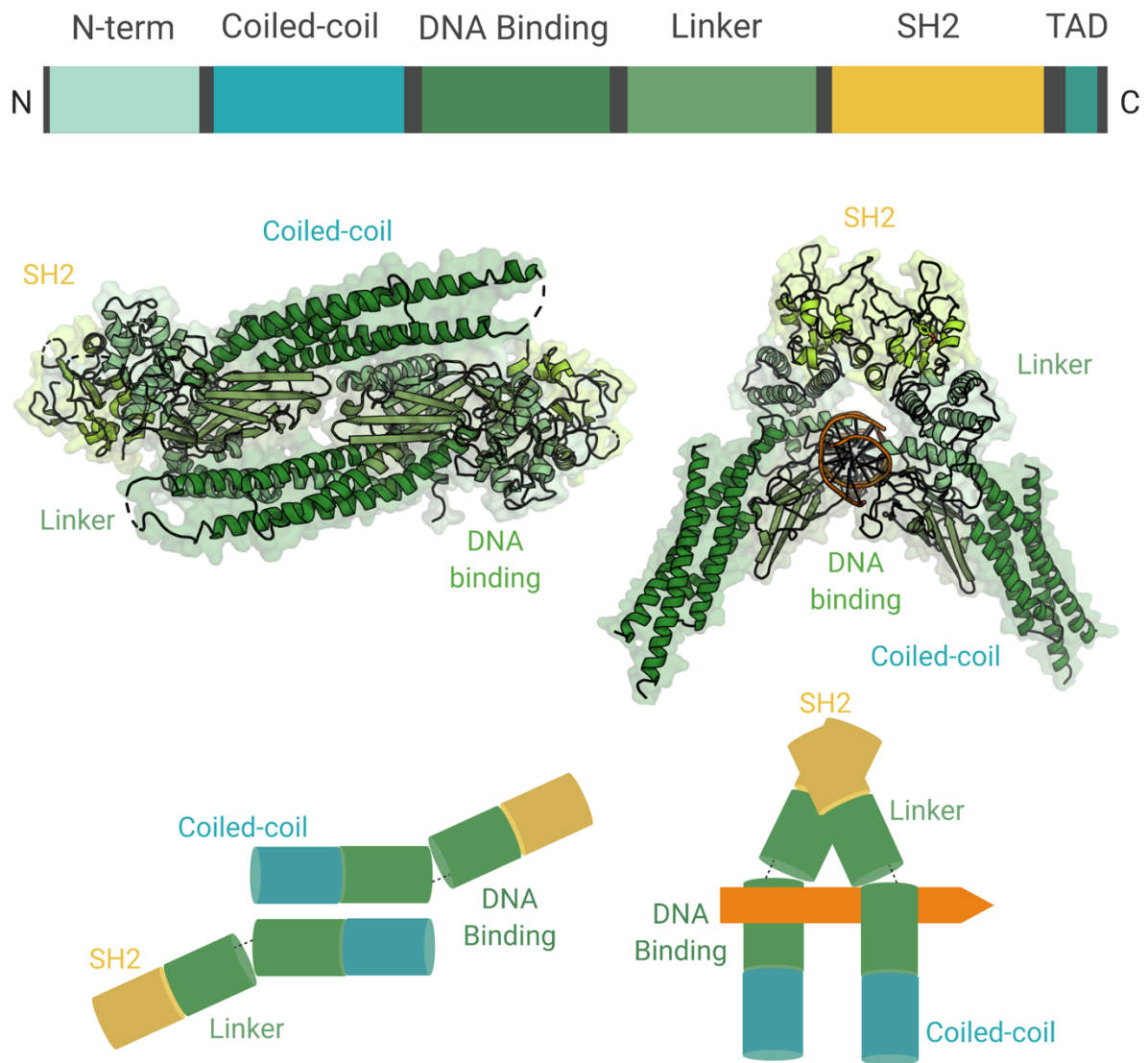


Figure 1.1.4 The Signal Transducer and Activator of Transcription (STAT) proteins. The STATs are a family of transcription factors involved in signal transduction via the JAK-STAT pathway. All STATs contain the same five domains, schematically shown above. Unphosphorylated STAT (uSTAT) exists as an inactive antiparallel dimer in the cytoplasm (left PDB ID: 1YVL (Mao et al. 2005)). The SH2 domain of uSTAT binds to phosphotyrosine residues in cytokine receptors. Once bound to the receptors, the JAKs phosphorylate the STATs at a tyrosine located in the transactivation domain (TAD). Phosphorylation of this tyrosine induces a conformational change into the active STAT form, where the SH2 domains of the STAT monomers form reciprocal interactions with the pTyr in the TAD. This allows phosphorylated STAT (pSTAT) to adopt its DNA-binding competent dimeric structure (right PDB ID: 1BF5 (Chen et al. 1998)).

1.2 Regulation of the JAK-STAT pathway

Signalling via the JAK-STAT pathway is a dynamic process that involves the rapid transmission of a signal from the cell membrane to the nucleus. This is followed by a highly organized cellular response and subsequent controlled downregulation and attenuation of the initial signal (Shuai, Ziemiecki, et al. 1993). Tight regulation of the JAK-STAT pathway is essential for the wellbeing of an organism as aberrant signalling via this pathway is associated with a variety of diseases and disorders (O'Sullivan et al. 2007). Negative regulation of this pathway is particularly important and can be achieved by one or more mechanisms, including downregulation of receptor/ligand expression, dephosphorylation of positive regulators, the upregulation of negative regulators, and the direct inhibition or degradation of signalling intermediates. Since the signalling cascade generated by cytokine-receptor engagement is effectively a wave of tyrosine phosphorylation driven by the JAK proteins, it is unsurprising that many of the proteins involved in the negative regulation of the JAK-STAT pathway interact with phosphorylated tyrosine residues.

This thesis will discuss the roles of the suppressor of cytokine signalling (SOCS) protein family, a group of protein tyrosine phosphatases, and the adaptor protein, LNK, in the negative regulation of this pathway (**Figure 1.5**). While the role of the SOCS proteins in regulating cytokine signalling is well understood, less is known about how PTP1B and LNK are involved in the regulation of signalling. Understanding the role of the latter two proteins will be the major focus of this thesis.

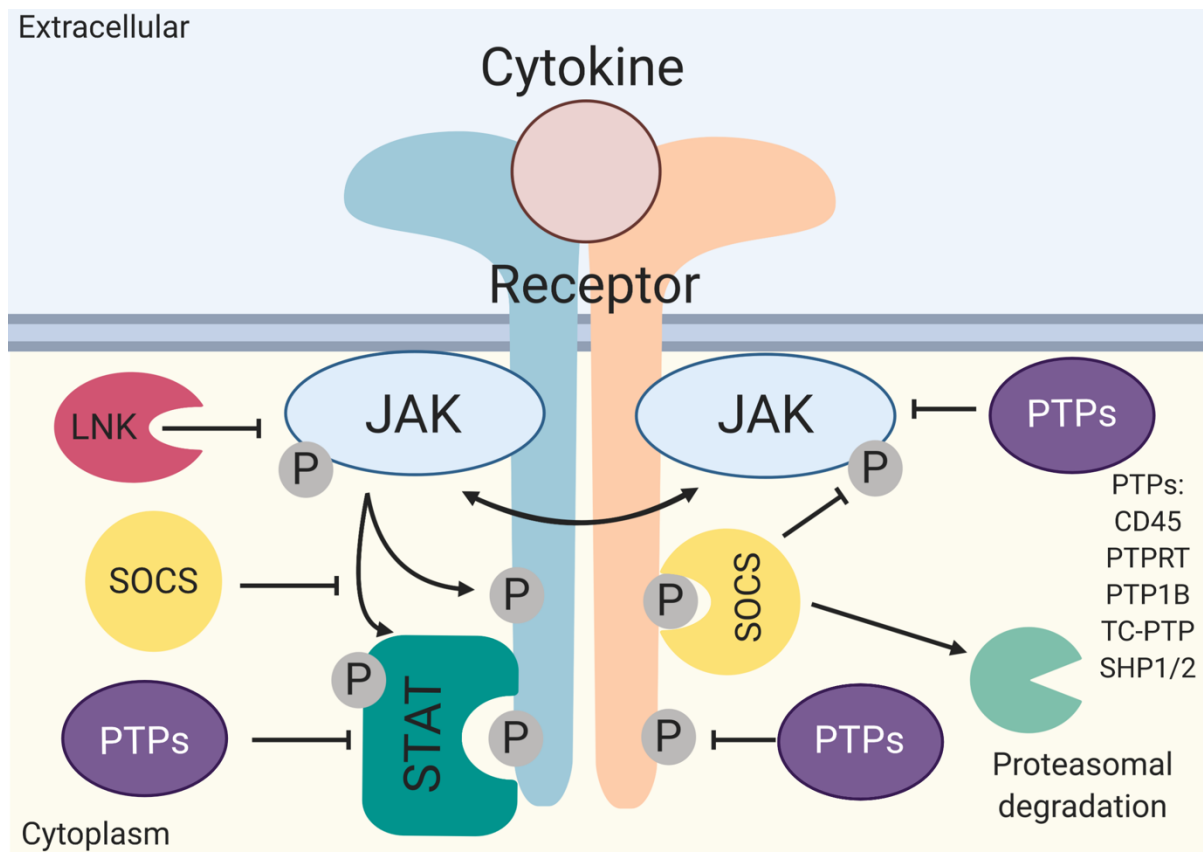


Figure 1.1.5 Negative regulators of cytokine signalling.

Six phosphatases have been identified to play a role in the regulation of JAK-STAT signalling, namely CD45, PTPRT, PTP1B, TC-PTP, SHP1 and SHP2. The phosphatases act by removing phosphate groups from phosphorylated tyrosine residues on the receptors, JAKs and STATs. The SOCS proteins are a family of eight proteins that recruit an E3 ubiquitin ligase complex which ubiquitinates the proteins of the JAK-STAT pathway, ultimately leading to proteasomal degradation. SOCS1 and SOCS3 are also able to directly inhibit JAK activity by blocking the substrate binding pocket with their kinase inhibitory region. LNK is an adaptor protein that contains an SH2 domain which binds phosphotyrosine residues in JAK2 and regulates activity downstream of EPO and TPO signalling.

1.2.1 Regulators of JAK-STAT signalling I. The Suppressor of Cytokine Signalling (SOCS) proteins

Humans express eight SOCS proteins, namely SOCS1-7 and CIS (Hilton et al. 1998). As their name suggests, the SOCS proteins play an important role in the negative regulation of cytokine signalling by controlling the signal duration (Endo et al. 1997; Starr et al. 1997; Naka et al. 1997). Each SOCS protein contains an N-terminal domain of varying length and unknown function, an SH2 domain which allows them to directly interact with their phosphorylated targets, which include the receptors and JAK proteins of the JAK-STAT pathway (Kershaw et al. 2013; Liao et al. 2018), and a C-terminal SOCS box (Hilton et al. 1998) (**Figure 1.6**). The expression of the SOCS proteins is induced upon cytokine signalling, as their genes are a target of STAT-induced upregulation (Starr et al. 1997).

In addition to binding to cytokine receptors and JAK proteins, the C-terminal SOCS box forms interactions with the adaptor proteins elongin B and C (Zhang et al. 1999; Babon et al. 2008), which then recruits Cullin-5, an E3 ligase scaffold (Babon et al. 2009). Cullin-5 is constitutively associated with RBX2, a RING domain protein, which recruits E2 ubiquitin conjugating enzymes that catalyse ubiquitination of the components of the JAK-STAT pathway (Kamura et al. 2004; Mahrour et al. 2008). Ubiquitinated substrates are then directed to the proteasome for degradation.

SOCS1 and SOCS3 also have the unique ability to directly inhibit the activity of the JAKs through the use of a motif termed the Kinase Inhibitory Region (KIR) (Sasaki et al. 1999; Yasukawa et al. 1999). The KIR is an unstructured extension of SOCS1 and SOCS3 (Babon et al. 2005; Babon et al. 2006) which is able to sit in the substrate binding groove of JAK1, JAK2 and TYK2, thus occluding substrate binding (Kershaw et al. 2013). This ultimately prevents the JAKs from phosphorylating and activating their substrates, resulting in inhibition of the JAK-STAT pathway.

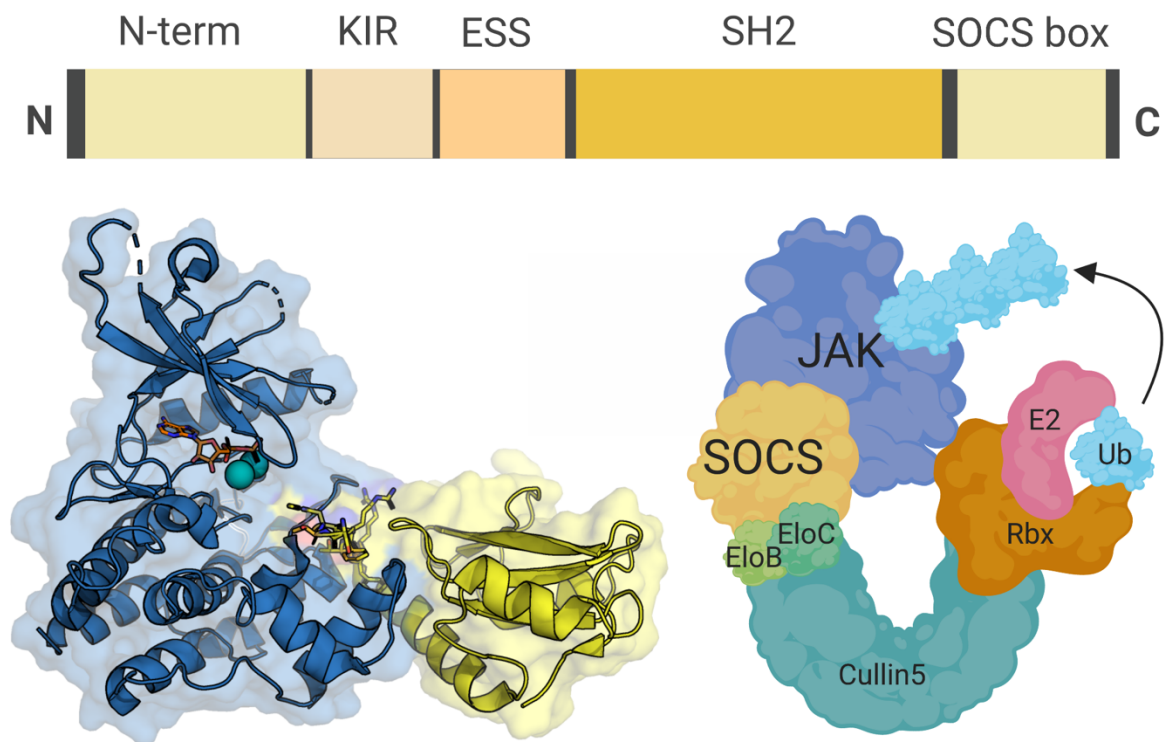


Figure 1.1.6 The Suppressor of Cytokine Signalling (SOCS) proteins.

Domain architecture of the SOCS proteins (KIR in SOCS1 and SOCS3). These proteins function as the substrate recruitment modules of an E3 ubiquitin ligase (modelled on the right) and promote the ubiquitination and degradation of cytokine receptors and potentially other substrates. Substrates bind to the SH2 domain of SOCS proteins (yellow) and ubiquitin is transferred via an E2 ubiquitin-conjugating enzyme that docks onto the RING-domain protein Rbx2. SOCS1 and SOCS3 have a unique motif termed the kinase inhibitory region (KIR) and can directly inhibit the JAK kinase domain by using their kinase inhibitory region to block the substrate binding site of the kinase, as shown on the left (PDB ID: 6C7Y (Liau et al. 2018)).

1.2.2 Regulators of JAK-STAT signalling II. Protein tyrosine phosphatases

Cytokine association with the appropriate receptors triggers a cascade of intracellular tyrosine phosphorylation events, therefore it is not surprising that tyrosine phosphatases play a crucial role in regulating signalling via the JAK-STAT pathway (Tonks and Neel 2001; Hunter 1995). Six tyrosine phosphatases in particular have been shown to regulate JAK-STAT signalling: the receptor tyrosine phosphatases CD45 and PTPRT, two related cytoplasmic phosphatases PTP1B and TC-PTP, and finally the SH2 domain containing phosphatases SHP1 and SHP2 (Neel 1993) (**Figure 1.7**). These phosphatases tend to restrain the amplitude of the signalling cascade, rather than control its duration. In other words, it is the balance between the action of these phosphatases and the activity of the JAKs that determines flux through the pathway.

Below is a short summary of the three major classes of phosphatases implicated in regulating cytokine signalling. All six phosphatases involved in the regulation of JAK-STAT signalling are cysteine based classical PTPs that specifically target phosphorylated tyrosine residues. Some phosphatases bind substrates via their SH2 domains and are expressed predominantly by haematopoietic cells and are able to dephosphorylate receptors as well as the JAK and STAT proteins (Klingmüller et al. 1995; Bone et al. 1997). These include SHP1 and SHP2. The other phosphatases involved in JAK-STAT signalling lack an SH2 domain and bind directly to their substrates via their phosphatase domain, which appears to provide their substrate specificity (Ubersax and Ferrell Jr 2007). These phosphatases include CD45, PTP1B, TC-PTP and PTPRT (**Figure 1.7 and 1.8**).

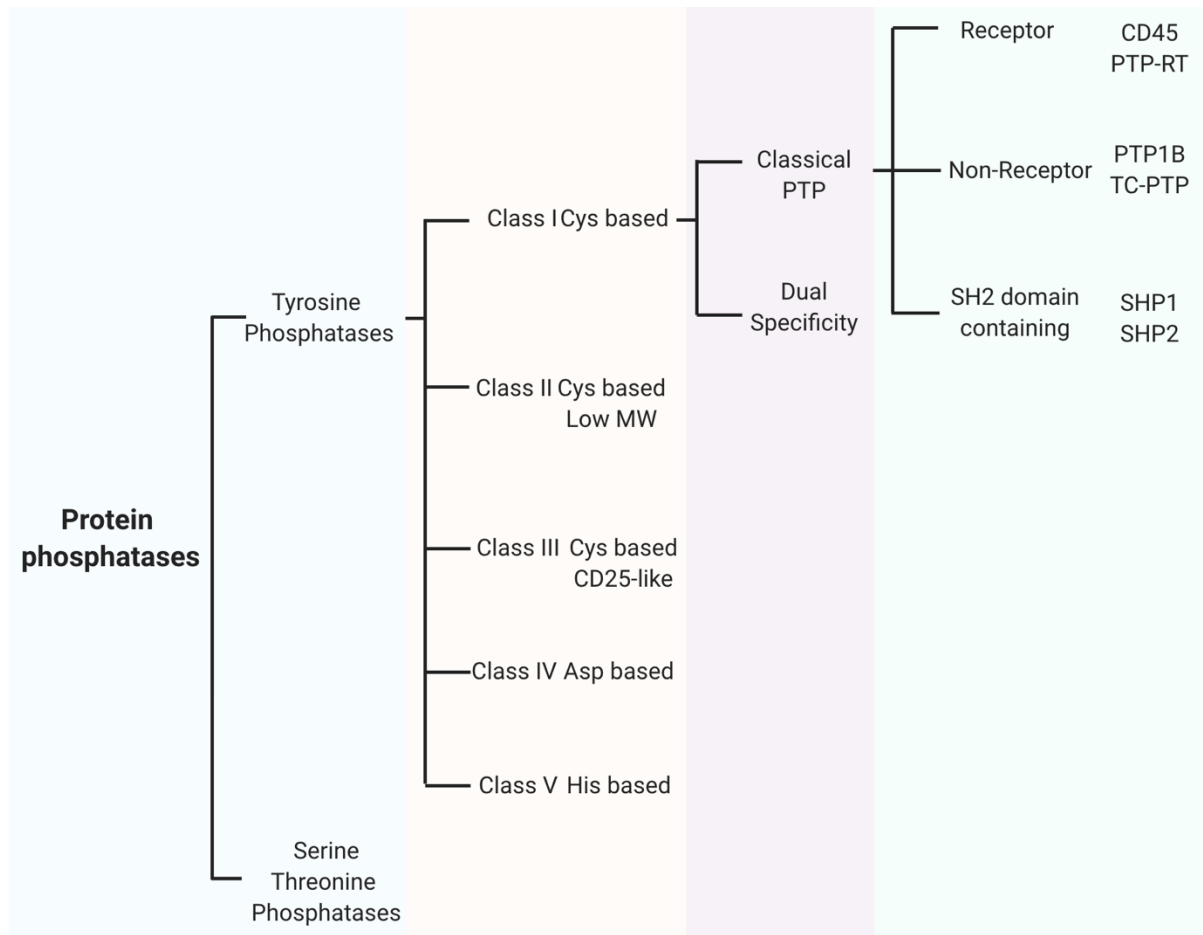


Figure 1.1.7 Classification of the phosphatases that are involved in the regulation of JAK-STAT signalling.

The six phosphatases involved in the regulation of JAK-STAT signalling are tyrosine specific phosphatases. They all contain a cysteine based catalytic pocket and can be broken up into receptor type (CD45 and PTPRT), non-receptor type (PTP1B and TC-PTP) and SH2 domain containing (SHP1 and SHP2) phosphatases.

1.2.2.1 The receptor phosphatases CD45 and PTPRT

CD45 and PTPRT are both receptor tyrosine phosphatases comprising an extracellular receptor-like region, a transmembrane domain and two intracellular tandem phosphatase domains. For both CD45 and PTPRT the first phosphatase domain is the catalytically active domain, whereas the second is a catalytically dead pseudophosphatase domain that is thought to have regulatory roles in both proteins. The extracellular region of CD45 also contains three FNIII domains prior to the transmembrane region that anchors the protein to the plasma cell membrane (Cahir McFarland, Pingel, and Thomas 1997). CD45 is highly expressed in haematopoietic cells and is thought to play vital roles in regulating antigen-receptor signalling by dephosphorylating all four JAK proteins (Irie-Sasaki et al. 2001). PTPRT is 1441 aa in length with an N terminal MAM domain followed by an Ig domain, four FNIII domains, a transmembrane domain and tandem PTP domains. PTPRT directly interacts with and dephosphorylates the critical tyrosine residue, pY705, in STAT3 (Zhang et al. 2007).

1.2.2.2 The SH2 domain containing phosphatases SHP1 and SHP2

The two cytoplasmic SH2 domain containing phosphatases, SHP1 and SHP2, are also known to be regulators of JAK-STAT signalling. Both SHP1 and SHP2 comprise two SH2 domains and a single PTP domain. The long form of SHP1 is 624 aa in length and expression is limited to the haematopoietic lineage and endothelial cells (Plutzky, Neel, and Rosenberg 1992; Tsui et al. 2002). Unlike SHP1, SHP2 is ubiquitously expressed (Feng, Hui, and Pawson 1993), however is also thought to play an essential role in the regulation of haematopoiesis. The 593 aa SHP2 protein binds pY759 on the IL6ST receptor (Lehmann et al. 2003) and negatively regulates JAK1, JAK2, and STAT3 (Cha and Park 2010; Huang et al. 2017). SHP2 can also negatively regulate STAT5 in response to IL-3 and IL-2 stimulation (Wheadon, Paling, and Welham 2002; Bone et al. 1997; Adachi et al. 1997).

1.2.2.3 The cytoplasmic phosphatases PTP1B and TC-PTP

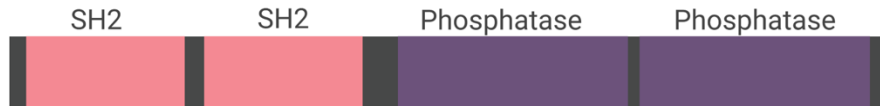
PTP1B and TC-PTP are two highly related cytoplasmic phosphatases that appear to have non-overlapping roles in cells. TC-PTP has two splice variants with differing cellular

localisation. The 415 aa TC-PTP is generally localised to the nucleus (Yamamoto et al. 2002), whereas the 387 aa protein interacts with the endoplasmic reticulum via a C terminal hydrophobic region (Haj et al. 2002). Both PTP1B and TC-PTP are expressed in a broad range of cell types including skeletal muscle, liver, adipose, tissue, brain and blood cells (Simoncic, McGlade, and Tremblay 2006; Zabolotny et al. 2008). PTP1B is a 435 aa protein comprising an N-terminal catalytic phosphatase domain followed by tandem proline rich motifs and an endoplasmic reticulum (ER) targeting motif that tethers this protein to the cytoplasmic facing side of the ER (Guan et al. 1990; Brown-Shimer et al. 1990; Frangioni et al. 1992). PTP1B has been shown to interact with JAK2 and TYK2 in cellular based assays (Myers et al. 2001) and PTP1B ^{-/-} mice have increased JAK2 phosphorylation (Zabolotny et al. 2002). The biological roles, structure and mechanism of PTP1B are discussed in detail below.

PTP1B & TC-PTP



SHP1 & SHP2



CD45



PTP-RT

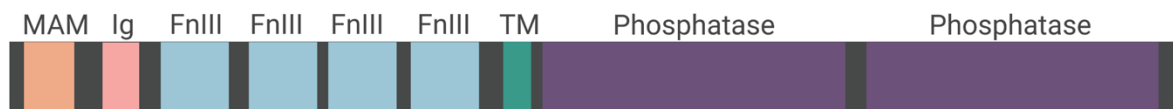


Figure 1.1.8 Domain architecture of the phosphatases involved in the regulation of JAK-STAT signalling.

(First) Protein tyrosine phosphatase 1B (PTP1B) and T-cell protein tyrosine phosphatase (TC-PTP) both have an N-terminal phosphatase domain and a C-terminal targeting motif that tethers the proteins to the cytoplasmic facing side of the endoplasmic reticulum (PTP1B) or nucleus (TC-PTP). (Second) SHP1 and SHP2 both contain two tandem N-terminal SH2 domains and two tandem C-terminal phosphatase domains. (Third) CD45 is a receptor tyrosine phosphatase with N-terminal receptor sequences and two C-terminal phosphatase domains. (Fourth) PTPRT is a receptor tyrosine phosphatase with two tandem C-terminal phosphatase domains.

1.2.3 PTP1B; the prototypical protein tyrosine phosphatase

1.2.3.1 Phosphatase structure

The overall structure of the protein tyrosine phosphatase domains share a common fold comprising several central beta strands surrounded by alpha helices (**Figure 1.9A and B**). All protein tyrosine phosphatases contain several conserved motifs that are required for catalysis and structure. The signature PTP loop in all family members forms the base of the catalytic pocket and comprises the sequence VHCSXGXGR[T/S]G (corresponding to residues 213 to 223 in PTP1B). The residues of the PTP loop coordinate the phosphotyrosine residue in the catalytic pocket, positioning the phosphate moiety for the dephosphorylation reaction. Binding of a phosphotyrosine containing substrate in the catalytic pocket results in a conformational change in a region termed the WPD loop. Upon substrate binding, the WPD loop moves over the phosphotyrosine residue to trap the bound residue, and brings the catalytic Asp residue in close proximity to the substrate (Kamerlin, Rucker, and Boresch 2007). The residues responsible for the recognition and specificity for phosphotyrosine residues comprises aromatic and non-polar residues which pack against the phosphotyrosine phenyl ring (Tyr 46 and Phe 182 in PTP1B). These residues are important for defining the depth and specificity of the catalytic pocket for phosphotyrosine over other phosphorylated residues (Flint et al. 1997).

1.2.3.2 Mechanism of dephosphorylation by PTP1B

Substrate dephosphorylation by PTP1B has been well characterised. Prior to dephosphorylation, the substrate phosphotyrosine enters the catalytic pocket and the phosphate group is coordinated and positioned by Arg 221 and the amide backbones of the residues comprising the PTP loop (Brandão, Hengge, and Johnson 2010) (**Figure 1.9B in catalytic pocket**). Following substrate binding the flexible WPD loop, which contains the catalytic residue Asp 181, closes over the phosphotyrosine (Barford, Flint, and Tonks 1994; Jia et al. 1995). The dephosphorylation reaction then occurs in two steps. In the first step, the reduced Cys 215 residue in PTP1B directs a nucleophilic attack at the phosphocenter of the phosphotyrosine residue, and the phosphate group is transferred to Cys 215 to form a cysteinyl phosphate intermediate (Brandão, Hengge, and Johnson 2010). During this first step Asp 181 acts as a general acid. Following the first step the

dephosphorylated tyrosine leaves the catalytic pocket (Brandão, Hengge, and Johnson 2010). In the second step of the reaction, the thiophosphate intermediate is hydrolysed, which is enabled by the catalytic residues Asp 181 acting as a general base catalyst and Gln 262 coordinating a water molecule. On completion of thiophosphate cleavage, the WPD loop then opens again, and the free phosphate is released from the catalytic site (**Figure 1.9C**). This completes the reaction and regenerates the active form of the enzyme.

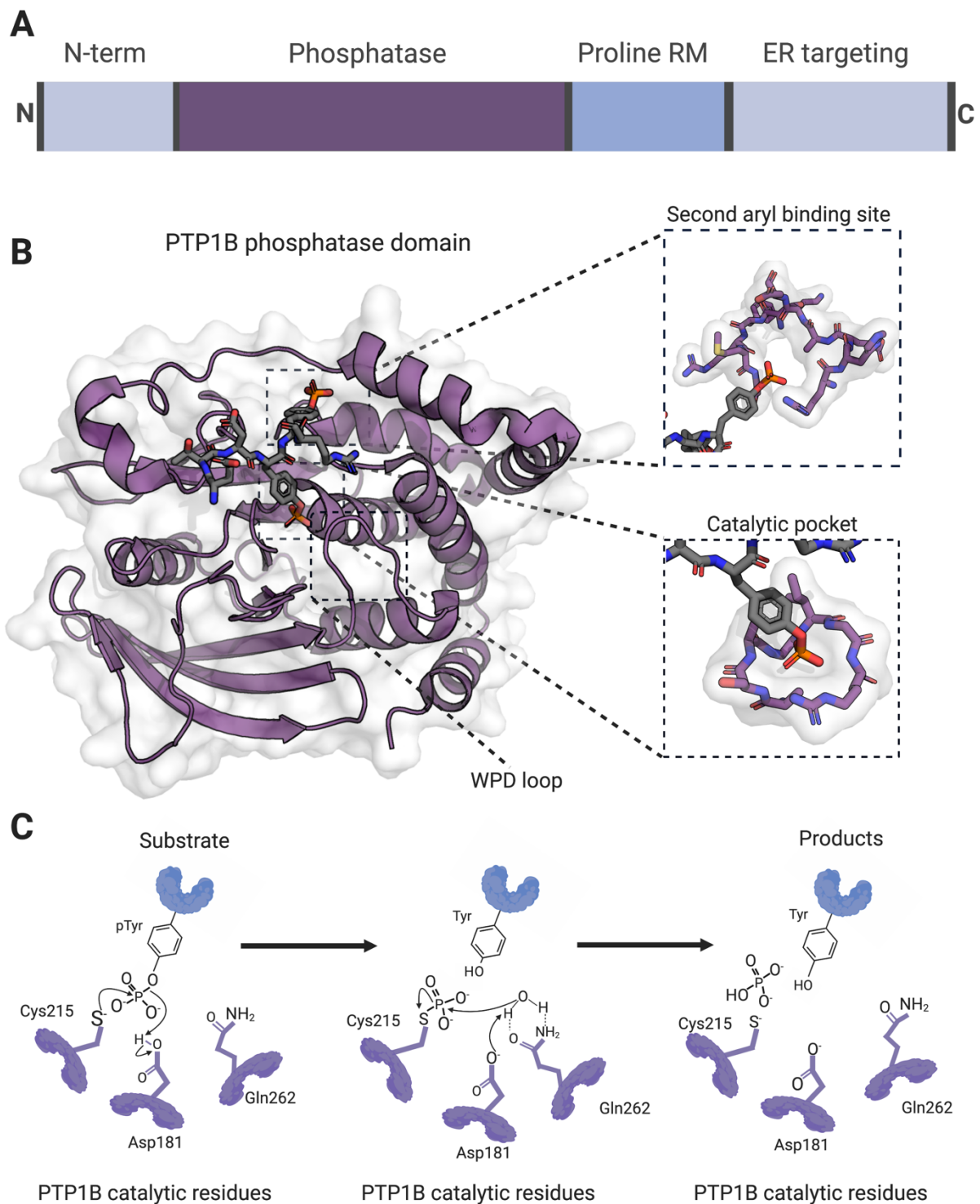


Figure 1.9 Protein Tyrosine Phosphatase 1B (PTP1B).

A) Protein tyrosine phosphatase 1B (PTP1B) is the prototypical phosphatase. PTP1B has an N-terminal phosphatase domain and a C-terminal endoplasmic reticulum (ER) targeting motif separated by a proline rich motif. **B)** PTP1B binds tandemly phosphorylated tyrosine residues, such as the IRK activation loop by interacting with the first phosphotyrosine at the catalytic site and the second phosphotyrosine in the second aryl binding site (PDB ID:1G1F (Salmeen et al. 2000)). **C)** General mechanism of dephosphorylation catalysed by PTP1B.

1.2.3.3 Regulation of insulin and leptin signalling by PTP1B

PTP1B plays a particularly significant role in the regulation of metabolic signalling (Bence and Tiganis 2013). Specifically, PTP1B is a key regulator of both insulin and leptin signalling (Zhang, Dodd, and Tiganis 2015; Tiganis 2013). PTP1B knock-out mice are hypersensitive to insulin, have low blood glucose levels (Elchebly et al. 1999; Dodd et al. 2019), and are resistant to obesity (Cheng et al. 2002), highlighting the essential role of PTP1B in the regulation of both signalling pathways. Insulin binding to the insulin receptor leads to the activation of IRK via phosphorylation. Leptin signals via the JAK-STAT pathway, specifically through JAK2. Upon leptin binding to the leptin receptor (LEPR), JAK2 is trans-phosphorylated and activated. For both pathways, activation of the kinase leads to activation of downstream signalling molecules and perpetuation of the signal. For both IRK and JAK2, PTP1B acts as an essential regulator of kinase activity by dephosphorylating the activated kinase (Zabolotny et al. 2002; Salmeen et al. 2000).

1.2.3.4 Discovering new substrates of PTP1B

The identification of phosphatase substrates would allow for a better understanding of their physiological roles and how they interact with their substrates. The development of substrate trapping mutants of PTP1B has been instrumental in defining phosphatase substrates. In PTP1B the three key catalytic residues are Asp 181, Gln 262 and Cys 215. Mutation of these residues to alanine or C215 to serine results in a reduction in catalytic activity, while having little effect on substrate affinity (Flint et al. 1997).

The interaction between PTP1B and the epidermal growth factor receptor (EGFR) was established through the use of the C215S substrate trapping mutant (Milarski et al. 1993). Shortly after, the crystal structure of the PTP1B phosphatase domain bound to a peptide corresponding to a phosphorylated segment of EGFR was determined (Jia et al. 1995). This structure revealed that PTP1B can interact with substrates that have negatively charged residues preceding the phosphotyrosine through salt-bridge interactions with Arg 47 (**Figure 1.10A**). The structure of PTP1B in complex with a peptide corresponding to pY1193 of Nephritin was also determined using the C215S mutant (Selner et al. 2014). This structure revealed that PTP1B binds the phosphotyrosine in the catalytic pocket and Arg 24 interacts with the glutamate two residues downstream of the phosphotyrosine (+2) (**Figure 1.10B**).

The crystal structure of PTP1B in complex with a peptide corresponding to the activation loop of the insulin receptor kinase (IRK) was also solved with a C215S mutant (Salmeen et al. 2000). This study notably showed how PTP1B interacts with substrates that contain tandem phosphotyrosine residues, binding the first phosphotyrosine in the catalytic pocket and the second in the second aryl binding site (**Figure 1.10C**). This study also showed that PTP1B has a higher affinity for proteins that contain tandem phosphotyrosine residues, and generates a highly specific interaction. Similarly to the IRK activation loop, JAK2 and TYK2 were shown to interact with PTP1B (Myers et al. 2001). These studies together suggested that PTP1B specifically regulates JAK2 and TYK2 along with IRK, because their activation loops have a consensus sequence comprising [D/E]pYpY[R/K]. PTP1B has also been suggested to dephosphorylate STAT5a and STAT5b (Aoki and Matsuda 2000), however, no structures of PTP1B in complex with any proteins of the JAK-STAT signalling pathway have been published and so we lack detailed information about how these interactions may occur.

The only structure of PTP1B in complex with another protein domain is between PTP1B and IRK (PDB ID: 2B4S), however, this structure does not represent the catalytic complex. Instead, it is believed that the structure may represent a non-catalytic mode of interaction important for *in vivo* specificity (Li et al. 2005) (**Figure 1.11**). There are two main interaction surfaces between the two proteins. The first interaction is seen between E1273 of IRK and K120 of PTP1B, and nearby residues including P170 and F182 of PTP1B, V1274 and P1269 of IRK, which form hydrophobic packing interactions (**Figure 1.11A**). The second interaction site involves E1022 of IRK and Y152 of PTP1B (**Figure 1.11C**). A number of Van der Waals interactions between surrounding residues in the two sites form a total buried surface area of 1725 Å² between the two proteins. Despite being unable to detect binding between unphosphorylated IRK and PTP1B in solution, this study argues that this interaction may be required for the co-localisation of the two proteins at the ER, allowing for rapid dephosphorylation upon IRK activation (Li et al. 2005). While this study may provide useful information regarding a novel interaction between these two proteins, we still lack a complete understanding of how PTP1B may interact with substrates through non-active site interactions when in complex for dephosphorylation.

While other substrates of PTP1B has been suggested, such as GRB2 (Rajala, Dilly, and Rajala 2013; Goldstein et al. 2000), MUNC18 (Bakke et al. 2013), VEGFR2 (Lanahan et al. 2014), beta-catenin (Balsamo et al. 1996), SRC (Zhu, Bjorge, and Fujita 2007), and PDGFR (Venkatesan et al. 2008), it is unlikely that the full spectrum of proteins PTP1B interacts with in a cellular context have been revealed.

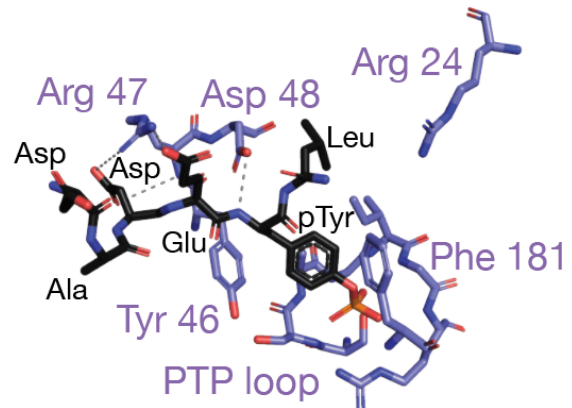
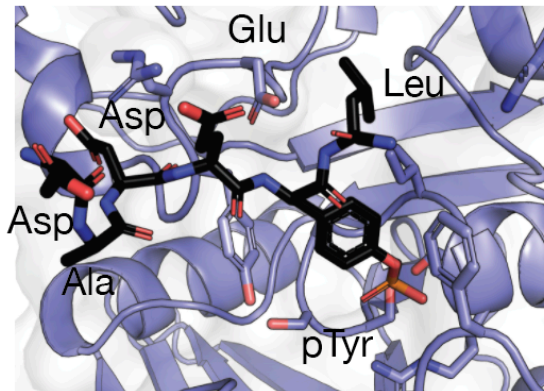
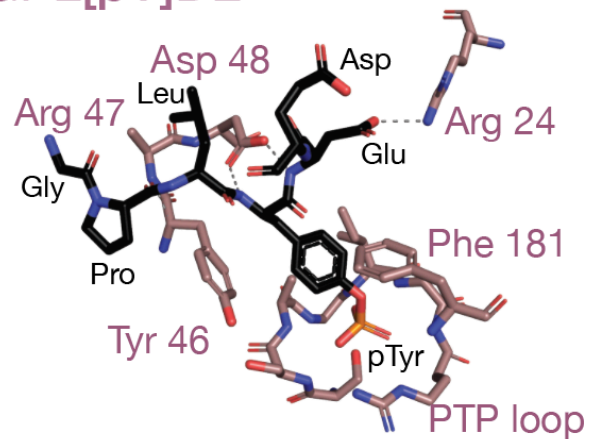
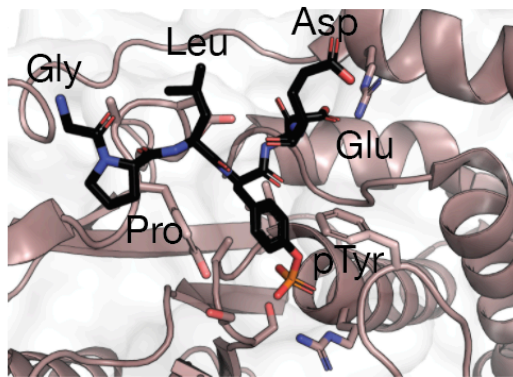
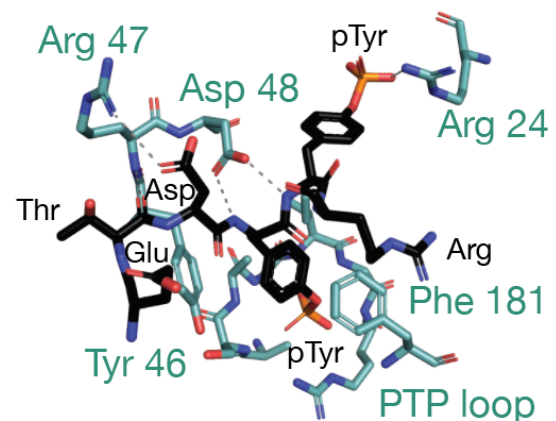
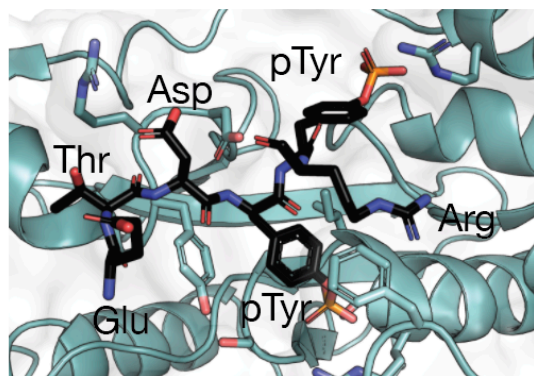
A**EGFR: DADE[pY]L****B****Nephrin: GPL[pY]DE****C****IRK ALP: ETD[pY][pY]R**

Figure 1.1.10 Structures of PTP1B in complex with peptides corresponding to three protein substrates.

PTP1B substrate trapping mutants in complex with peptides corresponding to substrate proteins. A) PTP1B C215S mutant in complex with an EGFR pY992 phosphopeptide. Asp 48 of PTP1B forms hydrogen bonds with the amide group of the pTyr. The guanidinium group of Arg 47 in PTP1B forms salt bridges with the -1 Glu and -2 Asp residues of the EGFR peptide. PDB ID: 1PTU (Jia et al. 1995). B) PTP1B C215S mutant in complex with a nephrin pY1193 phosphopeptide. Asp 48 of PTP1B forms hydrogen bonds with the amide groups of the pTyr and +1 Asp of the nephrin peptide. The guanidinium group of Arg 24 in PTP1B forms a salt bridge with the +2 Glu residue of the peptide. PDB ID: 4ZRT (Selner et al. 2014). C) PTP1B C215A in complex with the IRK activation loop peptide. Asp 48 of PTP1B forms hydrogen bonds with the amide groups of the pTyr and +1 Arg of the IRK peptide. The second phosphotyrosine residue sits in the second aryl binding site, where the phosphate moiety is coordinated by Arg 24 in PTP1B. PDB ID: 1G1F (Salmeen et al. 2000).

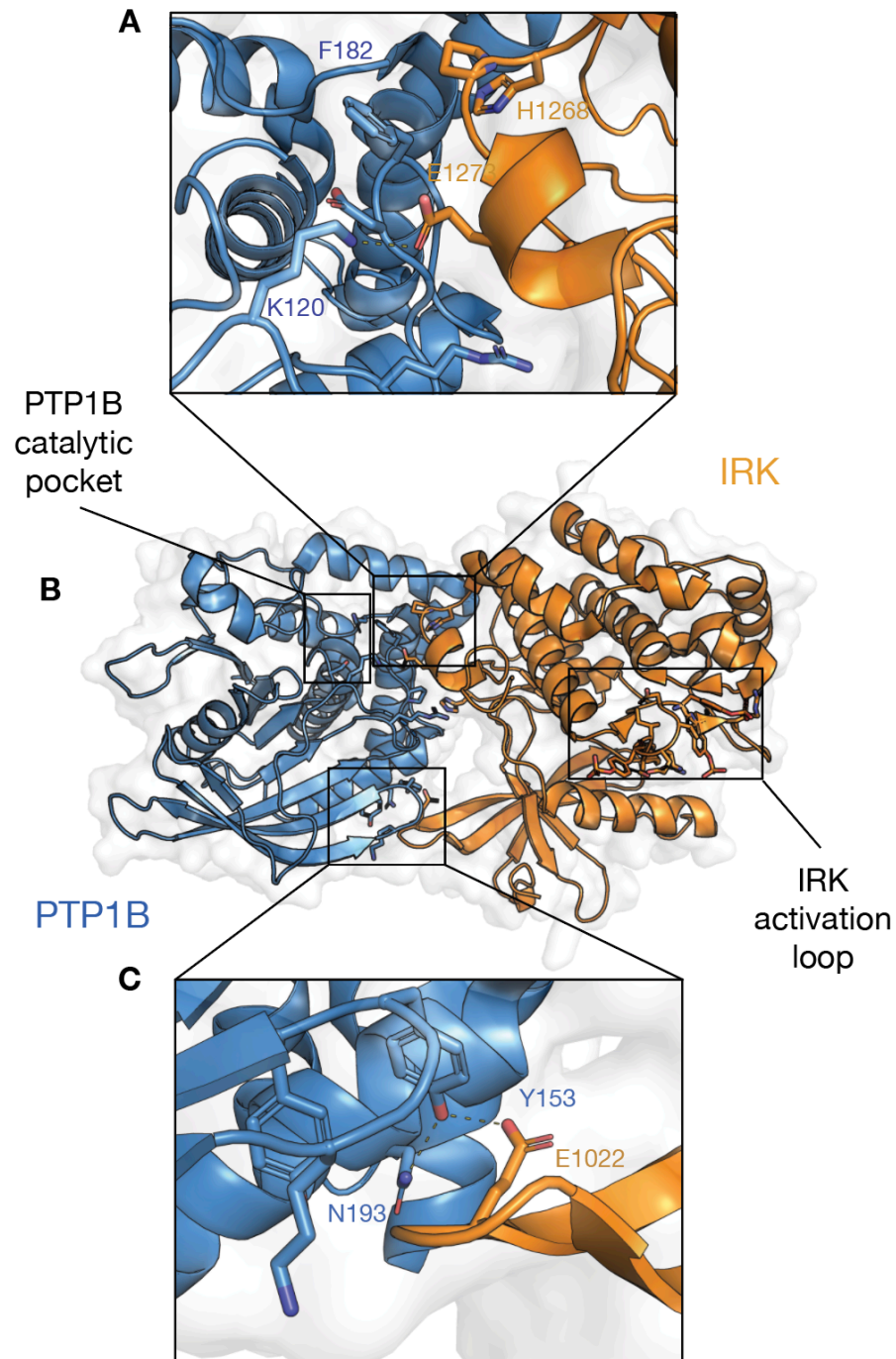


Figure 1.1.11 A complex between PTP1B and IRK.

The crystal structure of PTP1B in complex with IRK showing the non-catalytic interactions between the two proteins. PDB ID: 2B4S (Li et al. 2005). PTP1B is coloured in blue and IRK in orange. **A)** Interactions between Glu 1273 located in α J helix of IRK and Lys 120 of PTP1B. **B)** Full structure of the complex with the active site/catalytic pocket of PTP1B, and the activation loop of IRK highlighted. **C)** Interactions between the β 2- β 3 loop of IRK containing Glu E1022 and α 3 of PTP1B containing Asp 193, and β 9- β 10 turn containing Tyr 153.

1.2.3.5 Substrate specificity of PTP1B

One of the outstanding questions in the field of phosphatases is how their catalytic domains achieve substrate specificity. Much work has been done on deciphering the basis of PTP1B specificity. It is well established that PTP1B has a higher affinity for short peptide sequences over phosphotyrosine alone (Vetter et al. 2000; Zhang et al. 1994), and that the residues immediately preceding and following the pTyr can influence affinity and binding. One study investigating PTP1B specificity performed inverse alanine scanning to determine which residues PTP1B prefers at each position, the results of which are summarised in **Figure 1.13**. PTP1B displays an overall preference for negatively charged or acidic amino acid residues, and an aversion to positively charged residues surrounding the phosphotyrosine. They also demonstrated that the -1 and +1 positions showed the largest variability in preferences and aversions to various residues, and likely account for large proportion the specificity seen for PTP1B.

The residues that appear to be important for this preference in the -1 and +1 positions are Arg 47 and Arg 24 of PTP1B respectively. Arg 47 has been highlighted for its role in forming interactions with negatively charged residues in the -1 positions of peptides, and indeed mutation of Arg 47 to an alanine results in a decrease in the dephosphorylation rate for a phosphotyrosine peptide containing a -1 glutamate residue (Sarmiento et al. 1998). As indicated by the structures of PTP1B in complex with peptides corresponding to phosphotyrosine residues in IRK and nephrin, Arg 24 of PTP1B can coordinate negatively charged residues in the +1 and +2 positions (Li et al. 2005).

The two residues important for tyrosine specificity are Tyr 46 and Phe 182, both of which engage in pi-pi interactions with the aromatic ring of the phosphotyrosine in the catalytic pocket, and define the catalytic cleft along with the PTP loop residues that coordinate the phosphate group. Asp 48 of PTP1B also appears to be important for orienting the peptide in the catalytic pocket by forming interactions with the amide groups of the peptide backbone. Interactions between Arg 48 and peptide substrates has been shown to cause the phosphotyrosine in the catalytic pocket to adopt a right-handed α -helical conformation (Sarmiento et al. 1998). Together these residues form the basis of our understanding of PTP1B specificity.

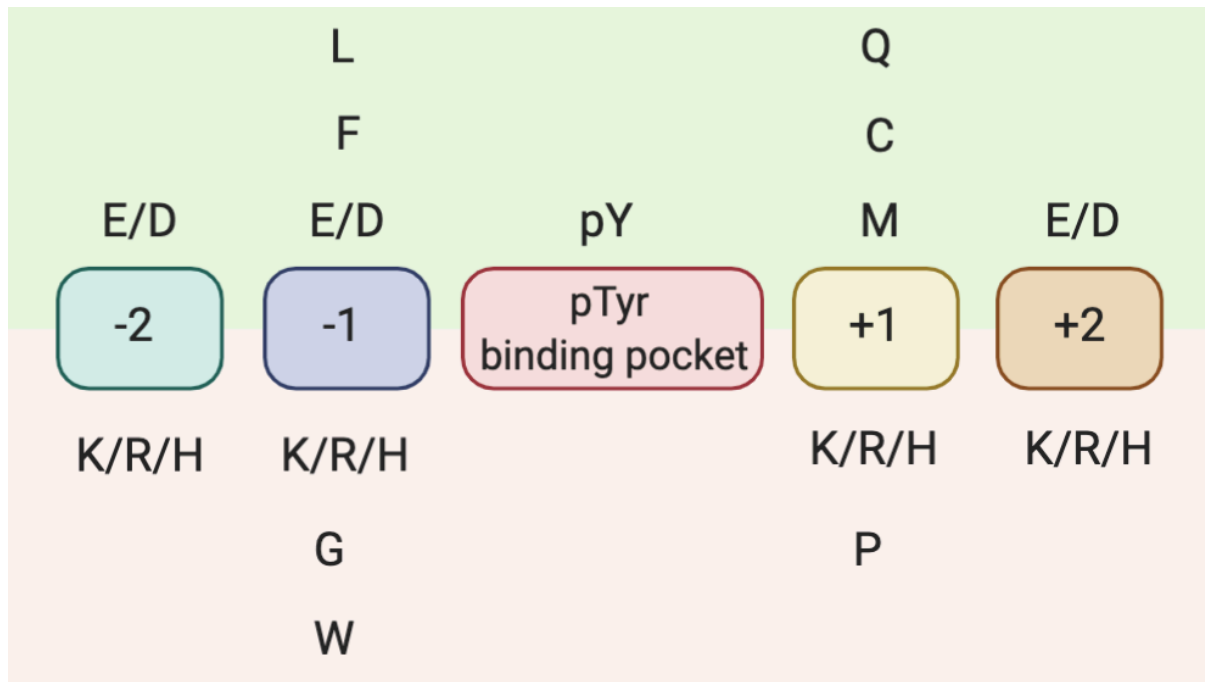


Figure 1.1.12 PTP1B substrate residue preferences.

Results from inverse alanine scanning for PTP1B substrate specificity presented in Vetter et al., 2000. The phosphotyrosine binding pocket is indicated by the red box, and the boxes preceding or following represent the position of the residues either side of the phosphotyrosine. Residues in the green section (top) increase the dephosphorylation rate when present at this position, and residues in the red section (bottom) decrease the dephosphorylation rate when present at the position. As a general rule, PTP1B prefers negatively charged residues near the catalytic pocket over positively charged residues.

1.2.3.6 Categories of protein tyrosine phosphatases based on presence of a second aryl binding site.

As discussed above in Figure 1.9, PTP1B has a second positively charged binding site known as the second aryl binding site (Puius et al. 1997). The presence of this second binding site allows PTP1B to interact with proteins that contain tandem phosphotyrosine residues. This second aryl binding site contains an arginine residue (Arg 24) that is able to coordinate the phosphate group of the second phosphotyrosine residue, generating the higher affinity seen for tandemly phosphorylated substrates.

The protein tyrosine phosphatases can be categorised into 5 groups based on the presence and accessibility of the second aryl binding site (Barr et al. 2009) (**Figure 1.13**). The first category of phosphatases comprises those that contain a second aryl binding site like PTP1B and an open gateway region that allows a second residue to interact with the second aryl binding site. Their aryl binding site contains a conserved arginine residue, which in PTP1B is Arg 24, responsible for coordinating the phosphotyrosine or negatively charged residue in the +1 position. The second category comprises phosphatases that also contain a second aryl binding site and open gateway region, however the conserved arginine in the aryl binding site is replaced with a cysteine residue. The third category comprises phosphatases that contain a second aryl binding site with an aspartate instead of an arginine, but also have a closed or blocked gateway region which may prevent interactions at the aryl binding site. The fourth category comprises phosphatases that have both a blocked second aryl binding site and blocked gateway that contain bulky aromatic or proline residues. The fifth category comprises phosphatases that have an open gateway but a bulky proline residue that precludes binding in the aryl binding site. Examples of each category are shown in (Barr et al. 2009)

These properties of the PTPs define which substrates they are able to interact with and dephosphorylate. Phosphatases such as PTP1B, SHP1, SHP2 and TC-PTP which regulate the JAK-STAT signalling pathway are all category I phosphatases that appear to contain a second aryl binding site which could potentially accommodate a second phosphotyrosine residue.

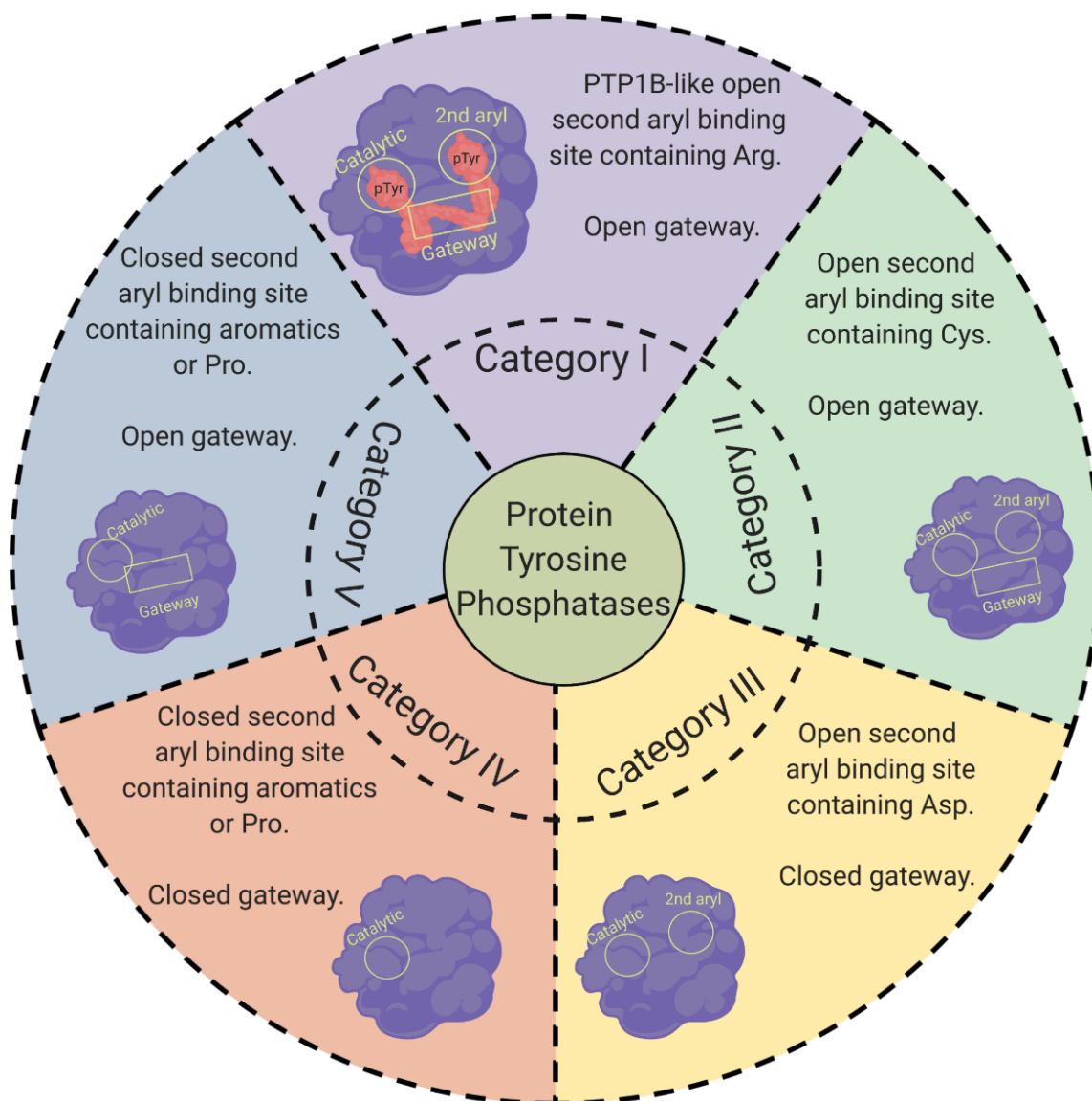


Figure 1.13 The five categories of protein tyrosine phosphatases based on presence of a second aryl binding site.

The protein tyrosine phosphatases can be grouped into five categories based on the presence and accessibility of the second aryl binding. Category I phosphatases have an open gateway and a second aryl binding site with an arginine residue. Category II phosphatases have an open gateway and second aryl binding site containing a cysteine. Category III phosphatases have an open second aryl binding site but the gateway is blocked by aromatic residues. Category IV phosphatases have neither a gateway or second aryl binding site as both are blocked by bulky residues. Category V phosphatases have an open gateway, but the second aryl binding site is blocked by a proline residue.

1.2.3.7 Regulation of PTP1B activity

Both the temporal and spatial activity of protein tyrosine phosphatases are tightly regulated in cells. As previously mentioned, PTP1B contains a short ER targeting motif that anchors the protein to the cytoplasmic facing side of the ER (Frangioni et al. 1992). Despite this localisation, it has been suggested that the ER can be quite mobile and flexible, allowing PTP1B to interact with the kinases at the cell surface (Haj et al. 2002), including IRK, JAK2 and TYK2.

Interestingly, *in vivo* the activity of PTP1B can also be regulated by the production of reactive oxygen species (ROS) in the cell. Due to the signature motif of PTPs containing the invariant cysteine (Cys 215 in PTP1B), the active site has a low pKa that allows this cysteine residue to act as a nucleophile for dephosphorylation. The cysteine is therefore highly susceptible to oxidation to form sulfenic acid, which then converts to cyclic sulfenamide. This oxidation ultimately leads to inhibition of catalytic activity. In response to IRK activation, an NADPH oxidase complex forms and generates ROS. This leads to the rapid oxidation of the invariant cysteine in the catalytic site (Cys 215), and temporary inhibition of enzymatic activity (Meng et al. 2004). This oxidation event causes a conformational change in PTP1B, which allows for the reduction of the cysteine. This reduction event returns PTP1B back to its catalytically active form.

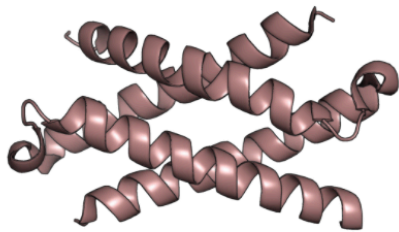
PTP1B has also been suggested to be regulated by a number of post-translational modifications. These include phosphorylation at serine and tyrosine residues (Shifrin, Davis, and Neel 1997), and sumoylation at two C-terminal lysine residues of PTP1B (Dadke et al. 2007). These modifications have been suggested to decrease PTP1B activity, however in comparison to cellular localisation and oxidation, post-translational modification appears to play only a minor role in the regulation of PTP1B.

1.2.4 Regulators of JAK-STAT signalling III. The SH2 domain containing adaptor family of proteins

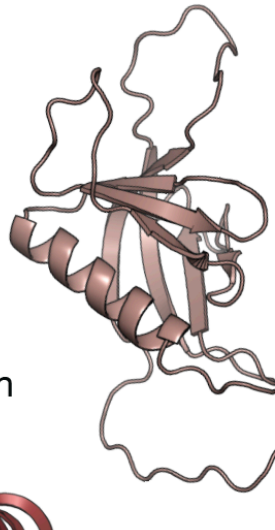
The SH2 domain containing adaptor family of proteins comprises APS (SH2B1), SH2B (SH2B2) and LNK (SH2B3). Each member of this family contains three domains. The first is a dimerization domain comprising ca. 60 residues, including a central phenylalanine zipper. The dimerization domain is thought to be important for protein-protein interactions and has been suggested to allow homo or heterodimerisation of the SH2B family of proteins (Dhe-Paganon et al. 2004). The second domain is the plekstrin homology (PH) domain, which comprises ca. 120 residues. The PH domain is involved in interactions with phosphatidylinositol lipids in the cell membrane (Harlan et al. 1994), and is thought to be important for localisation within the cell. Between the dimerization domain and the PH domain, there is a short Pro-x-x-Pro motif, which can be recognised by proteins containing SH3 domains, and may further aid in protein-protein interactions. The final domain is the SH2 domain, comprising ca. 100 residues, that bind phosphorylated tyrosine residues in other proteins. While APS and SH2B appear to propagate cytokine signalling via multiple pathways (Morris et al. 2009; Sheng et al. 2013; Kurzer et al. 2006), LNK is thought to primarily be a negative regulator of signalling (Gery et al. 2007; Gery, Cao, et al. 2009; Seita et al. 2007). There are some instances however, where APS has also been suggested to be a negative regulator, similar to the actions of LNK (O'Brien, O'Shea, and Carter-Su 2002).

Structures of the various domains of these proteins has provided insight into their functions in cells, and which proteins they interact with (**Figure 1.14**). The structure of all three domains of APS have been solved (Dhe-Paganon et al. 2004; McKercher et al. 2018), including a structure of the SH2 domain in complex with IRK, demonstrating how a dimer of the APS SH2 domain binds the IRK activation loop (Hu et al. 2003). Two structures of SH2B have been solved, one being the apo form of the SH2 domain, and the other the SH2 domain in complex with a phosphopeptide corresponding to pY813 of JAK2, revealing how the SH2B SH2 domain recognises and interacts with this residue of JAK2 (Hu and Hubbard 2006). No structures of LNK have been published, and so there is little information available on how LNK interacts with other proteins.

A APS
Dimerisation domain



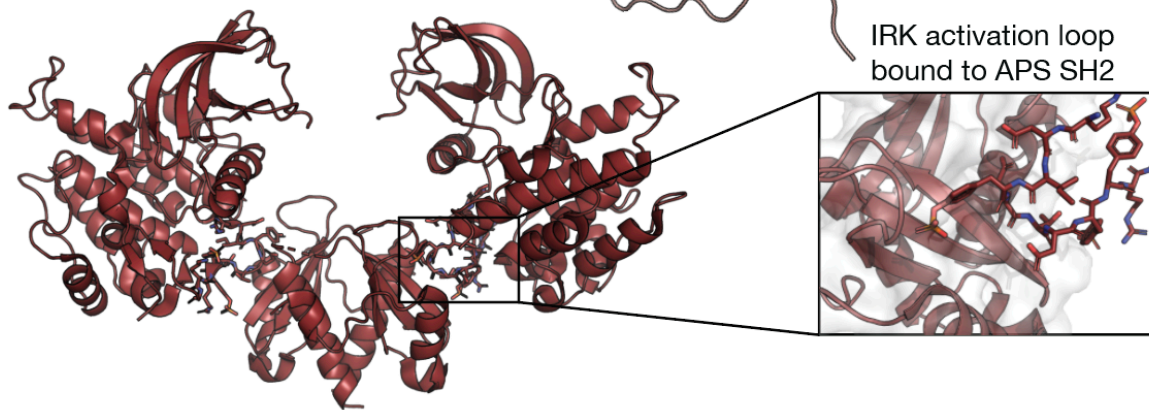
B APS
PH domain



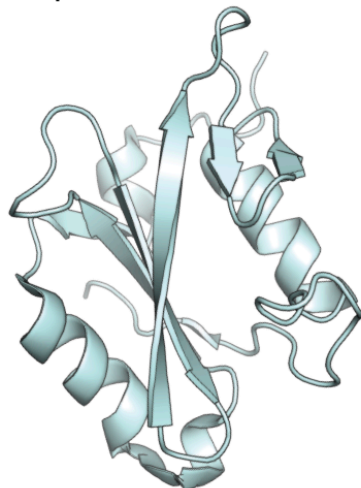
C APS
Apo SH2 domain



D APS SH2 domain with
IRK kinase domain



E SH2B
Apo SH2 domain



F SH2B SH2 with JAK2
pY813 bound

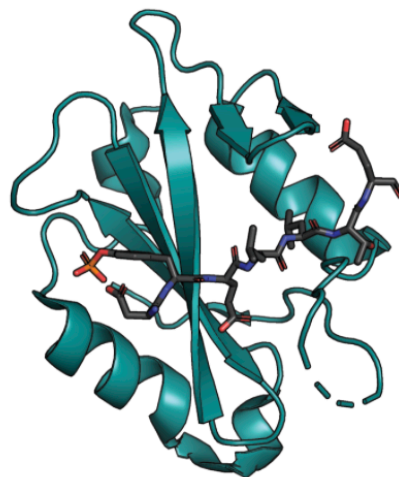


Figure 1.1.14 Structures of the SH2 domain containing family of adaptor proteins. Six structures of the SH2 domain containing family of adaptor proteins have been solved. **A)** The dimerization domain of APS solved by X-ray crystallography. PDB ID: 1Q2H (Dhe-Paganon et al. 2004). **B)** The PH domain of APS solved by solution NMR. PDB ID: 1V5M. **C)** The Apo SH2 domain of APS solved by X-ray crystallography. PDB ID: 5W3R (McKercher et al. 2018). **D)** Structure of the APS SH2 domain in complex with the insulin receptor kinase solved by X-ray crystallography. PDB ID: 1RQQ (Hu et al. 2003). **E)** The Apo structure of the SH2B SH2 domain solved by X-ray crystallography. PDB ID: 2HDV (Hu and Hubbard 2006). **F)** Structure of the SH2B SH2 domain with a phosphopeptide corresponding to pY813 of JAK2 bound solved by X-ray crystallography. PDB ID: 2HDX (Hu and Hubbard 2006).

1.2.4.1 The adaptor protein, LNK (SH2B3)

LNK was originally thought to be important in T-cell signalling, however generation of LNK deficient mice demonstrated that the mice display splenomegaly, an increase in B-cells, hyperplasia of megakaryocytes and increased erythrocyte numbers in comparison to their WT counterparts (Velazquez et al. 2002) with no effect on their T-cell numbers (Takaki et al. 1997). This phenotype is similar to that of a myeloproliferative neoplasm (MPN) phenotype. LNK has also been shown to be highly expressed in HSCs, and deletion of LNK from HSCs leads to an increase in cell number and proliferative capacity (Bersenev et al. 2008). The effect of LNK on HSC self-renewal and proliferation is due to the role of LNK in the regulation of TPO-mediated signalling (Seita et al. 2007) and suggests a role for LNK in regulating HSC self-renewal. Additionally, dose-dependent over-expression of LNK in lymphoid cells lead to impaired B-cell development, suggesting a role for LNK in the regulation of lymphopoiesis (Takaki et al. 2003). Without a comprehensive list of the proteins and pathways that LNK negatively regulates, it is difficult to fully understand its physiological roles. For this reason, many studies have focused on identifying candidate substrates of LNK using *in vitro* assays.

LNK has been shown to be particularly important in the negative regulation of the TPO and EPO signalling pathways (Tong and Lodish 2004; Tong, Zhang, and Lodish 2005). It was later determined that LNK is able to bind directly to JAK2, the primary JAK for EPO and TPO signalling, via its SH2 domain (Bersenev et al. 2008). The major binding site of the SH2 domain appears to be the JAK2 pY813 residue, and the pY613 residue may be a minor binding site. Further studies have suggested a role for LNK in the regulation of a number of proteins important for haematopoiesis including c-KIT (Gueller et al. 2008), FLT3 (Lin et al. 2012), JAK3 (Koren-Michowitz et al. 2010), EPOR (Tong, Zhang, and Lodish 2005), MPL (Gery et al. 2007), PDGFR (Gueller et al. 2011) and c-FMS (Gueller et al. 2010) by binding to various phosphotyrosine residues and modulating their activity (**Figure 1.15**). Although LNK's role in negatively regulating signalling has been established, it is still unknown how LNK performs this function. Structural and biochemical analyses of LNK may provide insight into the specificity of this protein for its substrates.

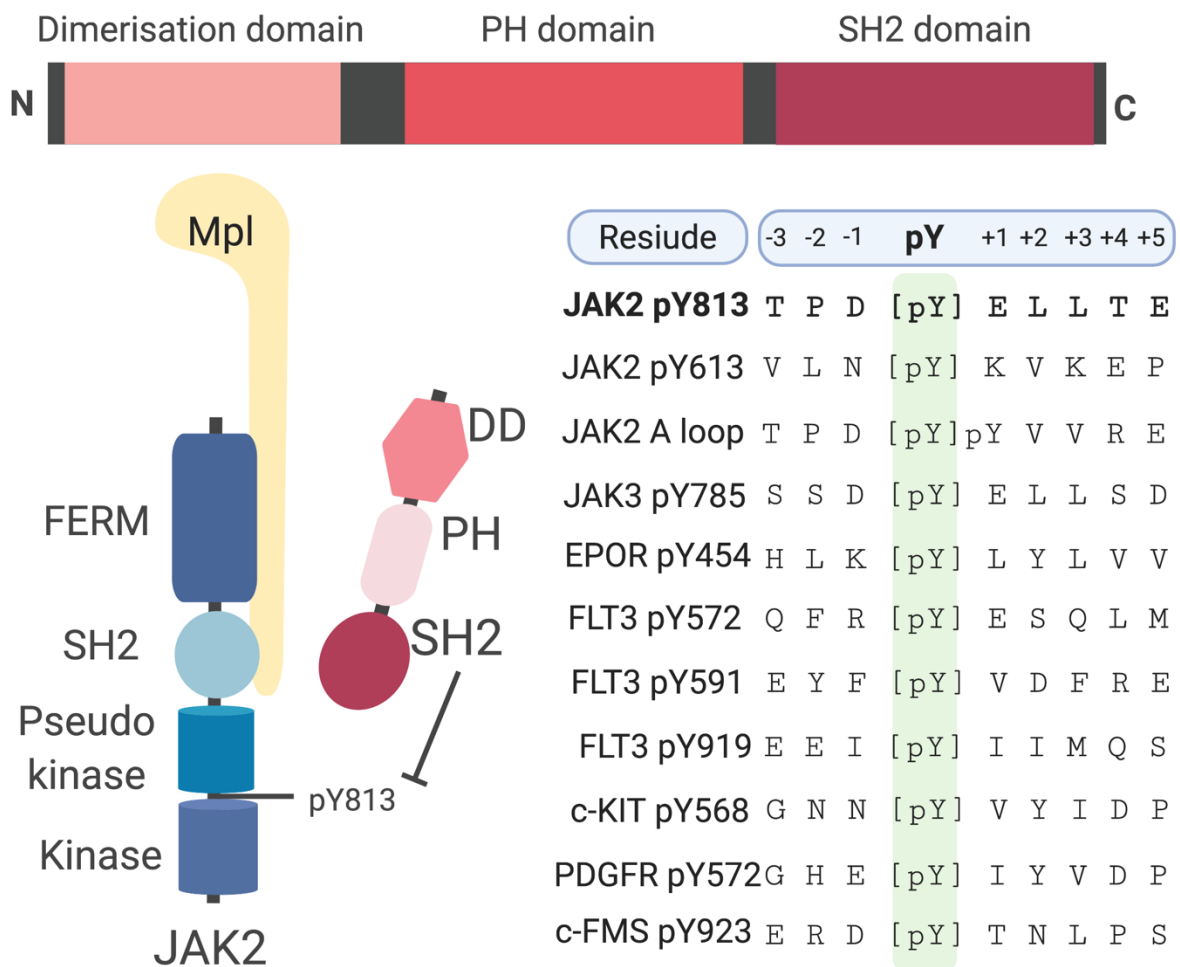


Figure 1.1.15 The Lymphocyte Adaptor Protein, LNK (SH2B3).

LNK belongs to the SH2 domain containing family of adaptor proteins which have various roles in regulating the activity of cytokine signalling. LNK has an N-terminal phenylalanine zipper which acts as a dimerization domain, followed by a plekstrin homology domain and a C-terminal SH2 domain. LNK is known to be a regulator of JAK2 downstream of TPO signalling. The sequences of some residues LNK has been suggested to interact with are listed on the right, with the phosphotyrosine highlighted in green.

1.3 Dysregulation of cytokine signalling in disease

The highly coordinated and tightly regulated interactions between the molecules that perpetuate signalling and those that negatively regulate signalling are important for maintaining homeostasis in cells. In the case of the JAK-STAT signalling pathway, when interactions between proteins are impaired, or the balance between the actions of the signalling molecules and negative regulators is lost, homeostasis cannot be achieved. Too much or too little cytokine signalling can result in diseases ranging from immune deficiencies to cancers (Schindler 2002; O'Sullivan et al. 2007). The complexities of the regulation of JAK-STAT signalling and the interplay with other pathways involved in cytokine signalling means that a single mutation or change in the activation of a protein can disrupt entire signalling cascades and have negative biological effects.

In the haematopoietic system, the JAK-STAT pathway is crucial for proliferation, development, differentiation and survival of blood and immune cells. The number and diversity of diseases associated with aberrant JAK-STAT signalling exemplifies the importance of studying this pathway at the molecular level in order to gain an in-depth understanding of how this pathway functions under steady-state conditions, and therefore how diseased states can occur when these interactions are altered. Yet while we have a grasp on which mutations lead to disease progression, we still lack a complete understanding of many of the proteins involved in regulating the pathway and therefore do not fully understand how these mutations cause disease. An example of this is the JAK2 V617F mutation (Krämer et al. 2007). While we know it is the leading cause of myeloproliferative neoplasms it is unclear why the mutation causes JAK2 to be constitutively active in cells. A number of diseases caused by mutations in the JAK-STAT pathway are discussed below and represented in **Figure 1.16**.

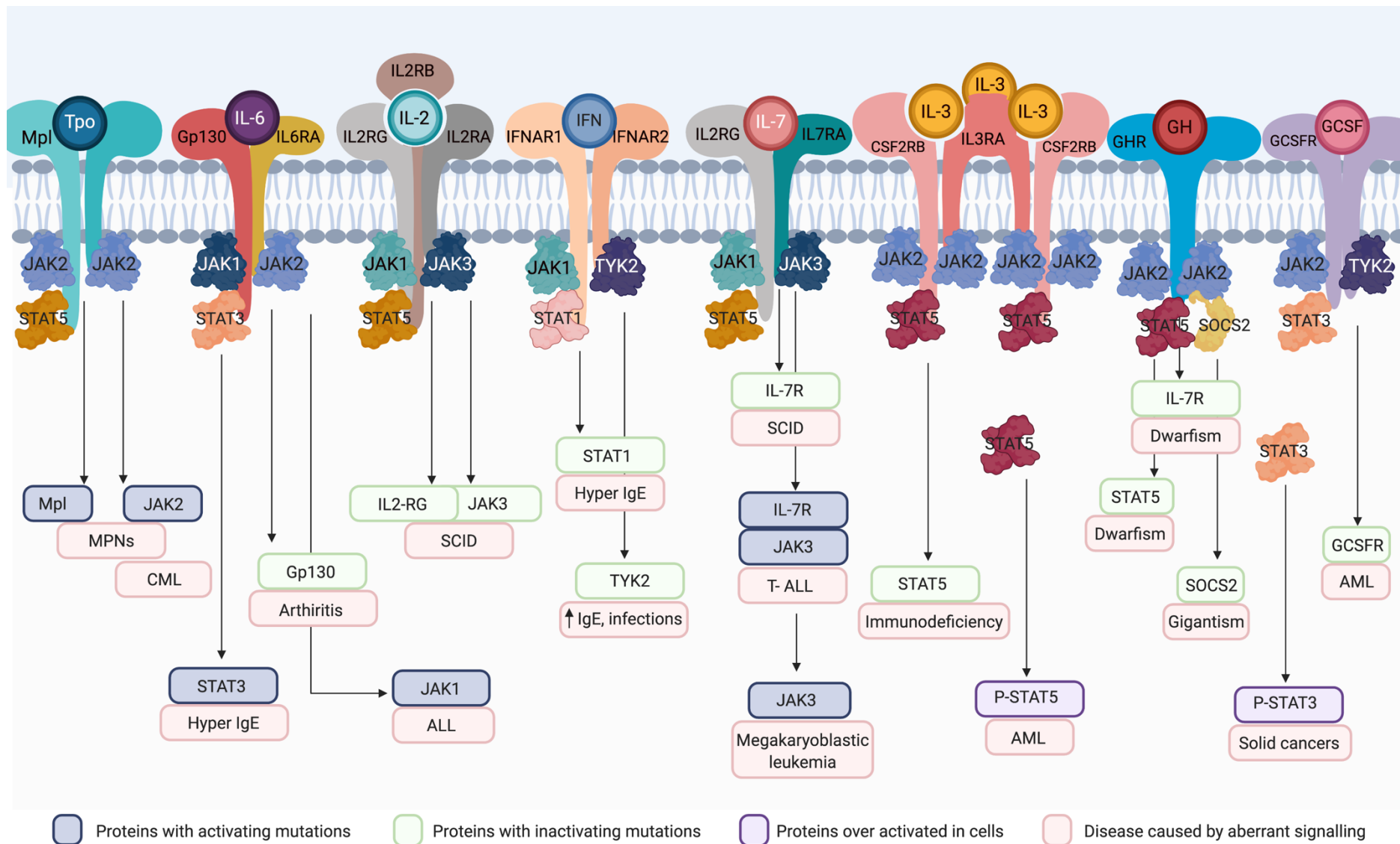


Figure 1.16 Mutations in the JAK-STAT pathway are implicated in disease.

Figure lists some of the mutations identified in proteins of the JAK-STAT signalling pathway that have been linked with disease. Blue boxes indicate activating mutations. Green boxes indicate inactivating mutations. Purple boxes indicate cases where a protein is not mutated, but constitutively activated by other proteins. Pink boxes indicate disease associated with mutation or change in signalling. SCID: severe combined immune deficiency, CML: Chronic myeloid leukaemia, ALL: acute lymphoblastic leukaemia, AML: acute myeloid leukaemia, T-ALL: T cell acute lymphoblastic leukaemia.

1.3.1 Immune dysfunction

The JAK-STAT signalling pathway is critical for appropriate immune responses and maintenance of the immune system. Inactivating mutations in the proteins of the JAK-STAT signalling pathway are therefore often associated with immune deficiencies. Inactivating mutations in the IL2RG and IL7RA receptors have been identified in patients with X-linked Severe combined immunodeficiency (SCID) (Puck et al. 1997; Puel et al. 1998), and inactivating mutations in the JAK proteins can lead to a decrease or inability to activate the JAK-STAT signalling pathway, which consequently can also result in immune deficiencies. JAK3 is associated specifically with the gamma common chain required for IL-2 family signalling. Similar to mutations in IL2RG, inactivating mutations in JAK3 have also been linked with SCID (O'Shea et al. 2004), and indeed most patients with SCID, who do not harbour an IL2RG mutation, harbour a JAK3 inactivating mutation. TYK2 inactivating mutations that result in immune deficiencies are much rarer than those for JAK3, however a small number have been previously reported to lead to elevated IgE levels and/or increased susceptibility to infection (Woellner et al. 2007; Watford and O'Shea 2006). Mutations in the STAT proteins can also lead to aberrant regulation of gene expression which can result in immune dysfunction. Dominant negative mutations in STAT3 have been implicated in autosomal dominant hyper IgE syndrome (Holland et al. 2007), mutations in STAT1 results in decreased interferon signalling (Sharfe et al. 2014) and mutations in STAT5 results in immunodeficiency and autoimmunity due to impaired IL-3 and IL-2 signalling (Bernasconi et al. 2006).

1.3.2 Blood cancers

Because cytokine signalling is intricately linked to haematopoiesis, it is no surprise that mutations in the proteins involved in JAK-STAT signalling can result in haematopoietic malignancies such as blood cancer. Contrariwise to the immune related disorders where most mutations are inactivating, the majority of mutations that are implicated in blood cancers are due to mutations that cause the JAK-STAT signalling pathway to be constitutively active (Hunter and Avalos 1999), which results in an increase in proliferation of blood cells. It should be noted however that some inactivating mutations have also been identified in the negative regulators of the JAK-STAT pathway.

A number of mutations in receptors have been linked to disease including activating G-CSFR mutations which can lead to the development of acute myeloid leukaemia in patients with severe congenital neutropenia (Hunter and Avalos 1999; Dong et al. 1997), and mutations in CRLF2 have been linked with B-cell acute lymphoblastic leukaemia (van der Veer et al. 2013; Yoda et al. 2010). As will be discussed further in the myeloproliferative neoplasms (MPN) section, activating mutations in MPL lead to constitutive signalling through the Tpo-JAK-STAT pathway (Bridgford et al. 2020), and in subsets of patients with childhood T-cell acute lymphoblastic leukaemia, gain of function IL-7R α mutations have been shown to drive an increase in T-cell numbers (Zenatti et al. 2011). FLT3 is another receptor involved in haematopoiesis and although it does not signal through the canonical JAK-STAT pathway, transformed FLT3 can constitutively activate STAT5a (Zhang, Fukuda, et al. 2000; Quentmeier et al. 2003; Choudhary et al. 2007). Mutations in FLT3 have been identified in up to 35% of AML cases, making it one of the most commonly mutated genes identified in AML (Kottaridis et al. 2001).

Mutations that constitutively activate the JAK proteins are found in a number of haematological malignancies. Although most common in the myeloproliferative disorders, the V617F mutation in JAK2 is also found in 20% of patients with atypical chronic myeloid leukaemia (Krämer et al. 2007), 10% of patients with chronic myelomonocytic leukaemia, 15% of patients with megakaryocytic acute myeloid leukaemia and 20% of cases of juvenile myelomonocytic leukaemia (Levine et al. 2005). Activating point mutations have also been identified in JAK1, JAK2 and JAK3 (Mullighan et al. 2009) in approximately 10% of patients with high-risk lymphoblastic leukaemia (ALL). Point mutations in JAK1 have also been identified in patients with acute myeloid leukaemia (AML) (Xiang et al. 2008). These point mutations are located in the kinase and pseudokinase domains of the JAKs. Activating mutations in the FERM and pseudokinase domains of JAK3 have also been shown to be associated with megakaryocytic leukaemia (Walters et al. 2006). JAK2 translocations leading to chimeric proteins capable of cellular transformation have also been associated with a number of leukaemias. For example, genetic recombination that leads to fusion of the catalytic domain of JAK2 with TEL, BCR or PCM1 are found in T-cell acute lymphoblastic leukaemia, atypical chronic myeloid leukaemia and T cell lymphoma, respectively (Laurence et al. 2012; Wilks 2008).

1.3.3 Myeloproliferative neoplasms

The myeloproliferative neoplasms are a group of diseases characterised by the clonal expansion of mature blood lineages. The classical myeloproliferative neoplasms can be split into two categories (**Figure 1.17**). The first are the Philadelphia chromosome positive MPN group, which comprises chronic myeloid leukaemia (CML)(Sawyers 1999). The Philadelphia chromosome is the translocation of part of chromosome 22 and chromosome 9 to form a fusion gene termed BCR-ABL. This results in a constitutively active form of the tyrosine kinase ableson (ABL), which drives the development of CML (Kurzrock, Gutterman, and Talpaz 1988).

The second group are the Philadelphia negative MPNs, which comprise essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF). Polycythemia vera is characterised by the over-proliferation of erythrocytes (Prchal 1974). Essential thrombocythemia is the over-proliferation of mature megakaryotic cells (Tefferi and Pardanani 2019), and primary myelofibrosis is the over-proliferation of many blood cells in the bone marrow causing scar tissue to form (Vannucchi 2011).

In addition to the four classic MPNs, a number of other diseases can be classified under the MPN category. These include chronic neutrophilic leukaemia, chronic eosinophilic leukaemia, not otherwise specified, hypereosinophilic syndrome, mast cell disease and unclassifiable MPNs.

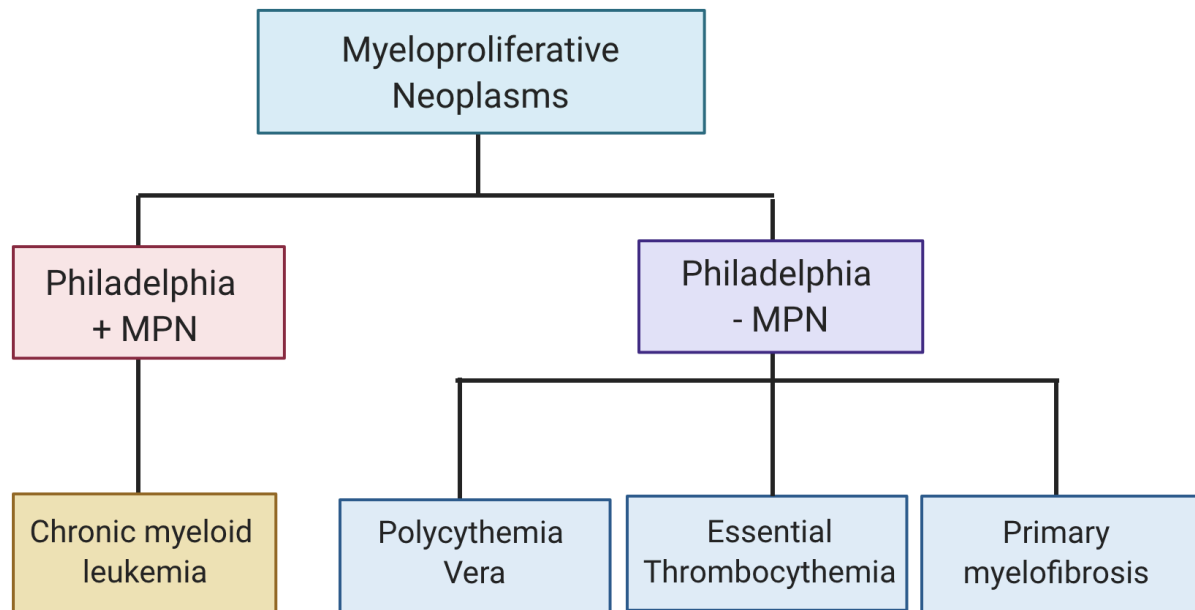


Figure 1.1.17 The myeloproliferative neoplasms.

Classification of the major myeloproliferative neoplasms based on their Philadelphia chromosome status. Chronic myelogenous leukaemia's are characterised by the presence of the Philadelphia chromosome. The Philadelphia negative MPNS include essential thrombocythemia, polycythaemia Vera and primary myelofibrosis.

1.3.3.1 Polycythemia vera (PV)

PV is characterised predominantly by the clonal expansion of cells of the erythroid lineage, however, hyperplasia of the white cells and platelets is also seen (Adamson et al. 1976; Barbui et al. 2016). The expansion of the erythroid cells results in elevated haemoglobin and haematocrit levels, without an increase in erythropoietin, suggesting that PV erythroid cells expand in an erythropoietin-independent manner (Prchal 1974; Cashman et al. 1983). The majority of patients with PV present after a thrombotic event as a consequence of increased blood viscosity, however other symptoms include hepatomegaly, splenomegaly and hypertension (Policitemia 1995).

1.3.3.2 Essential thrombocythemia (ET)

ET is characterised by the clonal expansion of megakaryocytes and platelets resulting in thrombocytosis (Fenaux et al. 1990; Tefferi and Pardanani 2019; Barbui et al. 2016). Patients with ET typically present with few symptoms, and most cases are diagnosed following a routine blood count. Other symptoms can include erythromelalgia, haemorrhage and thrombosis, with the risk of ET induced thrombosis exceeding 20% (Tefferi and Pardanani 2019).

1.3.3.3 Primary myelofibrosis (PMF)

PMF is characterised by scar tissue formation in the bone marrow resulting from excessive cellular proliferation (Barbui et al. 2018; Barbui et al. 2016). Patients with PMF present with severe anaemia and hepatosplenomegaly (Vannucchi 2011). PMF in many cases will ultimately lead to bone marrow failure as the bone marrow is replaced with fibrotic tissue (Dunn et al. 1998; Buesche et al. 2008).

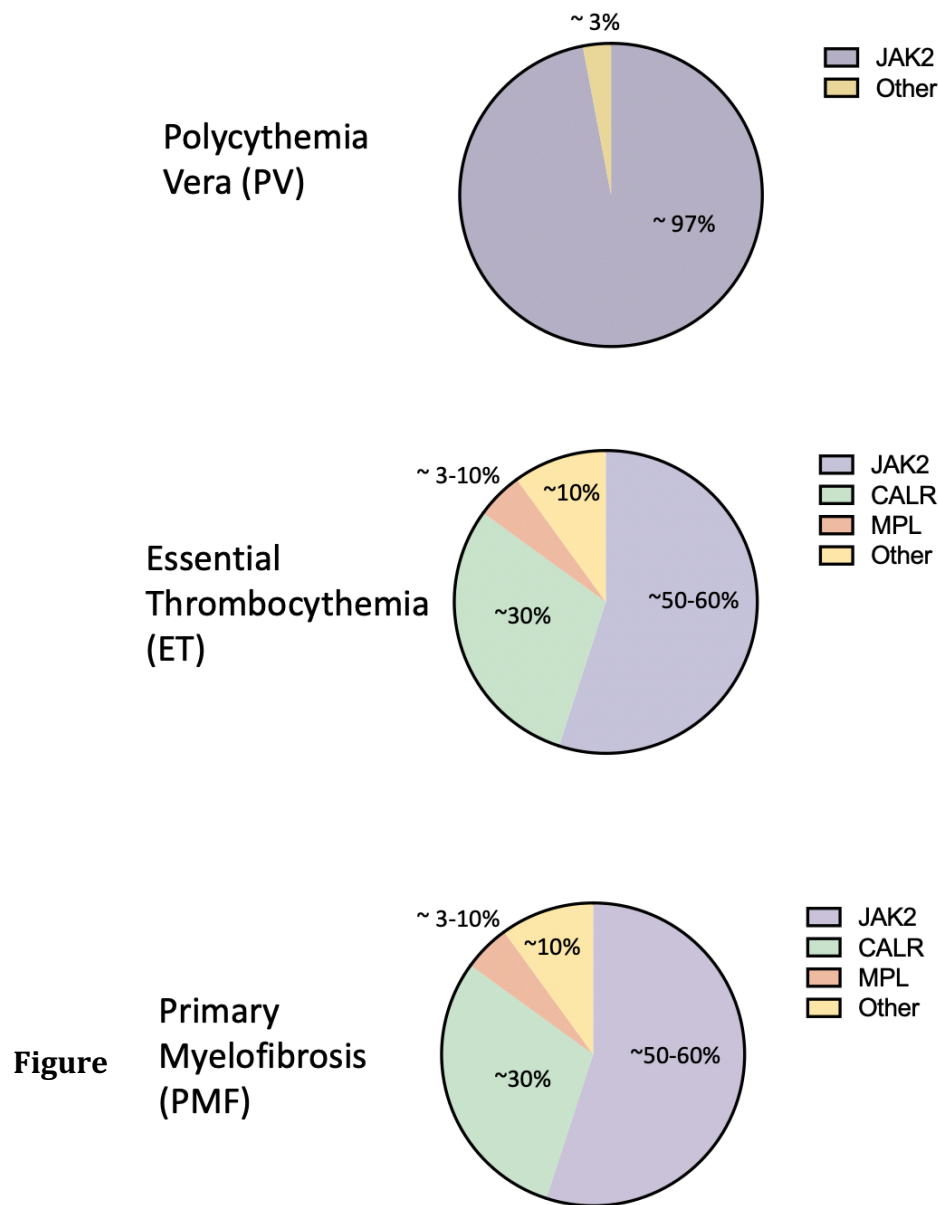
1.3.3.4 Molecular pathogenesis of MPNs

The causative mutations of the Philadelphia chromosome negative MPNs have been well characterised. The most common mutation for all three MPNs is the JAK2 V617F mutation, which occurs in the pseudokinase domain of JAK2, and causes the protein to be constitutively active. This mutation accounts for >95% of patients with PV and around 50% of the cases of PMF and ET (Levine et al. 2007; Koppikar and Levine 2008). Other mutations have also been identified with a lower incidence in JAK, and are located in exon

12. The second most commonly mutated protein is calreticulin (CALR), which is a quality control chaperone. Mutations in CALR are generally frameshift mutations that cause the protein to lose its endoplasmic reticulum retention signal and translocate with the receptor to the cell surface. The third protein most commonly mutated in MPNs is Mpl, the Tpo receptor. Mutations in Mpl cause constitutive activation of the JAK proteins, driving JAK-STAT5 signalling (Barbui et al. 2018; Tefferi and Pardanani 2019). Outside of these three common mutations, the remaining driving mutations make up less than 10% of all cases of myeloproliferative neoplasms (**Figure 1.18**). These include mutations in ASXL1, TET2 (Saint-Martin et al. 2009), SRSF2 (Zhang et al. 2012), U2AF1 (Tefferi et al. 2018), IDH1 and IDH2 (Pardanani, Lasho, Finke, Mai, et al. 2010), DNMT3A (Abdel-Wahab et al. 2011), IKZF1 (Jäger et al. 2009), PTP1B (Jobe et al. 2017) and LNK (Tefferi 2010; Oh et al. 2010). All of these mutations lead to a corresponding increase in signalling through the TPO-MPL-JAK-STAT pathway, including in the absence of any cytokine.

1.3.3.5 Progression into blast phase

All three Philadelphia chromosome negative myeloproliferative neoplasms have the ability to transform into blast phase. MPNs in blast phase are defined as having more than 20% myeloid blasts in either the bone marrow or peripheral blood (Tam et al. 2008; Tam et al. 2009). This is often preceded by an accelerated phase defined by 10-19% myeloid blasts in either the bone marrow or peripheral blood. The risk of leukemic transformation is highest for PMF with the yearly incidence of transformation being approximately 1.09%, whereas PV and ET have a 0.38% and 0.37% yearly incidence of progression respectively (Abdulkarim et al. 2009). It is not fully understood what factors cause this small proportion of patients to progress to blast phase, however as more research emerges, we are gaining a better insight into the combinations of mutations may drive blast phase progression. As an example, evidence has emerged that mutations in LNK may be associated with leukemic transformation of MPNs (Pardanani, Lasho, Finke, Oh, et al. 2010)



1.1.18 Common mutations in the myeloproliferative neoplasms.

For polycythaemia vera, approximately 97% of all mutations are JAK2 V617F mutations and approximately 3% of patients have other mutations. For Essential thrombocythemia, approximately half of mutations are the JAK2 V617F mutation, around 30% are CALR mutations, around 10% MPL mutations, and the other 3-10% are made up of other mutations. For primary myelofibrosis approximately half of mutations are the JAK2 V617F mutation, around 30% are CALR mutations, around 10% MPL mutations, and the other 3-10% are made up of other mutations.

1.3.4 Non-haematological diseases

The JAK-STAT signalling pathway also has many biological functions outside of the haematopoietic system. For this reason, many mutations in the proteins of the JAK-STAT pathway can also be associated with diseases of other tissues. Some of these are listed in **Table 1.2**.

Table 1.2 Examples of non-haematological diseases associated with JAK-STAT signalling.

Disease	Cause	Reference
Prostate cancer	Amplification of the STAT5A/B locus	(Haddad et al. 2013)
Ovarian cancer	Increased G-CSF receptor expression. Increases STAT1 activation	(Kumar et al. 2014; Stronach et al. 2011)
Myocardial hypertrophy	JAK-STAT activation my mechanical stretch.	(Pan et al. 1999; Ruwhof and van der Laarse 2000)
Gigantism	Aberrant SOCS2 activity – increased GH-IGF signalling	(Metcalf et al. 2000; Hannah-Shmouni, Trivellin, and Stratakis 2016; Suda et al. 2011)
Bronchial asthma	Dysregulated Th2 response	(Pernis and Rothman 2002; Wills-Karp et al. 1998)
Glioblastoma	Increased STAT3 due to decrease in Protein inhibitor of activated STAT 3 (PIAS3).	(Sherry et al. 2009; Brantley et al. 2008)
Hepatitis B-associated hepatocellular carcinoma	JAK1 mutations causing increased STAT1 and STAT3 levels.	(Liu et al. 2010)
Non-small cell lung cancer	Hyperexpression of the SOCS3 promoter leading to decreased SOCS3 expression	(He et al. 2003)
Head and neck squamous cell carcinoma	Increased IL-6 signalling resulting in increased STAT3 activation.	(Sriuranpong et al. 2003)

1.4 PTP1B and LNK in disease

1.4.1 The role of PTP1B in disease

Mutations in PTP1B have been implicated in a variety of disease states including various cancers. Because PTP1B is involved in the regulation of a diverse set of proteins with varied functions, the conditions under which PTP1B activates or inactivates these molecules in various cell types is unclear. This precludes our ability to understand what role PTP1B and its associated mutations play in cancer. In some types of cancers, such as leukaemias (Le Sommer et al. 2018), PTP1B levels are downregulated. On the other hand, PTP1B levels are reported to be upregulated in other cancers, such as breast cancer (Wiener et al. 1994) and colon cancer (Zhu, Bjorge, and Fujita 2007). For this reason, a better understanding of the proteins PTP1B regulates and how this regulation affects their activity is required. A list of cancer types and the change in PTP1B expression are listed in **Table 1.3**.

Table 1.3 Changes in expression of PTP1B in cancer.

Cancer type	PTP1B expression	Reference
Breast	upregulated	(Wiener et al. 1994)
Colon	upregulated	(Zhu, Bjorge, and Fujita 2007)
Gastric	upregulated	(Wang et al. 2012)
Leukaemia	downregulated	(Le Sommer et al. 2018)
Oesophageal	downregulated	(Warabi et al. 2000)
Prostate	upregulated	(Lessard et al. 2012)
Lymphoma	downregulated	(Gunawardana et al. 2014)

Due to its role in the regulation of metabolic signalling, PTP1B has been suggested to be a key player in various metabolism-related diseases, however much like its role in cancer, the role of PTP1B in metabolic disorders is contentious. Given that PTP1B is a key regulator of insulin signalling, mutations in the PTP1B gene *PTPN1*, have been suggested to be associated with type II diabetes. Multiple studies examining single nucleotide polymorphisms in PTP1B and whether they lead to an increase in type II diabetes have

implicated these mutations with an increase in susceptibility to developing type II diabetes (Bento et al. 2004; Palmer et al. 2004). Additional studies however reported no correlation between mutations in *PTPN1* and incidence of type II diabetes (Florez et al. 2005). PTP1B mutations have been studied further in terms of their role in other metabolic and inflammatory diseases, however more research is needed to definitively determine the role PTP1B plays in these diseases, if any.

1.4.1.1 PTP1B as a therapeutic target

The role of PTP1B in the negative regulation of insulin signalling has made it an attractive therapeutic target for treating diabetes. By blocking PTP1B, cells should become sensitised to insulin signalling, leading to increased uptake of glucose from the blood (Krishnan et al. 2018; Koren and Fantus 2007). More recently, following the identification of other PTP1B substrates, the use of PTP1B inhibitors to treat specific types of cancers has also been studied (Xu et al. 2019; Bartolomé et al. 2019). Because PTP1B is often amplified or upregulated in breast, colon, gastric and prostate cancers, trials of PTP1B inhibitors for treating these diseases are also being explored. Some studies have shown that inhibition of PTP1B can block cancer progression (Xu et al. 2019), while others have had limited effects. Efforts to drug PTP1B have largely focused on targeting the catalytic pocket using non-hydrolysable phosphotyrosine analogues, and exploiting the second aryl binding site of PTP1B to achieve high affinity and specificity interactions (Zhang and Zhang 2007). However, it has been challenging to generate efficacious compounds due to weak membrane permeability and poor specificity for PTP1B. Another avenue of current interest is the inhibition of PTP1B (Penafuerte et al. 2017) and similar phosphatases (Wiede et al. 2020) in order to enhance immunosurveillance and efficacy in immunotherapies.

1.4.2 The role of LNK in disease

Mutations in the *SH2B3* gene that encodes for LNK have been implicated in a number of diseases that can generally be split into two categories. The first is those diseases where inflammation is involved and the other is where over-proliferation of cells is involved. The majority of LNK mutations arise in the PH and SH2 domains, preventing localisation

and phosphotyrosine interaction, however mutations outside of these regions have also been identified.

1.4.2.1 Diseases that lead to the over-proliferation of cells

Mutations in LNK have been identified in a small population of patients with MPNs, with most found in conjunction with an activating mutation such as the JAK2 V617F mutation. Interestingly, the frequency of LNK mutations increases up to 10% in patients with blast phase transformation. It is therefore suggested that mutations in LNK, along with a JAK2 V617F, MPL or CALR mutation may exacerbate disease progression. LNK also appears to regulate the common MPN mutations JAK2 V617F (Gery, Cao, et al. 2009) and MPL W515L (Gery et al. 2007) *in vitro*, however the extent to which this occurs *in vivo* is unknown.

Idiopathic erythrocytosis (IE) is characterised by the expansion of erythrocytes with unknown cause where polycythemia vera has been ruled out. Mutations in LNK have been identified in patients with IE. More recently, mutations in LNK have been identified in patients with acute lymphoblastic leukaemias (Willman 2013). The mutation identified in the patients with acute lymphocytic leukaemia occurred in the PH domain of LNK and caused an increase in cellular proliferation. Studies of the mutant in mice showed that loss of LNK accelerated NOTCH-induced leukaemias (Perez-Garcia et al. 2013). Further studies identified patients with IL7R-high, LNK-low, high risk-ALL associated with Ikaros dysfunction, implicating IL7R/LNK/Ikaros in oncogenesis of high-risk leukaemia (Ge et al. 2016). LNK knock-out mice display a phenotype similar to the myeloproliferative neoplasms with increased numbers medullary and extramedullary myeloid and B-cell precursors, and HSCs with increased self-renewal capacity (Ema et al. 2005). Inversely, mice that overexpress LNK have decreased lymphoid expansion (Takaki et al. 2003), further highlighting the role of LNK in the regulation of cellular proliferation. Together these studies lead to the classification of LNK as a potential tumor suppressor.

1.4.2.2 Diseases that lead to inflammation in the body

Multiple studies have implicated the LNK R262W mutation in the PH domain in diseases including type I diabetes, coeliac disease and coronary heart disease. It is suggested that the R262W mutant results in increased blood cell numbers (Van Der Harst et al. 2012),

which may play a role in exacerbating inflammation. Other studies have suggested a role for LNK in the negative regulation of inflammatory signalling via TNF (Boulday et al. 2002). The inflammatory diseases that LNK mutations have been linked to include rheumatoid arthritis, cardiovascular disease (Wang et al. 2016; Dale and Madhur 2016), type 1 diabetes (Lavrikova et al. 2011), coeliac disease (Hunt et al. 2008) and multiple sclerosis (Alcina et al. 2010). Despite these reported associations, the mechanisms by which LNK mutations instigate or exacerbate disease has remained elusive.

1.4.2.3 LNK as a therapeutic target

Given the role of LNK in regulating haematopoietic stem cell quiescence there is interest in targeting LNK therapeutically to mobilize HSCs, or to increase progenitor expansion following bone marrow transplant (Takizawa et al. 2006). This therapeutic intervention would require an inhibitor of LNK, which would be aided by structural studies of the LNK SH2 domain, which may be druggable. As of 2020, pazopanib is the only drug suggested to interact with LNK (DRUGBANK 2020), however there is little to no evidence for this interaction. It has also been suggested that expression of LNK in leukemic cells could suppress their proliferation, however gene therapy is a challenging task and targeting cancer cells using gene therapy adds an extra element of difficulty.

1.4.3 Understanding protein-protein interactions to understand disease

To understand how disease occurs when protein-protein interactions are disrupted, it is first essential to understand how these interactions occur under steady-state conditions. Although the JAK-STAT signalling pathway is relatively simple in comparison to other pathways, requiring only a handful of components to transmit signal from the cell surface to the nucleus, complexities arise when considering the number of protein-protein interactions that must occur to maintain homeostasis in the system. In particular, it is the interplay between the proteins that perpetuate signalling through the JAK-STAT pathway and those that negatively regulate the pathway that maintains the balance of signalling in the cell and flux through the pathway. It is therefore essential to understand how these proteins interact with one another to ensure homeostasis is maintained.

1.5 Thesis Aims

Given the importance of JAK-STAT signalling in maintaining homeostasis, having a comprehensive understanding of the molecular interactions within this signalling pathway is crucial for being able to manage and treat disease. In the context of PTP1B and LNK, we lack detailed information about how both proteins recognize and interact with other proteins, which proteins they interact with and how both proteins are involved in disease when mutated or aberrantly involved in signalling. This project aims to study in detail the interactions between PTP1B and LNK with the proteins of the JAK-STAT pathway and investigate what happens when these interactions are altered using structural biology and biochemistry. To this end, there are two main aims for this project:

Aim 1: To determine how PTP1B interacts with and dephosphorylates the JAK kinase domain.

Despite PTP1B being the prototypical tyrosine phosphatase, little is known about the interactions that occur between the phosphatase domain of PTP1B and the proteins of the JAK-STAT pathway. This thesis addresses this question using structural biology and biochemical techniques to delineate the mechanisms by which the PTP1B phosphatase domain interacts with the JAK activation loop, thus negatively regulating the JAK proteins and down regulating JAK-STAT signalling.

Aim 2: To understand the substrate specificity of the LNK SH2 domain and how mutations in this domain may contribute to disease.

LNK (SH2B3) is a negative regulator of TPO and EPO signalling downstream of JAK2, however the mechanism of negative regulation and substrate recognition for this protein is largely unknown. This thesis presents the first crystal structure of any LNK domain and puts forward evidence for the mechanisms of specificity for JAK2. This thesis also demonstrates how mutations in the SH2 domain of LNK may contribute to disease burden by destabilizing and reducing the ability of the SH2 domain to bind phosphotyrosine residues.

Chapter 2 Materials and Methods

2.1 Mammalian cell culture

2.1.1 Cell lines used in thesis

2.1.1.1 M1 parental cell line

The murine myeloid leukaemia cell line, named M1, was originally isolated in 1969 from SL mice with spontaneous leukaemia (Ichikawa 1969). A sub-clone has been in culture at The Walter and Eliza Hall Institute for over 30 years and is termed M1P or the M1-Parental line. This is the cell line from which all of the M1 lines used in this thesis were made.

2.1.1.2 M1-Cas9 cell line

M1-Cas9 cells were generated by Andrew Jarratt in the Hilton laboratory (Walter and Eliza Hall Institute), by infecting M1P cells with Cas9-mCherry reporter lentivirus Cas9-2A-mCherry. Following infection, mCherry positive cells were sorted by flow cytometry, and subsequently plated in semi-solid agar. Single clones were picked and expanded to make clonal cultures and a clone that responded similarly to the M1 parental line when cultured in IL-6 was used for further experiments and was termed M1-Cas9.

2.1.1.3 M1-Cas9 + MPL cell line

M1-Cas9 cells were provided by Craig Hyland and Dr. Maria Kauppi in the Alexander laboratory (Walter and Eliza Hall Institute). Cells were generated by infecting M1-Cas9 cells with MPL construct with a GFP marker. Following infection, mCherry and GFP positive cells were sorted by flow cytometry, and subsequently plated in semi-solid agar. Single clones were picked and expanded to make clonal cultures and a clone that responded when cultured in IL-6 and in TPO was used for further experiments. This clone will be referred to as M1-Cas9 + MPL throughout this thesis.

2.1.1.4 Overexpression M1 cell lines

5×10^5 M1-Cas9 cells/well were seeded in a 24-well plate and 1 mL of lentivirus supernatant was added to a separate well of each plate followed by the addition of 4 mg/ml of polybrene. Cells were spin infected for 1-2 h at 2000 rpm in a Multifuge 2SR with a Swing-out 4 place rotor (Heraeus) at 37°C. Plates were then incubated overnight

at 37°C in 10% CO₂. The following day the cell suspensions were spun at 1500 rpm for 5 min. Cell pellets were resuspended in 1 mL of fresh DMEM-10% and seeded in a new 24-well plate. Following incubation overnight at 37°C in 10% CO₂, cell suspensions were transferred to a 12-well plate and 1 mL of DMEM-10%. For cells infected with lentivirus, DMEM-10% supplemented with puromycin was used to select for those cells the virus had infected. Cells were then incubated for 3 d at 37°C in a 10% CO₂ atmosphere prior to plating in semi-solid media.

2.1.1.5 HEK293T cells

Human embryonic kidney 293 cells containing the SV40 T-antigen were grown from cultures existing in the structural biology division at WEHI. These cultures are referred to as HEK293T cells throughout this thesis.

2.1.1.6 Maintenance

All M1 cells and HEK293T cell lines were routinely cultured in DMEM supplemented with penicillin/streptomycin (50 U/ml) (Pen/Strep; Thermo Fisher Scientific) and 10% (v/v) foetal calf serum (FCS; Thermo Fisher Scientific) (DMEM-10%) and maintained in an atmosphere of 5% or 10% CO₂ at 37°C.

2.2 Cytokines and antibodies

All cytokines used in this thesis are listed in **Table 2.1**.

Table 2.1 Cytokines.

Cytokine	Stock concentration
Interleukin 6 (IL-6)	0.8 mg/mL
Thrombopoietin (TPO)	1 mg/mL

Recombinant murine IL-6 and TPO stocks were made up to 0.8 or 1 mg/mL respectively in saline supplemented with 5% (v/v) FCS. For single concentration experiments, 100 µL of cytokine stock was used per 1 mL of media to give a final working concentration of 100 ng/mL. For titrations, cytokines were diluted 2-fold from 1 µg/mL to form an 11-point titration. All cytokines used were produced and generously provided by Dr. Jian-Guo

Zhang and Phillip Morgan from the division of Cancer and Haematology (Walter and Eliza Hall Institute) or generated by the Babon laboratory (Walter and Eliza Hall Institute).

2.3 Generation of constructs for mammalian cell expression

All constructs were generated using PCR amplification. Inserts and vectors were cut with their respective restriction enzymes for 2 h at 37°C or at 50°C in the case of Sfi1 and run on an agarose gel. Digested product was gel purified using a QIAquick gel extraction kit (QIAGEN) and ligated for 1 h using T4 DNA ligase prior to transduction into *E. coli* for expression. Constructs with kanamycin resistance were recovered in SOC broth for 1 h prior to plating onto agar plates supplemented with 50 µg/µL of kanamycin. Constructs with ampicillin resistance were plated directly onto plates supplemented with 100 ng/mL of ampicillin after transformation. All plates were incubated at 37°C overnight. Colonies were picked, grown in SB with their ampicillin before miniprepping of DNA using a ZR plasmid miniprep kit (Zymo research) for DNA sequencing.

2.3.1 Lentivirus production

10 mg of vector was added to 7.5 µg of psPAX packaging vector, 3 µg of VSV, 62.5 mL of CaCl₂ (2M), 500 mL of HBS made up to 1 mL with H₂O and mixed by bubbling the solutions together. Each mixture was added dropwise to a plate seeded with 2.5 x 10⁶ HEK293T cells. Cells were incubated overnight at 37°C in 10% CO₂ and the next day fresh DMEM-10% was added. Cells were again incubated overnight at 37°C in 10% CO₂. The next day, viral supernatant was harvested by filtering through a syringe filter and snap frozen in 1 mL aliquots. Virus was stored at -80°C and thawed slowly on ice before use.

2.3.2 Retrovirus production

All retroviral infections were carried out using constructs from a mouse brain cDNA library cloned into the pCFB vector. 14.4 µg of vector was added to 4.8 µg of MDI Gag-pol packaging vector, 0.6 µg of VSVg, 62.5 mL of CaCl₂ (2M), 500 mL of HBS made up to 1 mL with H₂O and mixed by bubbling the solutions together. Each mixture was added dropwise to a plate seeded with 3.5 x 10⁶ HEK293T cells pre-treated with chloroquine

(Sigma-Aldrich). Cells were incubated overnight at 37°C in 10% CO₂ and the next day fresh DMEM-10% was added. Cells were again incubated overnight at 37°C in 10% CO₂. The next day, viral supernatant was harvested by filtering through a syringe filter and snap frozen in 1 mL aliquots. Virus was stored at -80°C and thawed slowly on ice before use.

2.3.3 Selection of lentivirus infected cells

Two days following lentivirus infection, cells were transferred to media containing 5 ng/μL puromycin to select for cells containing lentivirus. Cells surviving puromycin selection were plated in semi-solid agar as described in the soft-agar assay (section 2.5). Cells were selected in puromycin for 7 d prior to scoring for colonies using a dissecting microscope as per section 2.5. Single colonies were picked from the semi-solid media and transferred to a 24-well plate, where they were grown in DMEM-10% supplemented with puromycin for 7 days. After 7 days, those cells that grew out were routinely cultured in DMEM-10% without puromycin.

2.4 DNA sequencing

All sequencing was carried out by the Australian Genome Research Facility (AGRF).

2.5 IL-6 agar titrations

2.5.1 Preparation

IL-6 titrations were prepared by Dina Stockwell in the Nicola and Babon laboratories (Walter and Eliza Hall Institute). Two-fold dilutions of cytokine from 100 ng/mL in saline supplemented with 5% FCS (v/v) formed an 11-point titration. 100 μL of each cytokine dilution was added to the center of a 2 cm petri dish. Saline supplemented with 5% FCS (v/v) was used as a control. Cells were suspended at approximately 400 cells/mL in modified double-strength DMEM supplemented with 40% FCS (v/v) and mixed with an equal volume of 0.6% (w/v) Bacto agar (Difco Labs). 1 mL of each suspension was added to each petri dish and mixed with the cytokine. Plates were incubated at 37°C in a humidified incubator with a 10% CO₂ atmosphere until scoring.

2.5.2 Colony scoring

After a 7-day incubation at 37°C in a humidified incubator with a 10% CO₂ atmosphere, agar assays were scored using a dissecting microscope by Professor Nicos Nicola (Walter and Eliza Hall Institute). Compact round colonies were scored as undifferentiated colonies whilst dispersed colonies of cells, or completely dispersed cells were scored as differentiated.

2.5.3 Imaging

Following scoring, cells from semi-solid media assays were photographed using a Zeiss Stemi SV11 microscope interfaced with an AxioCam 105 colour camera using 6.6x objective (Zeiss).

2.6 Cell-titre Glo assays

Cytokines and other reagents were mixed with media to desired concentration and 100 µL of each cytokine dilution was added to each well and approximately 1000 cells were added to each well. Plates were incubated at 37°C in a 10% CO₂ atmosphere. After 4 days, 40 µL of cell suspension was transferred to a white opaque 96-well plate and an equal volume of CellTiter-Glo® reagent (Promega) was added to each well. Contents were mixed together for 2 min on an orbital shaker, and plate was left to equilibrate for 30 minutes at room temperature. Luminescence was detected using a Chameleon multi-mode microplate reader (Hidex). All data was analysed using Prism (Prism).

2.7 Western blotting

2.7.1 Sample preparation

Samples were mixed with 4x reducing buffer and boiled for 5 min and subsequently centrifuged at 1300 rpm for 3 min.

2.7.2 Electrophoresis and transfer

Equal concentrations of protein were mixed with 4x reducing sample buffer. Each sample was loaded into a separate well of a 4-15% (w/v) Novex Nupage Tris-Glycine pre-cast gel (Invitrogen). Proteins were separated according to size at 140 V for 1 h in SDS running

buffer. Protein was transferred to a methanol activated Millipore PDVF membrane in transfer buffer at 80 V for 2 h or by semi-dry transfer using the iBlot system as per the manufacturer's instructions.

2.7.3 Blotting

Membranes were blocked in TBST containing 5% (w/v) skim milk powder and 0.1% (v/v) Tween-20 at room temperature for 10 min. Primary antibodies were used at dilutions listed in Table 2.6 in TBST with 5% (w/v) skim milk powder or 5% BSA. Membranes were incubated overnight in 1-5 mL of diluted antibody at 4°C. Membranes were washed 3 times in TBS with 1% (v/v) Tween-20 before probing with HRP-conjugated secondary antibody. Each membrane was incubated in 10 mL of diluted secondary antibody for at least 1 h at room temperature. Membranes were washed 6 times for 10 minutes and exposed to 1 mL of ECL detection reagent (Amersham; Thermofisher scientific) and imaged using a BioRad Chemidoc MP. Image analysis was performed using Image J software.

2.7.4 Densitometry

Western blots were quantified using Image J software (Schneider, Rasband, and Eliceiri 2012).

2.8 WT PTP1B purification

WT PTP1B was purified by Artem Laktyushin in the Babon laboratory (Walter and Eliza Hall Institute). DNA encoding human the human PTP1B phosphatase domain (residues 2-321) and an N-terminal His tag was clone into a pPROEX vector and transformed into BL21 (DE3) *E. coli* cells. *E. coli* were grown in Luria Broth (LB) supplemented with ampicillin and grown at 37°C until they reached an OD of 0.7, at this stage temperature was dropped to 18°C and when cells reached an OD of 1 expression was induced by addition of 1 mM IPTG. Induction proceeded overnight at 18°C and next day cells were harvested by centrifugation at 5000 rpm and pellets were stored at 4°C until purification. For purification, cell pellets were resuspended and lysed in lysis buffer (20 mM Tris (pH 8.0), 300 mM NaCl, 1 mM PMSF, 2 mM TCEP, and 20 mg lysozyme) containing EDTA-free protease inhibitor cocktail by sonication on an ice bath for 2 minutes (10 s on, 5 s off).

Lysate was clarified by spinning cells for 10 minutes at 20,000 g before loading supernatant onto 1 mL of glutathione affinity resin (GE Healthcare). Bound proteins were washed with Buffer A (20 mM tris (pH 8.0), and 300 mM NaCl, and 2 mM TCEP). Beads were resuspended in 20 mL of buffer A (20 mM tris (pH 8.0), and 300 mM NaCl, and 2 mM TCEP) with 0.5 g TEV protease and incubated on a roller at 4°C overnight. Cleaved PTP1B was separated from the GST tag through a gravity flow column. Eluate was then further purified using size exclusion chromatography (Superdex 200 16/600 from GE healthcare) in TBS plus 2 mM TCEP. Fractions were pooled and snap frozen and liquid nitrogen for storage.

2.9 PTP1B DQ and DQC mutant purification

DNA encoding human the WT human PTP1B phosphatase domain (residues 2-321, D181A/Q262A or D181A/Q262/C215A mutants) and an N-terminal His tag was clone into a pGEX-4T vector and transformed into tuner *E. coli* cells. *E. coli* were grown in Luria Broth (LB) supplemented with ampicillin and grown at 37°C until they reached an OD of 0.7, at this stage temperature was dropped to 18°C and when cells reached an OD of 1 expression was induced by addition of 1 mM IPTG. Induction proceeded overnight at 18°C and next day cells were harvested by centrifugation at 5000 rpm and pellets were stored at 4°C until purification. For purification, cell pellets were resuspended and lysed in lysis buffer (20 mM Tris (pH 8.0), 5 mM imidazole, 300 mM NaCl, 1 mM PMSF, 2 mM TCEP, and 20 mg lysozyme) containing EDTA-free protease inhibitor cocktail by sonication on an ice bath for 2 minutes (10 s on, 5 s off). Lysate was clarified by spinning cells for 10 minutes at 20,000 g before loading supernatant onto 1 mL of Nickel resin. Bound proteins were washed with buffer A (20 mM tris (pH 8.0), 10 mM imidazole (pH 8.0), 300 mM NaCl, and 2 mM TCEP) followed by buffer B (20 mM tris (pH 8.0), 30 mM imidazole (pH 8.0), 300 mM NaCl, and 2 mM TCEP) and eluted in buffer C (20 mM tris (pH 8.0), 250 mM imidazole (pH 8.0), 300 mM NaCl, and 2 mM TCEP). Eluate was then further purified using size exclusion chromatography (Superdex 200 16/600 from GE healthcare) in TBS plus 2 mM TCEP. Fractions were pooled and snap frozen and liquid nitrogen for storage.

2.10 Malachite green peptide dephosphorylation assay

All assays were performed using 2 nM human PTP1B and a Malachite green assay kit (Sigma Aldrich) in TBS, 2 mM TCEP and 0.01 mg/ml BSA in 96-well plates. 80 μ L reactions were set up with peptide at varying concentrations and reactions were started by the addition of PTP1B. After incubation times, reactions were quenched by the addition of working reagent provided in the assay kit. Plates were read using a chameleon V plate reader at 620 nm and results were analyzed using Prism (Prism). For all assays, two technical replicates were used and a peptide minus PTP1B was measured to determine the amount of free phosphate already in solution, and measurements of phosphate in the PTP1B minus sample was subtracted from the amount measured in the experimental reactions.

2.11 Dephosphorylation assay

PTP1B was added to JAK kinase domain in JAK GF buffer and samples were taken at each time point required for the assay and added directly to 4x loading dye to stop the reaction. JAK kinase domains were used at 13 μ M concentration unless otherwise specified. Reactions all contained JAK kinase domain, 5 mM EDTA, TBS + 0.5% BSA, and PTP1B and those performed with inhibitors contained 5 nM inhibitor or DMSO as a control. Samples were separated by size on 4-15% (w/v) Novex Nupage Tris-Glycine pre-cast gel (Invitrogen). Proteins were separated according to size at 140 V for 1 h in SDS running buffer. The rest of the assay followed the same protocol as the western blotting section detailed above.

2.12 NMR dephosphorylation analysis

2.12.1 Dephosphorylation of pYpY peptides

0.5 mg of JAK2 or IRK pYpY was dissolved in 500 μ L of 20 mM Tris (pH 7.5), 2 mM TCEP, and 100 mM NaCl. Dissolved peptide was then added to 25 μ L D₂O and 0.01% of sodium azide was added to the solution. NMR spectra were collected using a Bruker Advance 600 MHz or 800 MHz spectrometer. To start the dephosphorylation reaction, 2 nM PTP1B was added to the sample and spectra were recorded for up to 1 hour. The concentration of each species of peptide (pYpY, pYY, or YpY) in the reaction was calculated by integrating

the area under the peak for each representative species at each time point and normalising these values to the area under the peak for the starting pYpY peptide whose starting concentration prior to addition of enzyme was known. Data was analyzed using TopSpin v3.2 (Bruker) and normalised data was plotted in Prism.

2.12.2 Reaction rate calculations

0.5 mg of JAK2 or IRK pYpY, pYY and YpY were dissolved in 500 μ L of 20 mM Tris (pH 7.5), 2 mM TCEP, and 100 mM NaCl. Dissolved peptide was then added to 25 μ L D₂O and 0.01% of sodium azide was added to the solution. To start the dephosphorylation reaction, 2 nM PTP1B was added to each sample and spectra was recorded for up to 1 hour, for a total of 20 scans. Data was recorded using a Bruker Avance 600 MHz magnet equipped with a triple-resonance cryoprobe. The concentration of each species of peptide (pYpY, pYY, or YpY) in the reaction was calculated by integrating the area under the peak for each representative species at each time point and normalising these values to the area under the peak for the starting peptide whose starting concentration was known prior to addition of enzyme. These data were then normalised to enzyme concentration and reaction time to determine turn-over rate. Data was analyzed using TopSpin v3.2 (Bruker) and normalised data was plotted in Prism.

2.13 LNK SH2 domain purifications

DNA encoding human the mouse LNK SH2 domain and an N-terminal NusA fusion was cloned into an pET-50b(+) vector and transformed into tuner *E. coli* cells. *E. coli* were grown in Luria Broth (LB) supplemented with kanamycin and grown at 37°C until they reached an OD of 0.7, at this stage temperature was dropped to 18°C and when cells reached an OD of 1 expression was induced by addition of 1 mM IPTG. Induction proceeded overnight at 18°C and next day cells were harvested by centrifugation at 5000 rpm and pellets were stored at 4°C until purification. For purification, cell pellets were resuspended and lysed in lysis buffer (20 mM Tris (pH 8.0), 10 mM imidazole (pH 8.0), 300 mM NaCl, 1 mM PMSF, and 20 mg lysozyme) containing EDTA-free protease inhibitor cocktail (SigmaAldrich) by sonication on an ice bath for 2 minutes (10 s on, 5 s off, power 3). Lysate was clarified by spinning cells for 10 minutes at 20,000 g before loading supernatant onto Nickel resin. Bound proteins were washed with Buffer A (20 mM tris

(pH 8.0), 10 mM imidazole (pH 8.0) and 300 mM NaCl, 5 mM Phenyl Phosphate) followed by buffer B (20 mM Tris (pH 8.0), 30 mM imidazole (pH 8.0) and 300 mM NaCl, 5 mM Phenyl Phosphate I) and eluted in buffer C (20 mM Tris (pH 8.0), 250 mM imidazole (pH 8.0) and 300 mM NaCl, 5 mM Phenyl Phosphate). Eluate was then cleaved with TEV overnight at 4 °C. Eluate was then further purified using size exclusion chromatography (Superdex 200 26/600 from GE healthcare) in TBS + 2 mM BME + 5 mM Phenyl Phosphate. Fractions were pooled, snap frozen and liquid nitrogen for storage.

2.14 JAK kinase domains and full length JAK1 expression and purification

JAK kinase domain and full length JAK1 expressions and purifications were carried out by Nicholas Liao, Artem Laktyushin or myself (Walter and Eliza Hall Institute). *Sf21* insect cells were seeded into a Fernbach flask at $2.5\text{--}3.0 \times 10^6$ cells/mL and infected with baculovirus at a multiplicity of infection of approximately 3.0. Cells were incubated at 27°C for 48 h and subsequently harvested by centrifugation at 5,000 g for 30 min and pellets were stored at -30°C until purification. For purification, cells were resuspended and lysed in 10mM Tris pH 7.5, 150mM NaCl, 2mM TCEP, 1mM PMSF, 5U/mL DNase, by sonication on an ice bath for 3 minutes (10 s on and 10 s off). Lysate was clarified by spinning cells for 60 minutes at 40,000 g before being filtered through a 0.8 µm disposable filter unit. Filtered supernatant was loaded onto a HisTrap cartridge and washed with 20mL 2% of Nickel Buffer, and subsequently 15 mL of 7% Nickel Buffer. Full length JAK1 was eluted in 3 mL Nickel Buffer B. Eluate was then further purified using size exclusion chromatography (Superdex 200 16/600 from GE healthcare) in JAK gel filtration buffer (10% glycerol, 20 mM Tris pH 8.0, 500 mM NaCl and 2 mM TCEP). Fractions were pooled and snap frozen and liquid nitrogen for storage.

2.15 Thermostability assays

Proteins were desalted into 100 mM NaCl, 20 mM Tris (pH 8.0), 2 mM TCEP buffer and diluted to 100 µM. Where peptides were used, a five-fold molar excess of peptide was added to each sample. 10 µL of each sample was transferred into a capillary and measured from 35- 95°C using a Tycho N6T (Nanotemper). Data were analyzed in Prism.

2.16 Isothermal titration calorimetry

Proteins were buffer exchanged into TBS + 2 mM TCEP by PD-10 desalting column (GE Healthcare) or diluted 50-fold in the same buffer. Proteins were concentrated to 10-30 μ M in a 10kDa cut-off centrifugal concentrator (Millipore) and 280 μ L was loaded into the cell of a MicroCal iTC200 Isothermal Titration Calorimeter (ITC) (Malvern Scientific) set to 25°C with a syringe mixing speed of 750 rpm. 100-300 μ M peptide was loaded into the ITC syringe. Two initial injections of 0.4 μ L were followed by 3.15 μ L injections. Binding curves were fitted with Origin 7.0 software (OriginLab corporation) and binding parameters were quantified.

2.17 Quick change mutagenesis

The PTP1B D/Q/C 2-321 construct was generated by quick change mutagenesis using PTP1B D/Q 2-321 as the template. Using primers described in Table 2.11, following the manufacturer's instructions using 40 cycles. Colonies that grew were miniprep and sequenced to confirm presence of the C215A mutation.

2.18 DNA purification

1.5-2% molecular-grade agarose gels were made up in 1xTAE and used for DNA separation by electrophoresis. DNA bands were visualised with ethidium bromide or cybrgreen and excised with scalpel blades. DNA clean-up was performed using Miniprep, Maxiprep, and Gel Extraction kits from QIAGEN or DNA Recovery Kits from Zymo following manufacturer's instructions. All constructs were sent to the Australian Genome Research Facility (AGRF) for sequencing. Sequencing data was analysed using the Lasergene suite v.14 (DNASTAR).

2.19 Gel filtration experiments

PTP1B phosphatase domain constructs D/Q 2-298 and 2-298 and D/Q/C 2-321 were mixed together with the JAK kinase domains (1, 2, 3 and TYK2) and full length JAK1 at varying molar ratios and injected onto a Superdex 200 increase 10/30 (GE healthcare) for separation by size exclusion chromatography in TBS + 2 TCEP. Fractions under the peaks were collected and separated by SDS PAGE gel and stained with Coomassie blue

prior to imaging with a Bio-Rad imaging platform. Western blots were also performed for the PTP1B D/Q/C and full length JAK1 fractions as per the western blotting protocol. Where an inhibitor was used, 5 nM was added to the JAK kinase domain prior to being mixed with the PTP1B phosphatase domain.

2.20 Biacore binding experiments

All SPR experiments were performed on either a Biacore 4000 or 2000 (GE Healthcare) in biacore buffer and regeneration buffer using a streptavidin coated chip. Peptides were immobilised to chip by flowing over 1 mg/mL of peptide dissolved in Biacore buffer.

2.20.1 Direct binding assay

Direct binding experiments were carried out using the Biacore 2000. 4, 2, 1, 0.5 0.25 and 0 uM LNK SH2 domains were flowed over a streptavidin coated chip for 240 seconds at 30 μ L/min with immobilised biotinylated JAK2 pY813 bound to determine binding kinetics. A reference flow cell was included by passing buffer without protein over a single lane and the sensorgrams from the reference cell were subtracted from the experimental flow cell analyses. The curves were fitted to a simple 1:1 binding model to determine the dissociation constant (K_D) and subsequently plotted in Prism.

2.20.2 Competition assays

Competition assays were carried out using the Biacore 4000. 0.1-0.5 uM WT LNK SH2 domains were pre-incubated with 2, 1, 0.5, 0.25, 0.125 or 0 nM phosphopeptides or 1, 0.5, 0.25, 0.125, 0.0625 mM phenyl phosphate before being flowed over a streptavidin chip with immobilised biotinylated IL6ST pY757 for 240-720 seconds at 10-30 μ L/min. Data was analysed using Prism where the binding (raw data measuring how much LNK binds the immobilised peptide) of LNK in the presence of the peptide was normalised to an LNK only control. Normalised data was plotted as a percentage of binding to the immobilised peptide, relative to the LNK only control (where binding is 100%) against the $\log_{10}$ concentration of peptide. Measure of binding inhibition is shown as the concentration at which half the maximum binding to the IL6ST pY757 peptide is inhibited (IC_{50}), as determined by curve fitting to a non-linear regression in Prism.

2.21 Crystallography

2.21.1 Data collection and analysis

This research was undertaken in part using the MX2 beamline at the Australian Synchrotron, part of ANSTO, and made use of the Australian Cancer Research Foundation (ACRF) Eiger detector (McPhillips et al. 2002). Data reduction, scaling and integration was performed using XDS (Kabsch 2010). Matthews coefficient was estimated using MATTPROB (Kantardjieff and Rupp 2003; Weichenberger and Rupp 2014).

2.21.2 Structure visualization

All structures were visualized using Pymol (DeLano 2002). 2D representation of interactions between proteins and peptides was visualized using LigPlot+ (Laskowski and Swindells 2011). Composite omit maps were generated in PHENIX (Adams et al. 2010) with annealing at a sigma of either 0.8 or 1 and 1.5 Å carve.

2.21.3 Crystallization of PTP1B

Crystal trays containing PTP1B D/Q and D/Q/C 2-321 constructs with JAK activation loop peptides were set up using 10 mg/mL of PTP1B and a 2-fold molar excess of peptide using vapor diffusion hanging drop experiments (in house) or sitting drop experiments (C3) at 4°C. All PTP1B was exchanged into low salt buffer (20 mM tris (pH 8.0), 2 mM TCEP and 100 mM NaCl). Crystals trays at C3 CSIRO were set up robotically by Janet Newman or Bevan Marshall (CSIRO). All crystallization conditions for PTP1B structures are listed in **Table 2.2**. All crystals were cryo-protected in mother liquor supplemented with either 20% or 25% ethylene glycol and immediately snap frozen in liquid nitrogen.

Table 2.2 PTP1B crystallisation conditions

Protein and peptide	Conditions
PTP1B D/Q JAK1 pYpY	12% Peg 4000, 0.1 M Calcium acetate, 0.05 M MES (pH 6.5)
PTP1B D/Q JAK2 pYpY	14% PEG 8000, 0.10 M Mg Acetate, 0.1 M MES (pH 6.5)
PTP1B D/Q JAK3 pYpY	12% Peg 4000, 0.15 M Calcium acetate, 0.05 M MES (pH 6.5)
PTP1B D/Q TYK2 pYpY	0.2 M Ca Acetate, 12.5% PEG 4000, 0.05 M MES (pH 6.5)
PTP1B D/Q/C JAK2 pYpY	0.2 M Mg formate, 20% PEG 3350

PTP1B D/Q JAK2 pYY	12% PEG 8000, 0.15 M Mg Acetate, 0.1 M MES (pH 6.5)
PTP1B D/Q JAK2 YpY	14% PEG 8000, 0.20 M Mg Acetate, 0.1 M MES (pH 6.5)
PTP1B D/Q/C JAK2 pYY	14% PEG 8000, 0.20 M Mg Acetate, 0.1 M MES (pH 6.5)
PTP1B D/Q/C JAK2 YpY	25% w/v PEG 3350, 0.2 M NaCl, 0.1 M Tris Cl (pH 8.5)
PTP1B D/Q Apo	12% PEG 8000, 0.15 M Mg Acetate, 0.1 M MES (pH 6.5)
PTP1B D/Q/C TYK2 pYpY	14% PEG 8000, 0.20 M Mg Acetate, 0.1 M MES (pH 6.5)

2.21.4 X-ray structure determination for PTP1B constructs

Crystal structures of PTP1B were solved by molecular replacement (search model PDB ID: 1SUG or 4ZRT) using Phaser as implemented in PHENIX. All structures were refined using PHENIX (Adams et al. 2010) and model building was performed in COOT (Emsley and Cowtan 2004).

2.21.5 Protein crystallization of LNK

All LNK SH2 domain crystal trays were set up with 5 mg/mL of protein and a 2-fold molar excess of peptide using vapor diffusion hanging drop (in house) or sitting drop (C3) experiments. All LNK constructs were buffer exchanged into low salt buffer (20 mM tris (pH 8.0), 2 mM TCEP and 100 mM NaCl). The WT LNK SH2 domain with the JAK2 pY813 peptide crystallized in 0.2 M ammonium chloride, 20% w/v PEG 3350. The E372K SH2 domain crystallized in 18% w/v PEG 8000, 0.05 magnesium acetate, 0.1 M Tris pH 8.5. The WT LNK SH2 domain with the EPOR pY454 peptide 20% w/v PEG 8000, 0.05 magnesium acetate, 0.1 M Tris pH 8.5. All crystals were cryo-protected in paratone and immediately snap frozen in liquid nitrogen. Crystals trays at C3 CSIRO were set up robotically by Janet Newman or Bevan Marshall (CSIRO).

2.21.6 X-ray structure determination for LNK SH2 domains

Crystal structures of the LNK SH2 domain were solved by molecular replacement (search model PDB ID: 2HDX for WT LNK SH2 with JAK2 pY813 peptide and the for the E372K with pY813 and WT LNK SH2 with EPOR pY454, the WT with pY813 structure was used) using Phaser as implemented in PHENIX (Adams et al. 2010). All structures were refined using PHENIX and model building was performed in COOT (Emsley and Cowtan 2004).

2.22 Materials

2.22.1 Peptide sequences

The sequences of all peptides used in this thesis are listed in **Table 2.3**.

Table 2.3 Peptide sequences.

Name	Sequence	MW
JAK1 ALP pYpY	AIETDKE[pY][pY]TVKDDLD	2077
JAK2 ALP pYpY	VLPQDKE[pY][pY]KVKEPGE	2002
JAK3 ALP pYpY	LLPLDKD[pY][pY]VVREPGQ	1823
TYK2 ALP pYpY	VPEGHE[pY][pY]RVREDGD	1980
JAK2 ALP pYY	VLPQDKE[pY]YKVKEPGE	2001
JAK2 ALP YpY	VLPQDKEY[pY]KVKEPGE	2001
JAK2 ALP Ala	VLPQDKEA[pY]KVKEPGE	1780
IRK ALP pYpY	ETD[pY][pY]RKGGKGL	1658
IRK ALP pYY	ETD[pY]YRKGGKGL	1578
IRK ALP YpY	ETDY[pY]RKGGKGL	1578
JAK2 pY813	FTPD[pY]ELLTEND	1535
JAK2 pY813_1	FTPD[pY]ALLTEND	1478
JAK2 pY813_2	FTPD[pY]RLLTEND	1563
JAK2 pY813_3	FTPD[pY]ELFTEND	1579
JAK2 pY813_4	FTPA[pY]ELLTEND	1492
JAK2 pY813_5	FTPD[pY]ELFTAND	1478
JAK2 pY813_6	FTPD[pY]ELFAEND	1506
JAK2 pY613	LVLN[pY]GVCVCGD	1498
JAK3 pY785	ISSD{[pY]ELLSDPT	1418
MPL pY521	FPAH[pY]RRLRHAW	1689
MPL pY552	VLGQ[pY]LRDTAALS	1480
MPL pY626	ANHS[pY]LPLSYWQP	1655
MPL pY631	LPLS[pY]WQQP	1211
MPL pY591	AQDM[pY]RRLQPSC	1481
EPOR pY309	QLWL[pY]QNGCLWW	1689
EPOR pY386	AQDT[pY]LVLDKWL	1544

EPOR pY426	ASFE[pY]TILDPSS	1409
EPOR pY485	SDGP[pY]SNPYENS	1409
EPOR pY454	PHLK[pY]LYLVVS	1425
IL6ST pY767	STVHSG[pY]RHQVPSV	1732
IL6ST pY814	GILPRQQ[pY]FKQNCSQ	1890
IL6ST pY905	DEGMPKS[pY]LPQTVRQ	1828
IL6ST pY915	QTVRQGG[pY]MPQ	1344
IL6ST pY757	STASTVE[pY]STVVHSG	1607
FLT3 pY572	YKKQFR[pY]ESQLQM	1828
FLT3 pY591	SDNEYF[pY]VDFREY	1826
FLT3 pY919	YATEEI[pY]IIMQSC	1643
JAK2 ALP J2J1	VLPQDKEY[pY]TVKEPGE	1974
JAK2 ALP 1N	VLPQDKRYvKVKEPGE	2028
JAK2 ALP VA	VLPQDKEY[pY]KAKEPGE	1973
JAK2 ALP 2N	VLPQDEEY[pY]KVKEPGE	2002
JAK2 ALP 1C	VLPQDKEY[pY]EVKEPGE	2002
STAT3 pY705	SAAP[pY]LKTKFI	1317
STAT1 pY701	KGTG[pY]IKTELI	1301
JAK2 no pY	VLPQDKEYYKVKEPGE	1921

2.22.2 Expression vectors

Details of all expression vectors used for both mammalian cell over expression of proteins, *E. coli* expression and Insect cell expression of recombinant proteins are listed in **Tables 2.4, 2.5 and 2.6** respectively.

Table 2.4 Bacterial Expression vectors.

Vector	Antibiotic resistance
pPROEX	Ampicillin (50 µg/mL)
pGEX-4T	Ampicillin (100 µg/mL)
pET-50b(+)	Kanamycin (50 µg/mL)

Table 2.5 Mammalian expression Vectors.

Vector	Antibiotic resistance
pFB-LIC	Ampicillin (50 µg/mL)
pF-TRE3G	Ampicillin (100 µg/mL)

Table 2.6 Insect cell expression vectors.

Vector	Antibiotic resistance
Bacmid	Kanamycin (50 µg/mL), Gentamycin (10 µg/mL), Tetracycline (10 µg/mL)

2.22.3 Antibodies

All antibodies used in the experiments detailed in this thesis are listed in **Table 2.7**.

Table 2.7 Antibodies.

Antibody	Species	Dilution	Supplier	Code
<i>Primary Antibodies</i>				
Phospho-JAK1	Rabbit	1:1000	Santa Cruz Biotech	sc-10176
JAK2	Mouse	1:1000	Santa Cruz Biotech	sc-390539
PTP1B	Mouse	1:1000	Abcam	ab124375
<i>Secondary Antibodies</i>				
Anti-rabbit	Donkey	1:15,000	GE/Amersham	NA934
Anti-mouse	Sheep	1:15,000	GE/Amersham	NA931

2.22.4 Media

Media used to support growth of cellular cultures are detailed in **Table 2.8**.

Table 2.8 Media composition.

Media	Composition	Supplier
Super broth (SB)	35 g tryptone, 20 g yeast extract, 5 g NaCl, 2.5 mL 2 M NaOH up to 1 L with deionized water	WEHI media kitchen

LB agar	1% (w/v) tryptone, 0.5% (w/v) yeast extract 0.5% (w/v), NaCl, 0.2% (w/v) D-Glucose, 10mM Tris pH 7.4, 1 mM, MgCl ₂ , 1.5% (w/v) agar	WEHI media kitchen
DMEM	10% fetal bovine serum (FBS), 2 mM L- glutamine, 100 units/mL penicillin G, 100 µg/mL streptomycin	Gibco

2.22.5 Buffers

All buffers used in the experiments presented in this thesis are listed in **Table 2.9**.

Table 2.9 Buffer Composition.

Buffer	Composition
TBS	50 mM Tris-Cl, pH 7.5. 150 mM NaCl
TBST	50 mM Tris-Cl, pH 7.5. 150 mM NaCl, 1% v/v Tween 20
Malachite green assay buffer	50 mM Tris-Cl, pH 7.5. 150 mM NaCl, 0.1% w/v BSA, 2 mM TCEP,
Dephosphorylation assay buffer	50 mM Tris-Cl, pH 7.5. 150 mM NaCl, 0.1% w/v BSA, 2 mM TCEP, 5 mM EDTA
Transfer Buffer	25 mM Tris-Cl, 200 mM Glycine pH 8.3, 0.01% (w/v) SDS, 10% (v/v) methanol
SDS-PAGE reducing sample buffer	50 mM Tris-HCl pH 7.4, 200 mM β-ME, 10% (v/v) glycerol, 4% (v/v) SDS, 0.2% (w/v) bromophenol blue
MES running buffer	1X: 50 mM MES, 50 mM Tris-Cl, 0.1% SDS, 1 mM EDTA, pH 7.3
JAK gel filtration buffer	10% (v/v) glycerol, 20 mM Tris-Cl pH 8.0, 500 mM NaCl, 2 mM TCEP
PTP1B gel filtration buffer	50 mM Tris-Cl, pH 7.5. 150 mM NaCl, 2 mM TCEP
LNK gel filtration buffer	50 mM Tris-Cl, pH 7.5. 150 mM NaCl, 2 mM BME, 5 mM Phenyl phosphate
Biacore buffer	10 mM HEPES pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20

Biacore regeneration buffer	50 mM NaOH, 1 M NaCl
TE buffer	10mM Tris-Cl pH 8.0, 1mM EDTA
1X transfer buffer	20 mM Tris-Cl, 200 mM glycine, 10% methanol
Nickel Buffer A	20%(v/v) glycerol, 20mM Tris-Cl pH 8.0, 500mM NaCl, 5mM imidazole, 2mM TCEP
Nickel Buffer B	20% (v/v) glycerol, 20 mM Tris-Cl pH 8.0, 500 mM NaCl, 500 mM imidazole, 2 mM TCEP
2X reducing buffer	4% SDS, 20% glycerol, 200mM DTT, 0.01% bromphenol blue and 0.1 M Tris-HCl, pH 6.8
Blocking buffer	TBST, 5% w/v skim milk powder
NMR buffer	20 nM Tris-Cl pH 7.5, 2 mM TCEP, 100 mM NaCl, 0.01% azide, 20% v/v D ₂ O
Thermostability assay buffer	100 mM NaCl, 20 mM Tris-Cl pH 8.0, 2 mM TCEP
2% Nickel Buffer	98% (v/v) Nickel Buffer A, 2% (v/v) Nickel Buffer B
7% Nickel Buffer	93% (v/v) Nickel Buffer A, 7% (v/v) Nickel Buffer B

2.22.6 Protein constructs

All protein constructs used in this thesis are listed in **Tables 2.10 and 2.11**.

Table 2.10 Recombinant Protein Constructs.

Construct	Protein (Uniprot ref)	Residues
WT PTP1B phosphatase domain	P18031	2-321
PTP1B D/Q 2-298	P18031	2-298
PTP1B D/Q 2-321	P18031	2-321
PTP1B D/Q/C 2-321	P18031	2-321
LNK SH2 domain	O09039	324-446
LNK SH2 E372K	O09039	324-446
LNK SH2 V374M	O09039	324-446
LNK SH2 R387H	O09039	324-446
LNK SH2 R387C	O09039	324-446

JAK1 kinase domain	P23458	862-1154
JAK2 kinase domain	O60674	836-1132
JAK3 kinase domain	P52333	807-1124
TYK2 kinase domain	Q9R117	875-1183
Full Length JAK1	P23458	26-1154

Table 2.11 Mammalian Cell Protein Constructs.

Construct	Protein (Uniprot ref)	Residues
<i>Lentivirus</i>		
LNK	O09039	1-548
APS	Q9JID9	1-632
SH2B	Q91ZM2	1-756
<i>Retrovirus</i>		
TC-PTP	Q06180	1-406
CD45	P08575	1-1306
SHP2	P35235	1-593
SHP1.1	P29350-1	1-595
SHP1.2	P29350-3	1-597
PTP1B	P18031	1-435

2.22.7 Quikchange mutagenesis primers

Primers used for Quikchange mutagenesis are listed in **Table 2.12**.

Table 2.12 Primers for Quikchange mutagenesis.

Primer	Sequence
C215A F	gggcccgttggtgcacgccagtcaggcatcggcaggtct
C215A R	cctgccgatgcctgcactggcgtgcaccacaacgggcccgtg

Chapter 3 PTP1B Substrate Specificity and Dephosphorylation

3.1 Introduction

Reversible tyrosine phosphorylation is an essential form of post-translational modification in many signal transduction pathways (Graves and Krebs 1999). In the JAK-STAT signalling pathway, the JAK proteins phosphorylate specific tyrosine residues on target proteins, thereby activating the signalling cascade. For this reason, the JAKs can be considered the “writers” of the pathway. Proteins containing SH2 domains, such as STATs, then “read” these phosphotyrosine marks and coordinate a biological response. The STAT proteins are also phosphorylation targets of the JAKs. Phosphorylation of the STATs induces their activation, which ultimately mediates changes in gene expression. Working in opposition to the JAKs are the phosphatases. Phosphatases remove phosphate groups from tyrosine residues, returning activated proteins to their inactive state. These phosphatases can be thought of as the “erasers” of the pathway. The balance of kinase and phosphatase activity maintains cellular homeostasis (**Figure 3.1**). Six phosphatases have been implicated in the regulation of JAK-STAT signalling, including the prototypical tyrosine phosphatase, PTP1B.

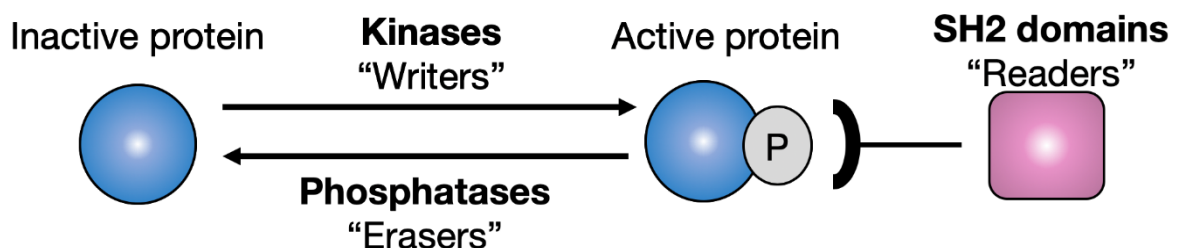


Figure 3.1 Readers, writers and erasers.

Kinases act as ‘writers’ by adding phosphate groups onto the tyrosine residues of proteins. SH2-containing proteins are ‘readers’ because they recognise and bind to phosphotyrosine-based motifs. Phosphatases act as ‘erasers’ by removing phosphate groups from tyrosine residues.

PTP1B is a negative regulator of JAK-STAT signalling, and specifically has been shown to interact with JAK2, TYK2 (Myers et al. 2001), STAT5a/b (Aoki and Matsuda 2000), and STAT6 (Lu et al. 2008), though the extent of PTP1B dephosphorylation of each target in cells is unclear. What is clear however is that the insulin receptor kinase and JAK2 are the main physiological targets of PTP1B *in vivo* as evidenced by PTP1B-deficient mice being

hypersensitive to insulin and resistant to obesity (Zabolotny et al. 2002; Elchebly et al. 1999). The mechanism of substrate recognition by the PTP1B phosphatase domain has been studied in most detail for the insulin receptor kinase (IRK), and the structure of the PTP1B phosphatase domain in complex with the tandemly phosphorylated activation loop of IRK was solved in 2000 (Salmeen et al. 2000). This structure revealed that the first (N-terminal) phosphotyrosine of the activation loop occupies the catalytic pocket of PTP1B, while the second phosphotyrosine, immediately downstream, crosses a gateway region and interacts with a positively charged patch on the surface of PTP1B referred to as the second aryl binding site (Salmeen et al. 2000; Barr et al. 2009). This interaction provides high specificity between the PTP1B phosphatase domain and tandemly phosphorylated substrates (X-pY-pY-X) such as the IRK activation loop. The kinase domains of JAK and IRK proteins have high sequence homology and similar mechanisms of action within the cell. Despite elucidating PTP1B's regulation of IRK in insulin signalling, much less is known about how PTP1B regulates the JAK proteins. As the JAK proteins also contain a tandemly phosphorylated activation loop, it has been proposed that PTP1B can dephosphorylate the activation loop of the JAKs (Zabolotny et al. 2002), however this remains to be demonstrated. A detailed analysis of the dephosphorylation of the JAK proteins by PTP1B would allow for a better understanding of the regulation of pathways induced by cytokines such as leptin (Zabolotny et al. 2002) , growth hormone (Gu et al. 2003) and IL-10 (Pike et al. 2014).

The aim of this chapter was to understand how PTP1B interacts with the JAK activation loop to negatively regulate JAK activity. This chapter presents the crystal structure of PTP1B in complex with peptides corresponding to the JAK activation loops, and details dephosphorylation differences between JAK2 and IRK. Further biochemical analyses reveal how JAK activation loop accessibility mediates the interaction with PTP1B and subsequent dephosphorylation.

3.2 PTP1B negatively regulates IL-6 signalling in the M1 cell line

The M1 cell line is a murine myeloid leukemic cell line that responds in a dose-dependent manner to IL-6 (Starr et al. 1997). Stimulation of M1 cells with IL-6 results in differentiation from monocytic-like progenitor cells into macrophages. When cultured in semi-solid media in the absence of IL-6, the M1 cells form tight bundles of colonies that arise from single cells, whereas increasing concentrations of IL-6 induce macrophage differentiation and dispersal through the culture medium. This differentiation occurs due to STAT3 activation upon IL-6 stimulation, making the M1 cell line ideal for studying proteins that may be involved in the regulation of JAK-STAT signalling (Starr et al. 1997; Richardson et al. 2002).

Six phosphatases regulate JAK-STAT signalling: PTP1B (Myers et al. 2001), PTPRT (Zhang et al. 2007), TC-PTP (Yamamoto et al. 2002), CD45 (Irie-Sasaki et al. 2001), SHP2 (Li et al. 2011) and SHP1 (Bone et al. 1997), and their splice variants. Five of these phosphatases were constitutively over-expressed in the M1 cell line to determine their ability to block macrophage differentiation upon IL-6 stimulation. M1 over-expression lines were plated in semi-solid media supplemented with increasing doses of IL-6, and colony differentiation was scored after 7 days (**Figure 3.2A**). Regulation of IL-6 signalling in M1 cells by a phosphatase is indicated by increased effective concentration at which half the maximal response to cytokine is observed (EC_{50}). The EC_{50} of IL-6 for the WT M1 parental cells is approximately 2 ng/mL. A similar EC_{50} was detected with SHP2, SHP1, CD45 and TC-PTP over-expression. In contrast, PTP1B over-expression required higher concentrations of IL-6 to differentiate into macrophages, with an EC_{50} of approximately 10 ng/mL (**Figure 3.2B**). This shift in responsiveness to IL-6 indicates PTP1B can inhibit IL-6 dependent differentiation of M1 cells.

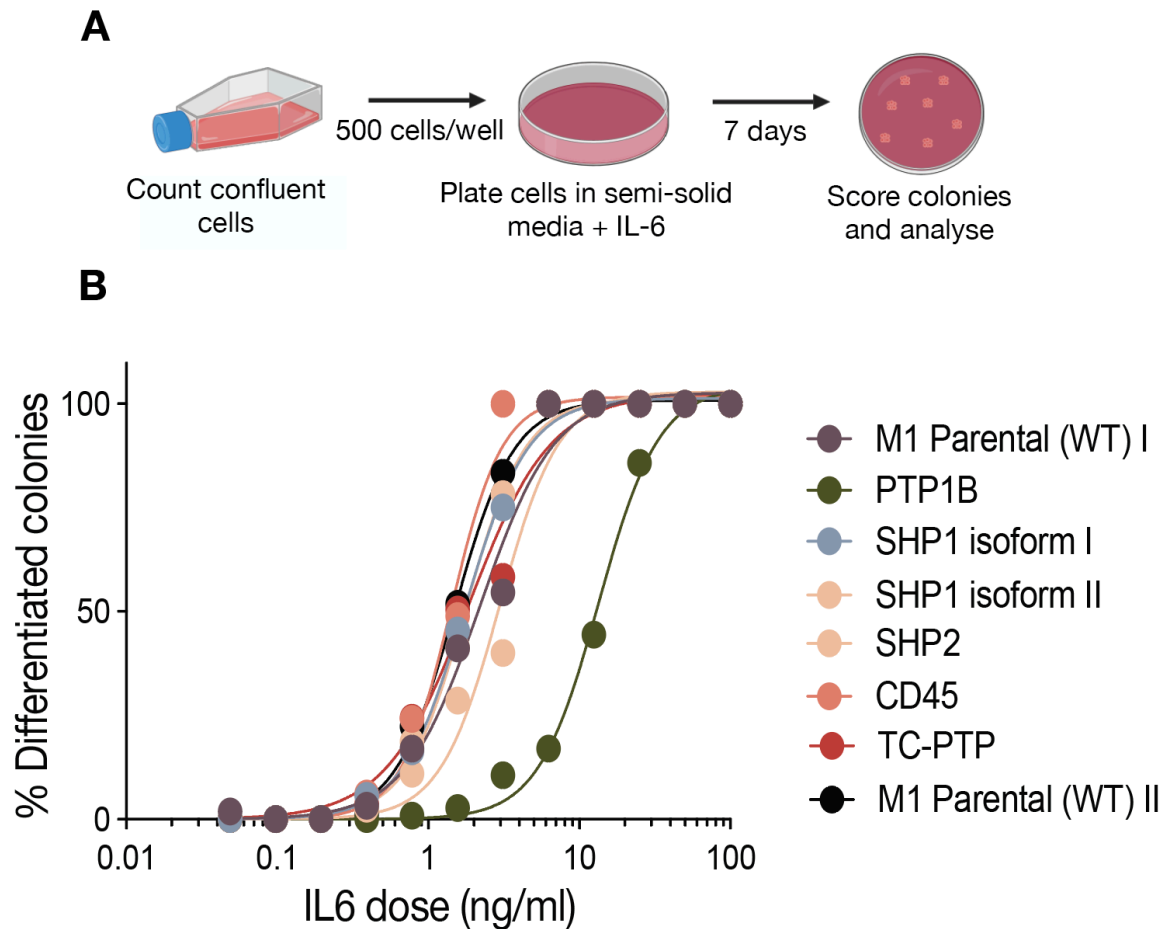


Figure 3.2 PTP1B causes a decrease in M1 cell response to IL-6.

A) Schematic of semi-solid media assay. 500 M1 cells were plated in semi-solid media containing a range of IL-6 concentrations and scored 7 days later for differentiated colonies.

B) IL-6 dose response curves for phosphatases overexpressed in the M1 cell line. The effective concentration at which half the maximum response is observed (EC_{50}) for the WT M1 cell line is approximately 2 ng/mL. Overexpression of PTP1B in the M1 cells caused a decrease in response to IL-6, with an EC_{50} of approximately 10 ng/mL. Overexpression of all other phosphatases led to a similar EC_{50} to the WT M1 cell line.

3.3 PTP1B dephosphorylates the JAK activation loop

The ability of PTP1B expression to decrease IL-6 induced M1 cell differentiation implies that PTP1B dephosphorylates one or more IL-6 pathway components. Prior work suggested that PTP1B dephosphorylates both JAK (Zabolotny et al. 2002; Myers et al. 2001) and STAT proteins (Aoki and Matsuda 2000; Lu et al. 2008). As PTP1B is known to interact with and dephosphorylate the IRK activation loop (Kuperman et al. 1998), it was hypothesised that the JAK activation loop is another PTP1B substrate.

PTP1B-mediated dephosphorylation of the activation loop in the four JAK kinases (JAK1, JAK2, JAK3, TYK2) was analyzed by dephosphorylation assay. 900 nM PTP1B phosphatase domain was mixed with 10 μ M recombinant activated (phosphorylated) JAK kinase domain and incubated for 4 hours with regular sampling. Samples were subsequently separated by SDS-PAGE, transferred to a membrane and probed by an antibody specific to the phosphorylated JAK activation loop (P-JAK). The high sequence homology of the JAKs and antibody cross-reactivity allowed use of the same P-JAK antibody for all samples. All four JAK activation loops were dephosphorylated in a PTP1B-dependent manner, with most of the phosphorylation signal undetectable by two hours (**Figure 3.3**). JAK1 was dephosphorylated to a lesser extent than JAK2, JAK3 and TYK2 at all time points, suggesting that the JAK1 activation loop is a poorer substrate than the other JAKs. These results indicate that the activation loop of the JAK proteins is a substrate for PTP1B *in vitro*, representing a possible mechanism by which PTP1B regulates JAK activity.

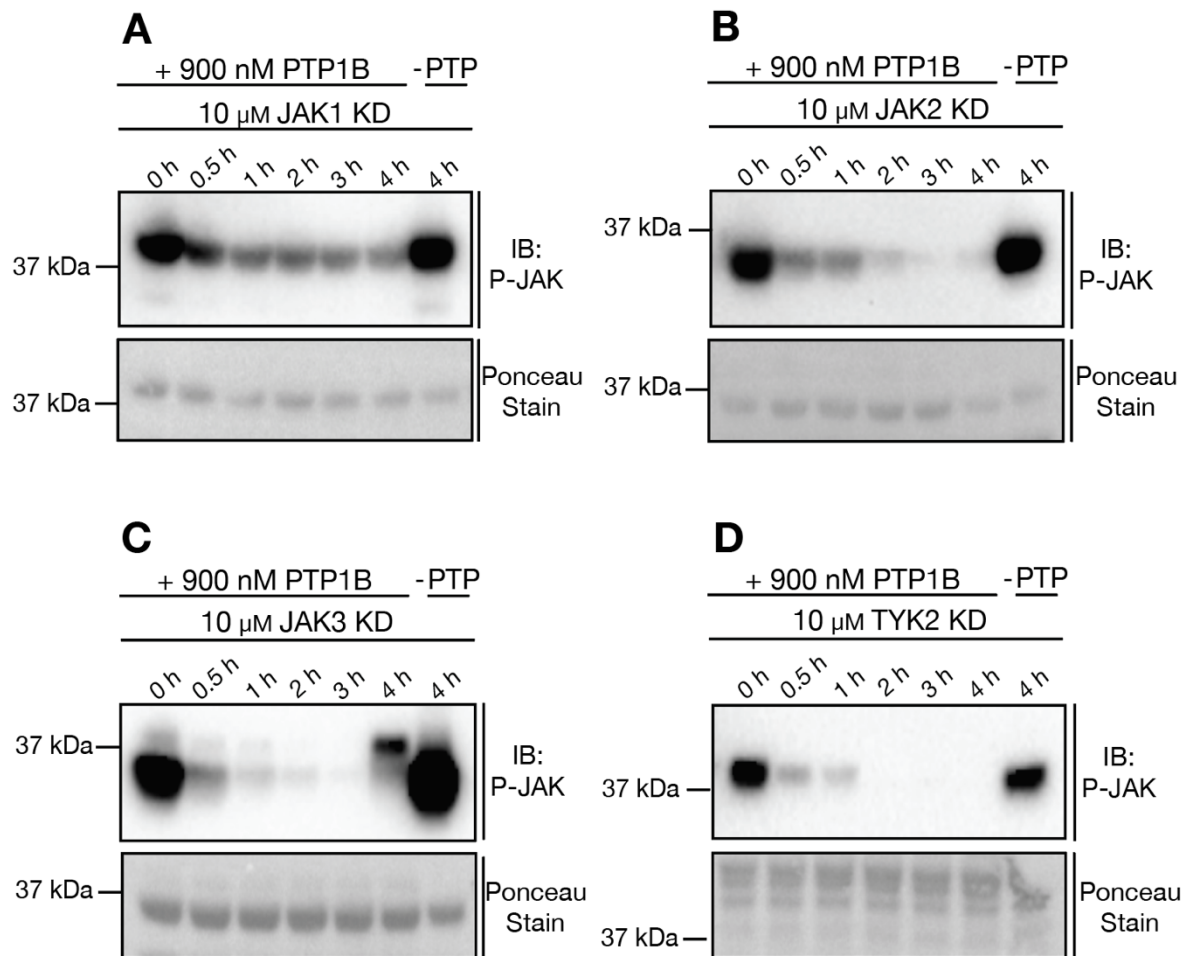


Figure 3.3 PTP1B can dephosphorylate all four JAK kinase domains.

Dephosphorylation of the four JAK kinases; **A)** JAK1, **B)** JAK2, **C)** JAK3 and **D)** TYK2 in a PTP1B-dependent manner. -PTP indicates sample where no PTP1B was added. (Top) Immunoblot probing for phosphorylated-JAK activation loop. (Bottom) Ponceau stained membrane to confirm equal input of proteins on gel. Data is representative of 3 independent experiments.

3.4 Substrate trapping mutants of PTP1B

Having determined that the JAK activation loop is a PTP1B substrate, I next investigated the interaction mechanism/interface. To date, no structures of PTP1B in complex with another protein have been solved, and so there is little information about how PTP1B associates with a substrate protein for dephosphorylation.

Studying the interaction between the PTP1B phosphatase domain and the JAK kinase domain requires a form of PTP1B where the substrate affinity remains similar, but the catalytic properties have been significantly reduced or abolished. PTP1B has three key catalytic residues; Aspartate 181, Glutamine 262 and Cysteine 215, and previous work has shown that mutation of any number of these residues can give rise to catalytically inactive or partially active forms of the protein while retaining their substrate-binding ability (Flint et al. 1997; Xie, Zhang, and Zhang 2002; Boubekour et al. 2011; Zhang, Yao, et al. 2000). These altered versions of PTP1B have been termed substrate trapping mutants. Two different length substrate trapping mutants of the PTP1B phosphatase domain were expressed to study the interaction between PTP1B and the JAK kinase domain (residues 2-298 and 2-231). Both constructs contained alanine replacements of the Asp 181 and Gln 262 catalytic residues, (Xie, Zhang, and Zhang 2002). Mutated proteins were expressed using a Tuner (DE3) *E. coli* expression system, then purified first by nickel affinity chromatography, exploiting the PTP1B N-terminus His-tag, followed by Superdex S200 16/60 size exclusion chromatography (**Figure 3.4**). See section 2.9 for purification method details.

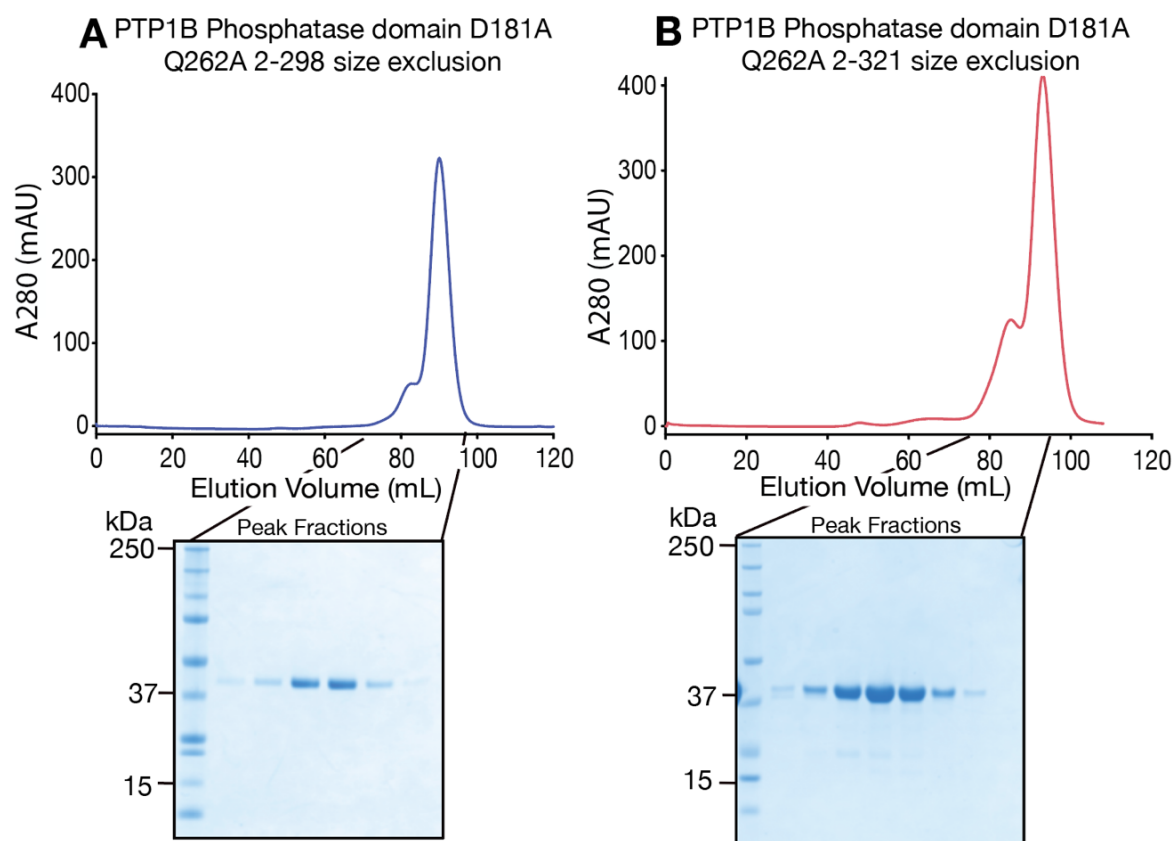


Figure 3.4 Purification of PTP1B DQ phosphatase domains.

Representative chromatographs from A) PTP1B D181A/Q262A 2-298 size exclusion chromatography purification and corresponding coomassie stained SDS-PAGE gel of peak fractions B) PTP1B D181A/Q262A 2-321 size exclusion purification and corresponding coomassie stained SDS-PAGE gel of peak fractions. Both the 2-298 and 2-321 DQ PTP1B constructs are approximately 38 kDa and eluted at ca. 90 mL.

3.4.1 The PTP1B D181A/Q262A mutant has reduced catalytic activity

Reduced catalytic activity of PTP1B D181A/Q262A mutant (hereafter referred to as PTP1B DQ) relative to WT PTP1B was confirmed in dephosphorylation assays. 0 nM, 100 nM, 500 nM and 2000 nM of PTP1B phosphatase domain were mixed with 10 μ M activated JAK kinase domain and incubated for 30 minutes at 27°C. Samples were separated by SDS-PAGE gel, transferred to a membrane and probed with P-JAK antibody corresponding to the activation loop to determine remaining levels of phosphorylation in the presence of PTP1B (**Figure 3.5**).

Increasing concentrations of the WT PTP1B phosphatase domain led to a decrease in phosphorylated JAK1 kinase domain, with approximately 80% of JAK1 activation loop dephosphorylated after 30 minutes of treatment with 2000 nM PTP1B. Conversely, increasing concentrations of the DQ mutant had no observable effect on JAK1 kinase domain phosphorylation levels, indicating this mutant has significantly reduced enzymatic activity, consistent with the literature (Xie, Zhang, and Zhang 2002).

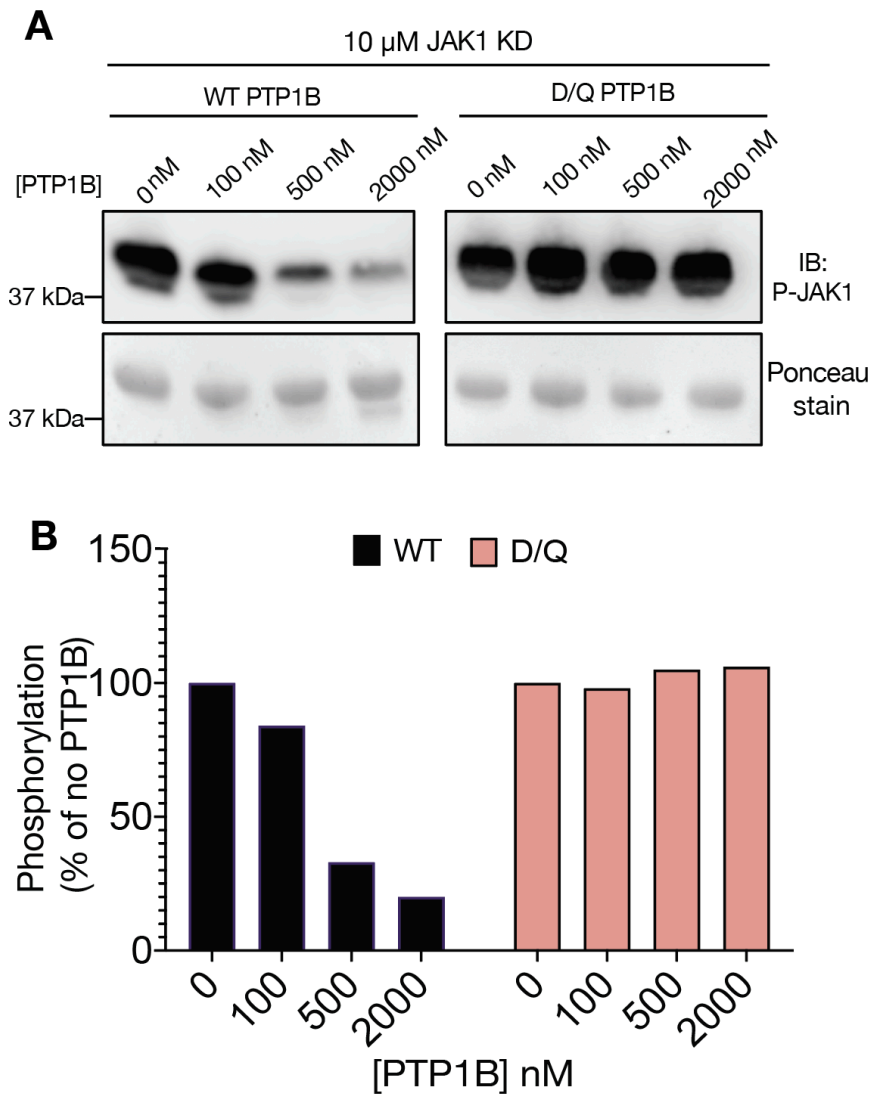


Figure 3.5 PTP1B DQ cannot dephosphorylate JAK.

A) (Top) Immunoblot of phospho-JAK1 activation loop to determine dephosphorylation levels. Increasing concentrations of WT PTP1B corresponded to an increasing loss of phosphorylated JAK1 kinase domain, whereas increasing concentrations of the DQ mutant (residues 2-321) had no effect on P-JAK1 signal. (Bottom) Ponceau stained blots to indicate equal loading in each sample. **B)** Quantification of JAK1 phosphorylation normalised to the sample in the absence of PTP1B.

Generating a PTP1B-JAK kinase domain complex

Having determined that the PTP1B DQ mutant had reduced catalytic activity, it was used in an attempt to isolate a complex between PTP1B and the JAK kinase domain to investigate their interaction using structural techniques. These data would provide information about how the PTP1B phosphatase domain and the JAK kinase domain associate for the dephosphorylation reaction to occur.

To generate a stable complex between PTP1B DQ and JAK kinase domain, equimolar amounts of each domain were mixed together and separated by size exclusion chromatography using an S200 10/30 column (**Figure 3.6A**). Peak fractions were analysed by SDS-PAGE to determine if stable complexes were being formed. It is expected that a complex of the two proteins would elute prior to the monomeric proteins. As shown in **Figure 3.6B**, the elution profile for the mixture of PTP1B DQ 2-298 and JAK kinase domains overlays with PTP1B alone and JAK kinase domain alone (with the exception of an additional peak in the void volume due to some protein aggregation). This indicates that PTP1B and JAK kinase domain were unable form a complex that was stable throughout the course of gel filtration. Despite attempts with numerous protein constructs (both of JAK and PTP1B) and techniques (gel filtration and anion exchange), no stable complex was formed under any conditions tested.

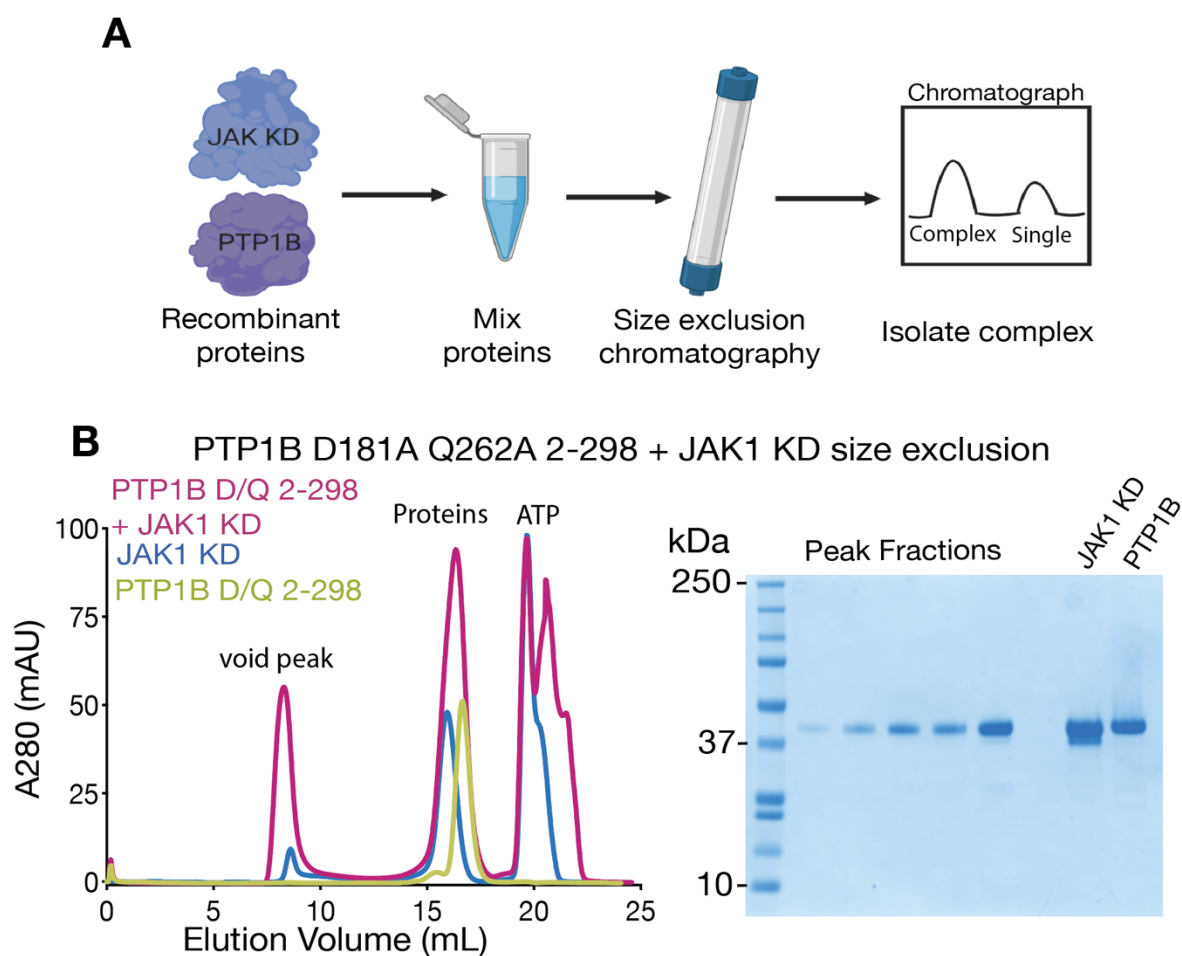


Figure 3.6 Gel filtration of PTP1B and JAK kinase domains in an attempt to isolate a complex.

A) Schematic diagram of gel filtration experiments. JAK kinase domains were mixed with PTP1B DQ phosphatase domain and injected onto an S200 10/30 size exclusion column or anion exchange column to isolate a PTP1B-JAK complex. **B)** A mixture of PTP1B DQ (2-298) and JAK1 kinase domain (purple) eluted at the same volume as each protein individually (PTP1B, yellow; JAK kinase domain, blue).

3.5 Structures of a complex between PTP1B and the JAK activation loop

Given that it was not possible to generate and isolate a complex between PTP1B and a JAK kinase domain by size exclusion or ion exchange chromatography, alternate techniques were employed to study this interaction. Previous work has employed the use of phosphopeptides corresponding to the IRK activation loop (Salmeen et al. 2000) to understand how PTP1B interacts with IRK. Therefore, in a similar manner, phosphopeptides corresponding to the activation loops of all four JAK (JAK1,2,3, TYK2) were designed and used for crystallographic studies. A pre-crystallisation test (Watson and O'Callaghan 2005) (Hampton) determined that the appropriate PTP1B concentration for crystallisation was approximately 10 mg/mL. The DQ 2-321 mutant was used for crystallisation as this construct is a more accurate representation of the full phosphatase domain. Crystal trays were set up using 10 mg/mL of the 2-321 DQ mutant and a two-fold molar excess of the JAK2 and TYK2 activation loop peptides. Crystallisation trays were set up robotically at the Collaborative Crystallisation Centre (C3) at The Commonwealth Scientific and Industrial Research Organisation (CSIRO) in 96 well sitting drop vapour diffusion plates with 150 nL protein solution and 150 nL precipitant over a 50 μ L reservoir volume.

Initial screening of the PTP1B DQ mutant with the JAK2 and TYK2 activation loop peptides in 768 crystallisation conditions produced crystals in 10 conditions. The largest crystals grew to a size of approximately 50x50x200 μ m in 100 mM MES pH 6.5, 12% (w/v) PEG 8000, 200 mM MgAc or 50 mM MES pH 6.5, 12.5% (w/v) PEG 8000. Crystals were confirmed to be protein by UV fluorescence (**Figure 3.7A**). Crystal trays for PTP1B DQ 2-321 with the JAK1 and JAK3 activation loop peptides were then manually set up in hanging drop vapour diffusion plates using a 1 μ L- to-1 μ L ratio of protein to precipitant, over 450 μ L of reservoir solution. The concentrations of the PEG 8000 and MgAc or CaAc were varied for the aforementioned condition, and crystals grew in multiple drops. Crystals of interest were flash-frozen in liquid nitrogen, using mother liquor containing an additional 20-25% (v/v) ethylene glycol as cryoprotectant. Diffraction data for all four complexes were collected on the MX2 beamline at the Australian Synchrotron (**Figure**

3.7B-E). Data were processed in XDS with a resolution cut off of 2.3 Å for JAK1, 2.2 Å for JAK2, 2.35 Å for JAK3 and 2.15 Å for TYK2.

For the PTP1B DQ + JAK1 and PTP1B DQ + TYK2 datasets, we employed a $CC_{1/2}$ based cut-off allowing the inclusion more data in the final models when compared to using R_{meas} or I/σ based cut offs as was used for the other data sets. I/σ is a measure of the signal to noise ratio for reflections within a resolution shell and data are usually truncated as the I/σ drops below 2.0. R_{meas} describes the precision of individual measurements, independent of their multiplicity and high quality data sets typically have an overall R_{meas} around 5%. Using $CC_{1/2}$, the data are split in half at random, and Pearson's correlation coefficient is determined, and a Student's t-test can determine where significance ends and where it is appropriate to cut the data. A $CC_{1/2}$ correlation of 10% is generally considered statistically significant and can improve the model quality (Karplus and Diederichs 2012). All four datasets had a completeness of 97% or higher. Full statistics are detailed in **Table 3.1**.

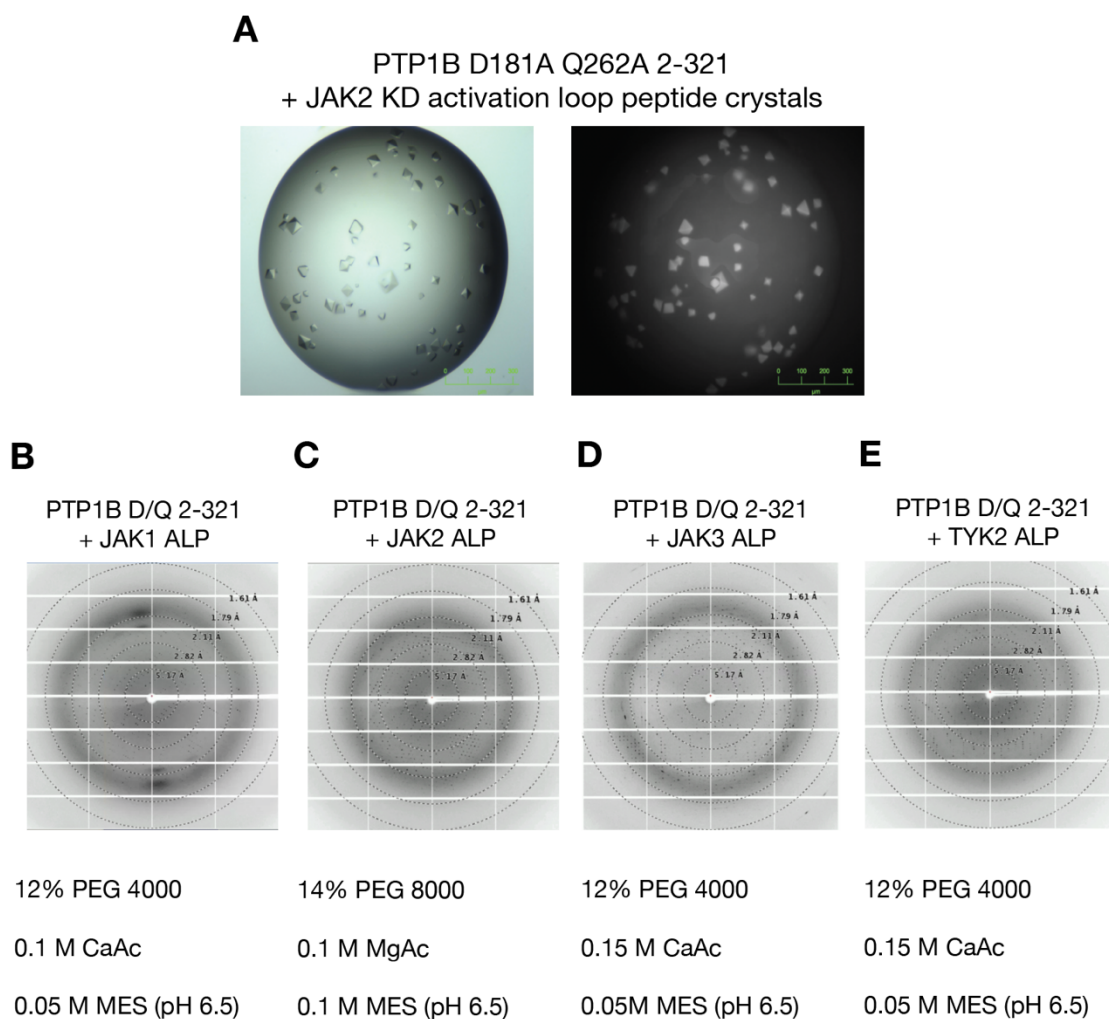


Figure 3.7 Crystals of PTP1B DQ 2-321 with peptides bound.

Crystals of PTP1B D181A/Q262A 2-321 with JAK activation loop phosphopeptides. A) Protein crystals generated at C3 from of PTP1B D181A/Q262A 2-321 and the JAK2 activation loop phosphopeptide in bright-field (left) and UV (right) light. B-E) Representative diffraction patterns of PTP1B DQ 2-321 with the JAK1, JAK2, JAK3 and TYK2 activation loop peptides. Crystallization condition are indicated below diffraction patterns.

Table 3.1 Data collection and refinement statistics for PTP1B D181A/Q262A 2-321 with JAK activation loop peptides.

	PTP1B DQ + JAK1 pYpY	PTP1B DQ + JAK2 pYpY	PTP1B DQ + JAK3 pYpY	PTP1B DQ + TYK2 pYpY
Data Collection				
Space group	P 4 ₃ 2 ₁ 2	P 4 ₃ 2 ₁ 2	P 4 ₃ 2 ₁ 2	P 4 ₃ 2 ₁ 2
Cell dimensions				
a, b, c (Å)	88.57 88.57 132.43	88.48 88.48	88.59 88.59	88.32 88.32
α, β, γ (°)	90 90 90	130.41	132.16	131.23
R _{meas} (%)	16.12 (471)*	90 90 90	90 90 90	90 90 90
I / sigma(I)	9.56 (0.27)	5.93 (84.21)	5.36 (56.53)	9.82 (318)*
CC _{1/2} (%)	99.8 (11)*	21.46 (2.14)	27.61 (3.21)	11.38 (0.76)
Completeness (%)	97.33 (73.58)	99.9 (83)	100 (91)	99.8 (27)*
Redundancy	97.33 (73.58)	99.77 (98.72)	99.49 (95.84)	99.54 (97.94)
	8.3 (5.2)	7.4 (7.2)	11.4 (8.4)	7.3 (6.4)
Refinement				
Resolution (Å)	45.5 - 2.30	44.25 - 2.09	39.62 - 2.35	45.24 - 2.15
	(2.38 - 2.30)	(2.17 - 2.09)	(2.43 - 2.35)	(2.23 - 2.15)
No. reflections	23449 (1743)	26961 (2623)	22507 (2118)	28847 (2809)
R-work (%)**	20.66 (41.52)	21.00 (28.36)	20.61 (26.29)	21.69 (38.05)
R-free (%)**	23.86 (43.63)	25.02 (30.49)	24.76 (30.08)	24.04 (38.13)
No. atoms				
protein	2426	2412	2393	2408
ligand	21	5	13	21
solvent	53	70	86	74
B factors				
protein	69.06	51.44	61.62	64.31
ligand	81.12	54.30	64.76	72.21
solvent	62.18	47.33	58.01	61.04
r.m.s. deviations				
RMS(bonds)	0.008	0.007	0.007	0.006
RMS(angles)	0.89	0.83	0.78	0.79
Ramachandran favored (%)	93.36	95.64	92.62	94.24
Ramachandran allowed (%)	6.31	3.36	7.05	5.42
Ramachandran outliers (%)	0.33	1.01	0.34	0.34
Rotamer outliers (%)	4.28	1.95	2.78	5.04

Values in parentheses are for highest-resolution shell. *Criteria for data cut-off were from (Karplus and Diederichs 2012) using the split-half correlation coefficient (CC_{1/2}).

** Data are for structures where second tyrosine occupies the catalytic pocket.

For the remainder of this chapter, the position of the residues comprising the JAK activation loop peptides will be referred to as in the +/- (plus or minus) positions relative to the phosphotyrosine occupying the catalytic pocket. **Figure 3.8** uses the JAK2 activation loop as an example of this nomenclature.

Amino acid position	-2	-1	pTyr in catalytic pocket (CP)	+1	+2
JAK2 pTyr 1 in CP	Lys	Glu	pTyr	pTyr	Lys
JAK2 pTyr 2 in CP	Glu	pTyr	pTyr	Lys	Val

Figure 3.8 Plus (+) and minus (-) residues in the PTP1B binding site.

The phosphotyrosine in the catalytic pocket defines the + and – residues for all peptides. For the JAK2 activation loop peptide, when the first tyrosine is in the catalytic pocket, the +1 residue will be the second pTyr, and the -1 the glutamate. When the second phosphotyrosine is in the catalytic pocket, the -1 residue will be the first phosphotyrosine, and the +1 residue will be the lysine.

For all four datasets, Apo PTP1B phosphatase domain (PDB ID: 1SUG) was used as a search model in PHASER (McCoy et al. 2007) and a solution was found in the space group $P4_3 2_1 2$ with one chain in the asymmetric unit. Models were built using COOT (Emsley and Cowtan 2004) and refined in PHENIX (Adams et al. 2010).

Overall, the structures of PTP1B DQ 2-321 bound to each of the JAK activation loop peptides are very similar, with a root-mean-square deviation (RMSD) of atomic positions between 0.2 and 0.3 Å over 298 residues (**Figure 3.9**). The overall RMSD was determined using DALI (Holm and Sander 1995). For all four structures, residues 2-299 could be resolved, however the final 22 residues (300-321) were not visible.

Although the PTP1B DQ mutant has significantly reduced catalytic activity in comparison to WT PTP1B (**Figure 3.5**), both tyrosine residues of all four JAK activation loop peptides had been dephosphorylated. For each structure, a phosphate molecule was present in the

catalytic pocket, which was modelled at an occupancy of approximately 70% in all cases. This implies that not all copies of the protein within the crystal contained a phosphate group in the catalytic pocket (**Figure 3.9C**). Several waters are often found in the catalytic pocket of PTP1B and substrate binding leads to their expulsion. Initially we thought the phosphate in the catalytic pocket could instead be one of these waters, however replacement of the phosphate with water resulted in an observed residual peak of 8.84 sigma in the Fo-Fc map (See appendix IV) indicating this density was more accurately represented by the phosphate. It should be noted that in all of these structures, the phosphate in the catalytic pocket is not covalently bound to the active site cysteine, indicating that both steps of the dephosphorylation reaction had occurred (see Figure 1.9 of introduction). While the density indicates the phosphate is likely not in a fixed conformation within the catalytic pocket, for the purpose of this thesis several interactions between PTP1B and the phosphate group have been shown in **Figures 3.9D and E** to highlight the position of the free phosphate in the context of surrounding residues. Based on these structures it is not possible to tell whether the dephosphorylation of the peptides occurred prior to or after crystal formation, and it was not possible to determine which register the peptide was in. For purposes of **Figures 3.9C, D and E**, the second phosphotyrosine was modelled in the catalytic pocket, however as seen in the omit map (**Figure 3.9F and G**) it was unclear as to which tyrosine occupied the catalytic pocket. This may suggest that a mixture of both the first and second tyrosine can occupy the catalytic pocket. This was true for all four DQ structures.

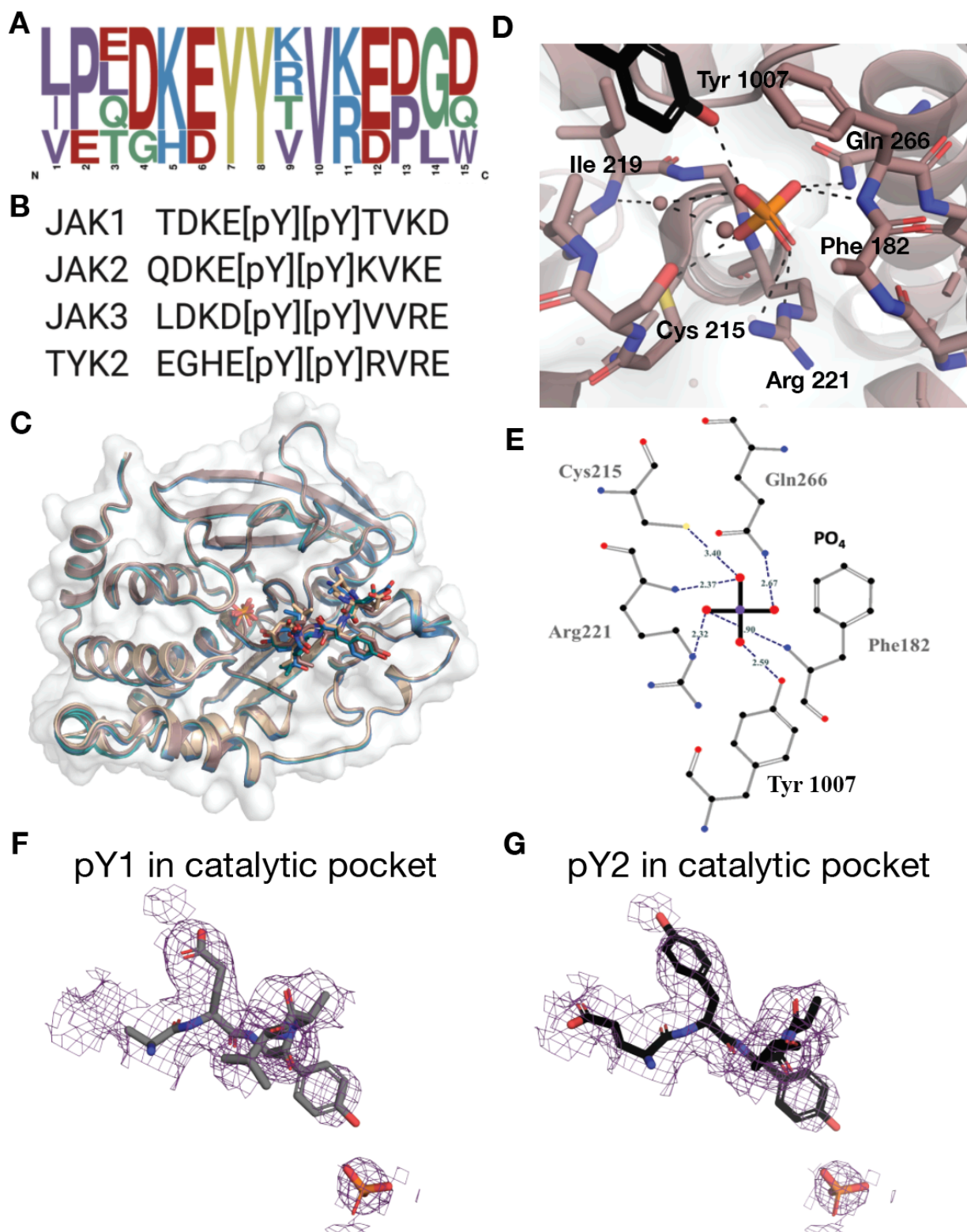


Figure 3.9 Structures of PTP1B DQ 2-321 mutant with all four JAK activation loop peptides bound.

A) Sequence logo (Crooks et al. 2004) of the four JAK activation loops, highlighting the most common amino acids at each position. Negatively charged residues in red, positively charged residues in blue, hydrophobic residues in yellow, uncharged residues in purple, tyrosine residues in pink and others in green. **B)** Sequences of each individual JAK activation loop. **C)** Overlay of all four structures of the PTP1B D181A/Q262A 2-321 mutants with JAK1, JAK2, JAK3 and TYK2 peptide bound in the catalytic pocket. **D)** Interactions between the PTP1B phosphatase domain, JAK2 activation loop peptide and free phosphate. The free phosphate can be coordinated by the side chains of Cys 215, Arg 221, Gln 266, Tyr 1007 of the peptide and the backbone amide of Phe 182 and Ile 219 through a water molecule. **E)** 2D representation of the interactions between PTP1B and the free phosphate from the JAK2 activation loop peptide. Interactions are indicated by dashed lines, with the distance between the residues in angstroms indicated. Dotted lines represent hydrogen bonds or electrostatic interactions. Visualized using LigPlot+ (Laskowski and Swindells 2011). **F)** 2Fo-Fc composite omit map with simulated annealing, contoured to 1 σ for the JAK2 pYpY activation loop peptide with first tyrosine in catalytic pocket. **G)** Simulated annealing 2Fo-Fc composite omit map, contoured to 1 σ for the JAK2 pYpY activation loop peptide with second tyrosine in catalytic pocket.

3.6 The PTP1B D181A Q262A and C215A mutant

Despite not observing catalytic activity for PTP1B DQ 2-321 in the dephosphorylation assay (**Figure 3.5**), this mutant still hydrolysed the phosphate groups of JAK activation loop peptides, as shown in the crystal structures (**Figure 3.9**). This is presumably due to the remaining Cys 215 residue, allowing formation of the cysteinyl phosphate intermediate. Therefore, a second mutant was generated to remove this residual activity. In this PTP1B mutant, all three catalytic residues were mutated to alanines, including Cys 215. The C215A mutation was introduced into the PTP1B DQ 2-321 construct by QuikChange mutagenesis (Wang and Malcolm 2002) then expressed using a Tuner (DE3) *E. coli* expression system, as per the DQ mutants (see section 3.4) to yield purified protein. The DQC mutant eluted at approximately 90 mL and peak fractions were separated by SDS-PAGE gel and stained with coomassie (**Figure 3.10**). All fractions were pooled, concentrated and frozen for future experiments. This construct will be termed the PTP1B DQC construct hereafter.

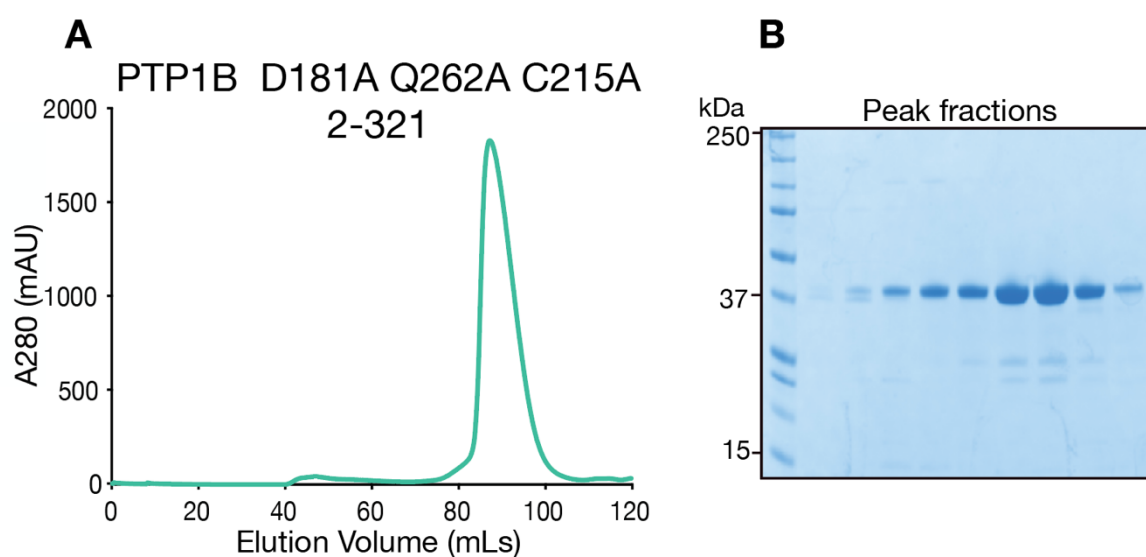


Figure 3.10 Purification of PTP1B DQC phosphatase domain.

A) Representative chromatograph from PTP1B D181A/Q262A/C215A 2-321 size exclusion purification. PTP1B 2-321 DQC eluted at 90 mL on a Superdex S200 16/60 column. PTP1B DQC 2-321 is approximately 38 kDa. **B)** Coomassie stained SDS-PAGE gel of PTP1B DQC from gel filtration fractions indicating correct size of protein at approximately 38 kDa and relative purity.

3.6.1 Structures of the PTP1B DQC mutant and the JAK2 activation loop peptide

Although previous experiments showed that PTP1B is able to dephosphorylate all four JAK proteins and the sequences are similar (**Figure 3.9B and C**), it has been reported that JAK2 and TYK2 are the two family members dephosphorylated by PTP1B *in vivo* (Myers et al. 2001). For this reason, their corresponding activation loop phosphopeptides were used for all further crystallisation experiments. Crystal trays were set up robotically at the C3 at CSIRO as in section 1.5. Initial screening of 192 crystallisation conditions produced crystals in four conditions (**Figure 3.11**). Conditions from one drop were further manually optimised by ranging the MgCl_2 and PEG 3350 concentrations as in section 1.5.

Crystals of PTP1B DQC and the JAK2 activation loop peptide were cryo-protected in mother liquor containing 20% (v/v) ethylene glycol for cryoprotection, and flash-frozen in liquid nitrogen. Diffraction data were collected on the MX2 beamline at the Australian Synchrotron, and data were processed in XDS to a resolution cut off of 2.1 Å. Apo PTP1B phosphatase domain (PDB ID: 1SUG) was used as a search model in PHASER (McCoy et al. 2007) and a solution was found in the space group $P3_2 2 1$ with two copies in the asymmetric unit.

Crystals of PTP1B DQC and the TYK2 activation loop peptide crystals were cryoprotected with paratone and flash-frozen in liquid nitrogen. The structure of the PTP1B phosphatase domain with the peptide chain removed (PDB ID: 4ZRT) was used as a search model in PHASER (McCoy et al. 2007) and a solution was found in the space group $P2_1 2_1 2_1$ with one chain in the asymmetric unit.

Both models were built using COOT (Emsley and Cowtan 2004) and refined in PHENIX (Adams et al. 2010). Further statistics for both structures are listed in **Table 3.2**.

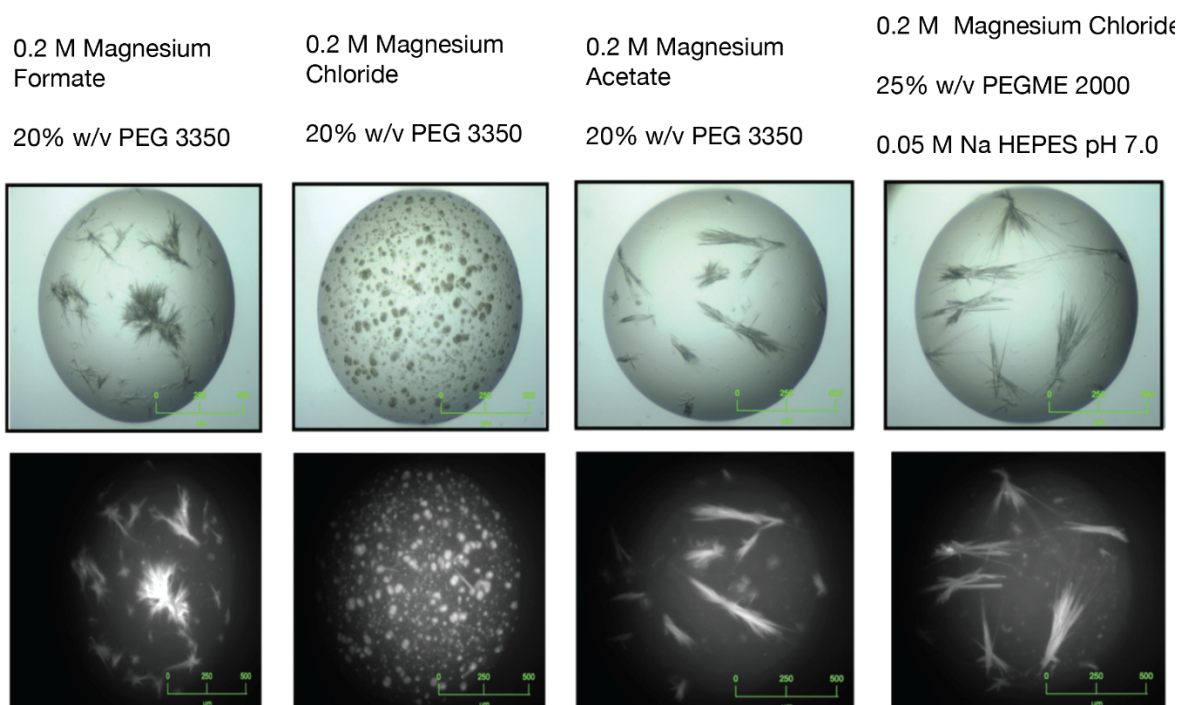


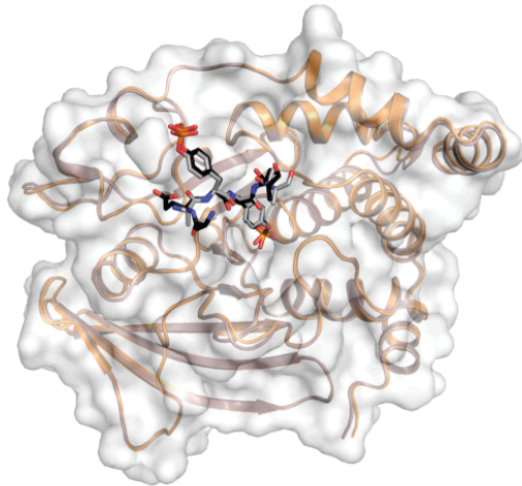
Figure 3.11 Crystals of PTP1B DQC 2-321 phosphatase domain with activation loop peptides bound.

(Top) Four conditions yielding crystals of PTP1B D181A/Q262A/C215A 2-321 with the JAK2 activation loop phosphopeptide. (Bottom) Images of crystals generated and UV fluorescence of each drop.

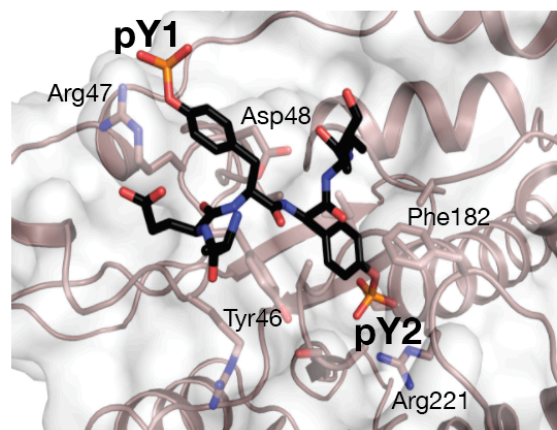
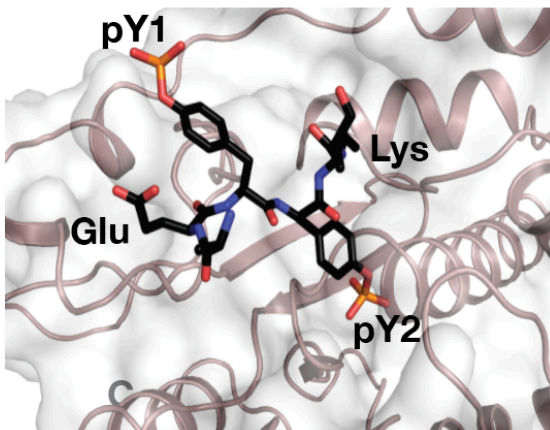
Few differences in the protein backbones are evident when overlaying the two structures (**Figure 3.12A**) with the overall RMSD for the two structures being 0.4 Å (DALI (Holm and Sander 1995)) over 298 residues. The structures of PTP1B bound to the JAK2 and TYK2 activation loop peptides revealed that no dephosphorylation had occurred. In both cases, the second phosphotyrosine occupies the catalytic pocket (pTyr 1008 for JAK2 and pTyr 1046 for TYK2), and the first (pTyr 1007 for JAK2 and pTyr 1045 for TYK2) occupies the -1 position. In both DQC structures, the aromatic ring of the second phosphotyrosine occupying the catalytic pocket is flanked by the aromatic side chains of Tyr 46 and Phe 182 of PTP1B, which define the catalytic pocket depth and substrate specificity for PTP1B (Flint et al. 1997) (**Figure 3.12B and C**). The bottom of the PTP1B catalytic site contains the residues of the PTP loop, comprising the conserved/signature PTP motif (I/V)HXXAGXXR(S/T)G. The phosphate group of the second phosphotyrosine residue is coordinated by the amide groups of these residues, which in PTP1B includes Ser 216, Ala 217, Gly 218, Ile 219, Gly 220 and Arg 221, and the side chain of Arg 221 in PTP1B (**Figure 3.12 and 3.13**), consistent with other structures of PTP1B (e.g. IRK activation loop peptides (Salmeen et al. 2000), EGFR peptide (Milarski et al. 1993)). Ala 217 and Ile 219 of PTP1B may also coordinate the tyrosine in the catalytic pocket through Van der Waals interactions.

In both the JAK2 and TYK2 activation loop structures, the first phosphotyrosine (pTyr 1007 of JAK2 and pTyr 1045 of TYK2) in the -1 position is coordinated by Arg 47 of PTP1B (**Figure 3.12 and Figure 3.13**). The Guanidinium group of Arg 47 forms a salt bridge with the phosphate group of the first phosphotyrosine of each activation loop, and the backbones of both residues form hydrogen bonds with one another. The sidechain of Asp 48 coordinates the backbone amide of the pTyr (1008 of JAK2 and 1046 of TYK2) and the +1 residue (Lys 1009 of JAK2 and Arg 1047 of TYK2). No other interactions between the activation loop peptides and the PTP1B phosphatase domain could be resolved from the data. A consistent observation for both structures is the preferential binding of the second phosphotyrosine in the catalytic pocket rather than the first. This differs from previously published work which indicated that PTP1B has a preference for the first phosphotyrosine of the IRK activation loop (Salmeen et al. 2000).

A
 JAK2 ALP: QDKE [pY] [pY] KVKE
 TYK2 ALP: EGHE [pY] [pY] RVRE



B PTP1B D181A Q262A C215A + JAK2 ALP



C PTP1B D181A Q262A C215A + TYK2 ALP

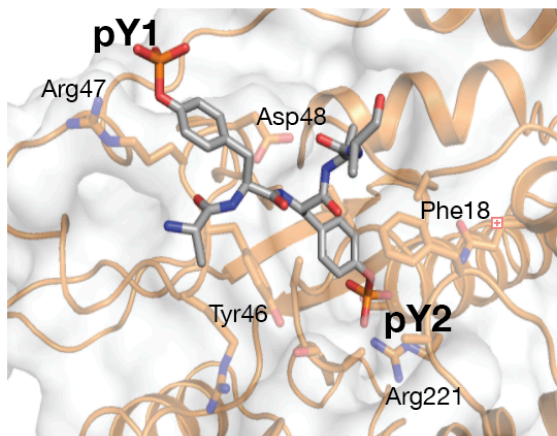
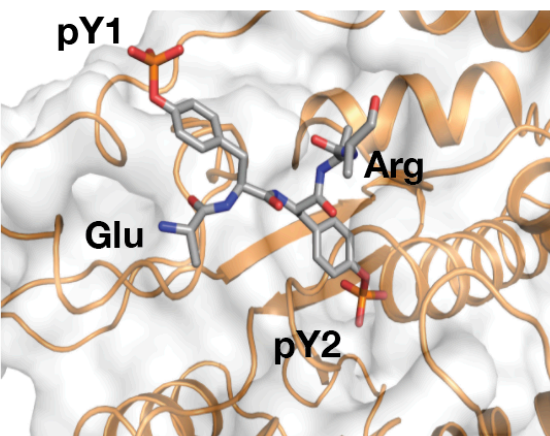


Figure 3.12 PTP1B DQC 2-321 phosphatase domain with JAK2 and TYK2 activation loop peptides bound.

A) PTP1B DQC 2-321 mutant bound to JAK2 (pink) and TYK2 (orange) activation loop phosphopeptides aligned (left), and separate (right). Both structures have the second phosphotyrosine in the catalytic pocket and the first in the -1 position. **B)** (Right) Active site of PTP1B with the JAK2 activation loop peptide bound. The second phosphotyrosine (pTyr 1007) is in the catalytic pocket. (Left) Nearby residues of PTP1B that contribute to activation loop peptide-binding are highlighted. The -2 Glu residue of the peptide is present, however the +1 Lysine side chain could not be resolved. **C)** (Right) Active site and of PTP1B with the TYK2 activation loop peptide bound. The second phosphotyrosine (pTyr 1046) is in the catalytic pocket. (Left) Nearby residues of PTP1B that contribute to activation loop peptide-binding are highlighted. The other side chains of the TYK2 peptide could not be resolved. For both structures, Tyr 46 and Phe 182 of PTP1B sandwich the aromatic ring of the phosphotyrosine in the catalytic pocket, and Asp 48 coordinates the peptide backbone.

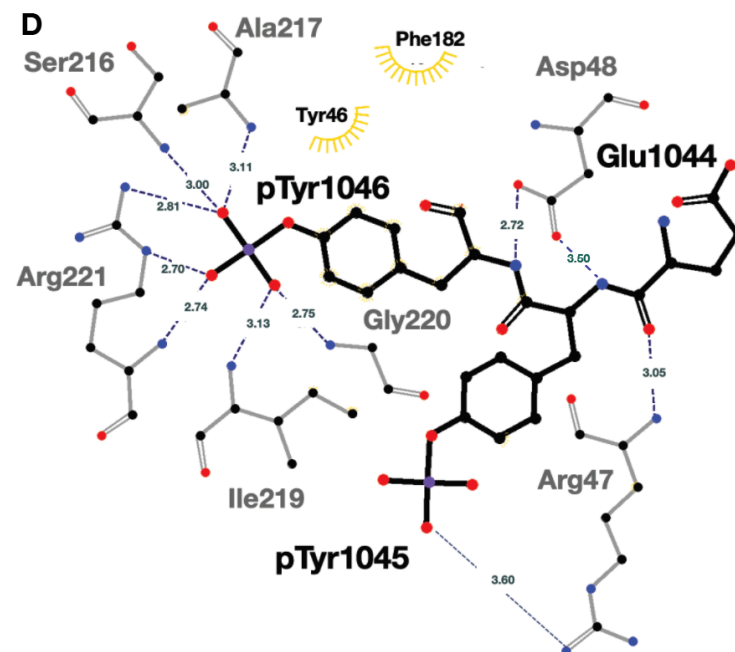
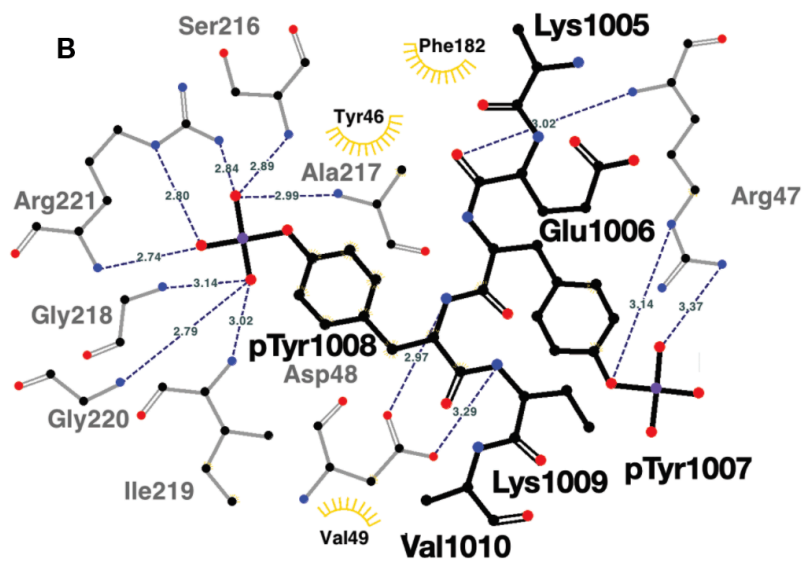
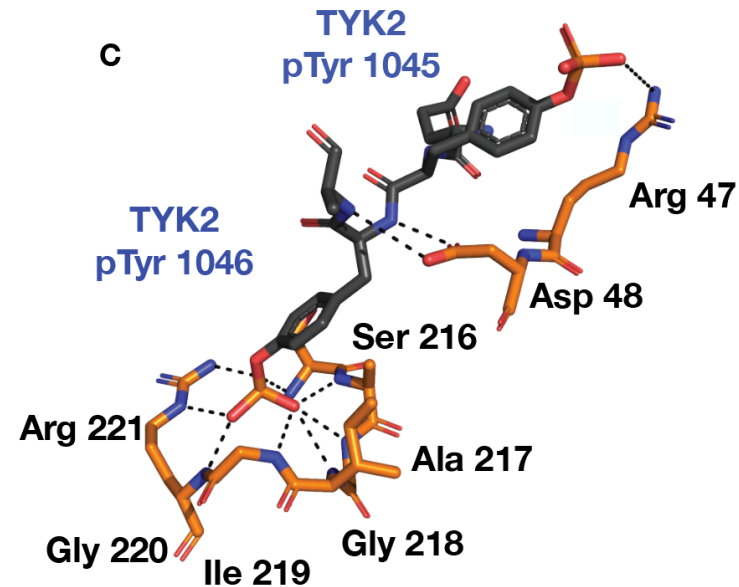
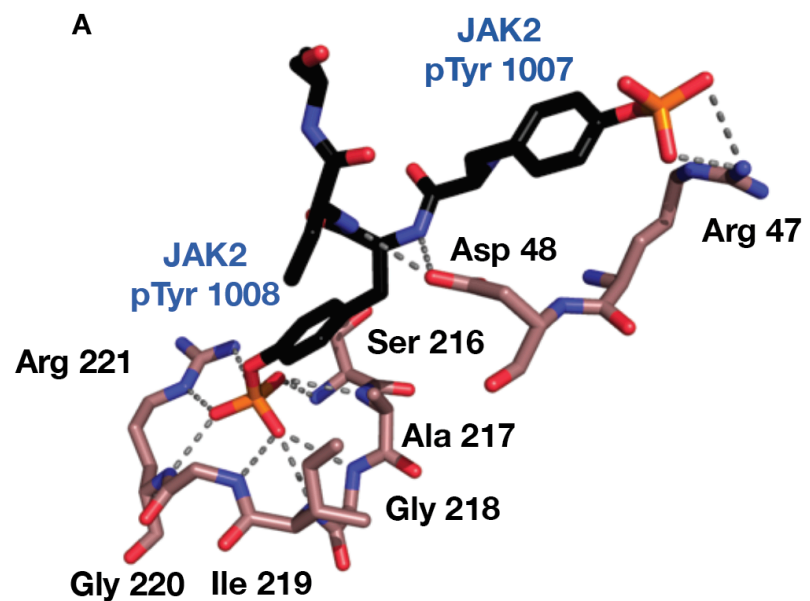


Figure 3.13 Interactions between PTP1B DQC 2-321 and the JAK2 and TYK2 activation loop phosphopeptides.

A) Structure of the PTP1B DQC 2-321 mutant with the JAK2 activation loop peptide bound. Arg 47 of PTP1B interacts with the first pTyr of the JAK2 activation loop peptide (pTyr 1007), Asp 38 with the amide bonds of the peptide backbone and the residues in PTP1B that make up the PTP loop, interact with the second phosphotyrosine of the JAK2 activation loop peptide (pTyr 1008). **B)** 2D representation the interactions between PTP1B and the JAK2 activation loop peptide. **C)** Structure of the PTP1B DQC 2-321 mutant with the TYK2 activation loop peptide bound. Asp 38 interacts with the amide bonds of the peptide backbone and the residues in PTP1B that make up the PTP loop interact with the second phosphotyrosine of the TYK2 activation loop peptide (pTyr 1046). **D)** 2D representation of the interactions between PTP1B and the TYK2 activation loop peptide. For both 2D representations, interactions are indicated by dashed lines, with the distance between the residues in angstroms indicated. Dotted lines represent hydrogen bonds or electrostatic interactions. Yellow dashes crescents represent hydrophobic interactions. Visualized using LigPlot+ (Laskowski and Swindells 2011). Omit map for the PTP1B DQC + TYK2 pYpY peptide can be found in appendix II.

Table 3.2 Data collection and refinement statistics for PTP1B D181A/Q262A/C215A 2-321 with JAK2 and TYK2 activation loop peptides and apo PTP1B D181A/Q262A.

	PTP1B DQC + JAK2 pYpY	PTP1B DQC + TYK2 pYpY	Apo PTP1B DQ
Data Collection			
Space group	P 3 ₂ 2 1	P 2 ₁ 2 ₁ 2 ₁	P 3 ₁ 2 1
Cell dimensions			
a, b, c (Å)	88.83 88.83 197.45	44.48 88.22 97.66	88.72 88.72 104.65
α, β, γ (°)	90 90 120	90 90 90	90 90 120
R _{meas} (%)	6.05 (73.42)	16.08 (245.6)*	4.89 (121.8)
I /sigma(I)	16.67 (2.58)	10.44 (0.91)	23.89 (1.84)
CC _{1/2} (%)	99.9 (85)	99.7 (37.7)*	100 (67.7)
Completeness (%)	99.67 (99.26)	99.56 (97.73)	99.87 (98.88)
Redundancy	5.0 (5.2)	6.5 (6.3)	9.0 (9.1)
Refinement			
Resolution (Å)	43.33 - 2.09 (2.17 - 2.01)	48.84 - 2.79 (2.89 - 2.79)	44.36 - 1.59 (1.65 - 1.59)
No. reflections	53432 (5267)	64878 (6043)	63292 (6181)
R-work	21.70 (33.12)	22.16 (38.84)	19.05 (27.36)
R-free	25.85 (40.68)	27.00 (46.36)	21.20 (28.79)
No. atoms			
protein	4914	2364	2412
ligand	23	-	12
solvent	175	7	162
B factors			
protein	53.65	83.32	31.28
ligand	62.88	-	48.49
solvent	52.48	71.11	38.37
r.m.s. deviations			
RMS(bonds)	0.006	0.005	0.006
RMS(angles)	0.76	0.75	0.83
Ramachandran favored (%)	95.81	95.61	97.64
Ramachandran allowed (%)	3.69	4.05	2.03
Ramachandran outliers (%)	0.50	0.34	0.34
Rotamer outliers (%)	1.17	1.69	1.16

Values in parentheses are for highest-resolution shell. *Criteria for data cut off were from (Karplus and Diederichs 2012) using the split-half correlation coefficient (CC_{1/2}).

3.7 Comparison of PTP1B bound to the IRK and JAK activation loops

Studies of PTP1B in complex with the activation loop of IRK showed the first phosphotyrosine (pTyr 1162) occupying the catalytic pocket of PTP1B while the second phosphotyrosine (pTyr 1163) is coordinated by Arg 24 and Arg 254 in the second aryl binding site (**Figure 3.14A, E and F**). This has been widely recognized as the way by which PTP1B recognizes and interacts with tandemly phosphorylated tyrosine residues (Salmeen et al. 2000; Barr et al. 2009). Crystal structures of the PTP1B DQC mutant in complex with the JAK2 and TYK2 activation loop peptides presented in this thesis have the second phosphotyrosine occupying the catalytic pocket (pTyr 1008 for JAK2 and pTyr 1046 for TYK2). This places the JAK activation loop peptides in a different register when compared to the IRK activation loop peptide and suggests multiple arrangements for PTP1B interaction with tandemly phosphorylated substrates.

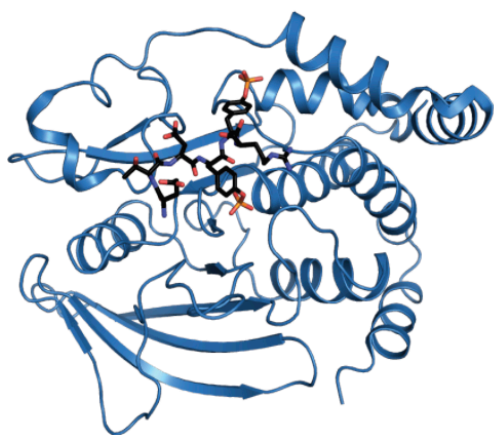
Overlaying the structure of PTP1B in complex with the IRK activation loop peptide versus JAK2 activation loop peptide reveals that despite the two activation loop peptides being in different registers, the peptide backbones sit in almost identical positions. The overall RMSD for both structures is 0.4 Å over 297 residues. In the PTP1B DQC + JAK2 activation loop peptide structure, pTyr 1007 is coordinated in the -1 position by Arg 47 of PTP1B, (**Figure 3.14A, C and D**). In the PTP1B + IRK activation loop peptide structure however, the preceding aspartate occupies this position (**Figures 3.14A and 3.15A**). The second phosphotyrosine (pTyr 1163) of the PTP1B + IRK structure crosses the gateway region and occupies the second aryl binding site of PTP1B where it is coordinated by Arg 24 and Arg 254. While in the PTP1B + JAK2 activation loop peptide structure the density for both phosphotyrosine residues is clear, our analysis of the density for the phenyl ring containing pTyr 1163 for the published PTP1B + IRK structure indicates poor resolution (**Figure 3.15B and C**). The limited density for the second phosphotyrosine of the IRK activation loop peptide may suggest this residue can adopt multiple conformations in the second aryl binding site. Additionally, if the second phosphotyrosine was to occupy the catalytic pocket as per the JAK2 structure, the +1 residue would be an arginine.

In both the PTP1B DQC + JAK2 and PTP1B C215A + IRK structures, Asp 48 of PTP1B coordinates the amide backbone of the of the pTyr occupying the catalytic pocket (1008 of JAK2 and 162 of IRK) and following residue (Lys 1009 of JAK2 and pTyr 163 of IRK) to stabilise the molecular interaction. Both phosphotyrosine residues occupying the catalytic pocket (pTyr 1008 of JAK2 and pTyr 162 of IRK) are coordinated by the amide groups of Ser 216, Ala 217, Gly 218, Ile 219, Gly 220 and Arg 221, and the side chain of Arg 221 of the PTP loop. Additionally, in both structures, Gln 266 and the phosphate group of the phosphotyrosine occupying the catalytic pocket also coordinate a water molecule.

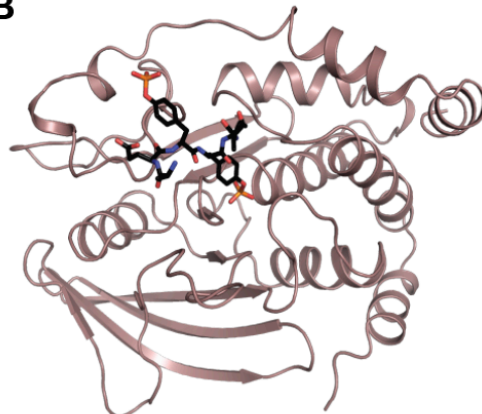
PTP1B D181A Q262A C215A + JAK2 ALP: DKE[PY][PY]KVKE

PTP1B C215A + IRK ALP: ETD[PY][PY]RKGG

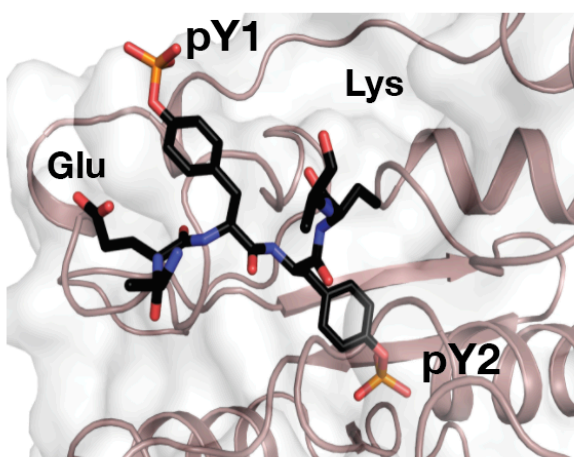
A



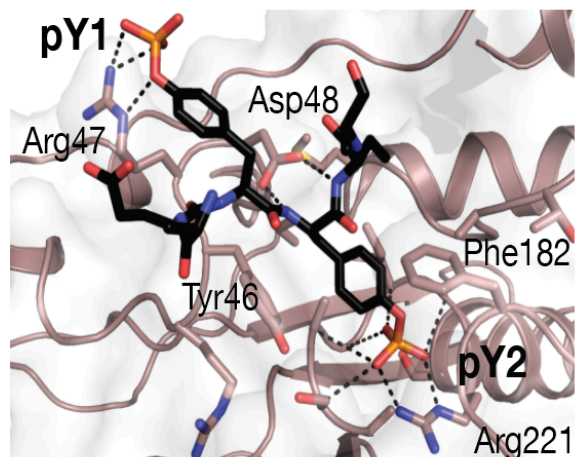
B



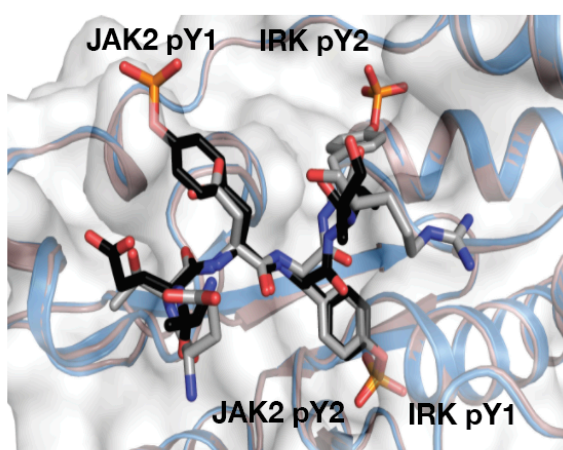
C



D



E



F

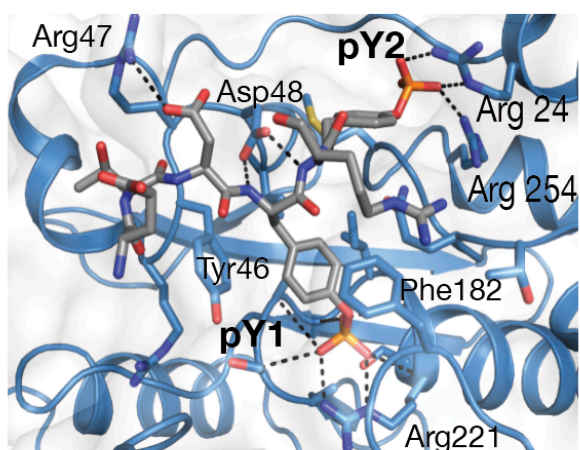


Figure 3.14 PTP1B DQC with the JAK2 activation loop peptide bound compared with PTP1B with IRK activation loop peptide bound.

A) Structure of the PTP1B C215A mutant with an IRK activation loop phosphopeptide bound (PDB ID: 1G1F (Salmeen et al. 2000)). **B)** Structure of the PTP1B DQC mutant with a JAK2 activation loop phosphopeptide bound. **C)** Active site and surrounding residues of PTP1B with the JAK2 activation loop peptide bound, the second phosphotyrosine (pY2) is in the catalytic pocket. **D)** Active site and surrounding residues of PTP1B with the JAK2 activation loop peptide. **E)** Active site of PTP1B with the IRK activation loop peptide bound, the pY1 is in the catalytic pocket. **F)** Active site and surrounding residues of PTP1B with the IRK activation loop peptide.

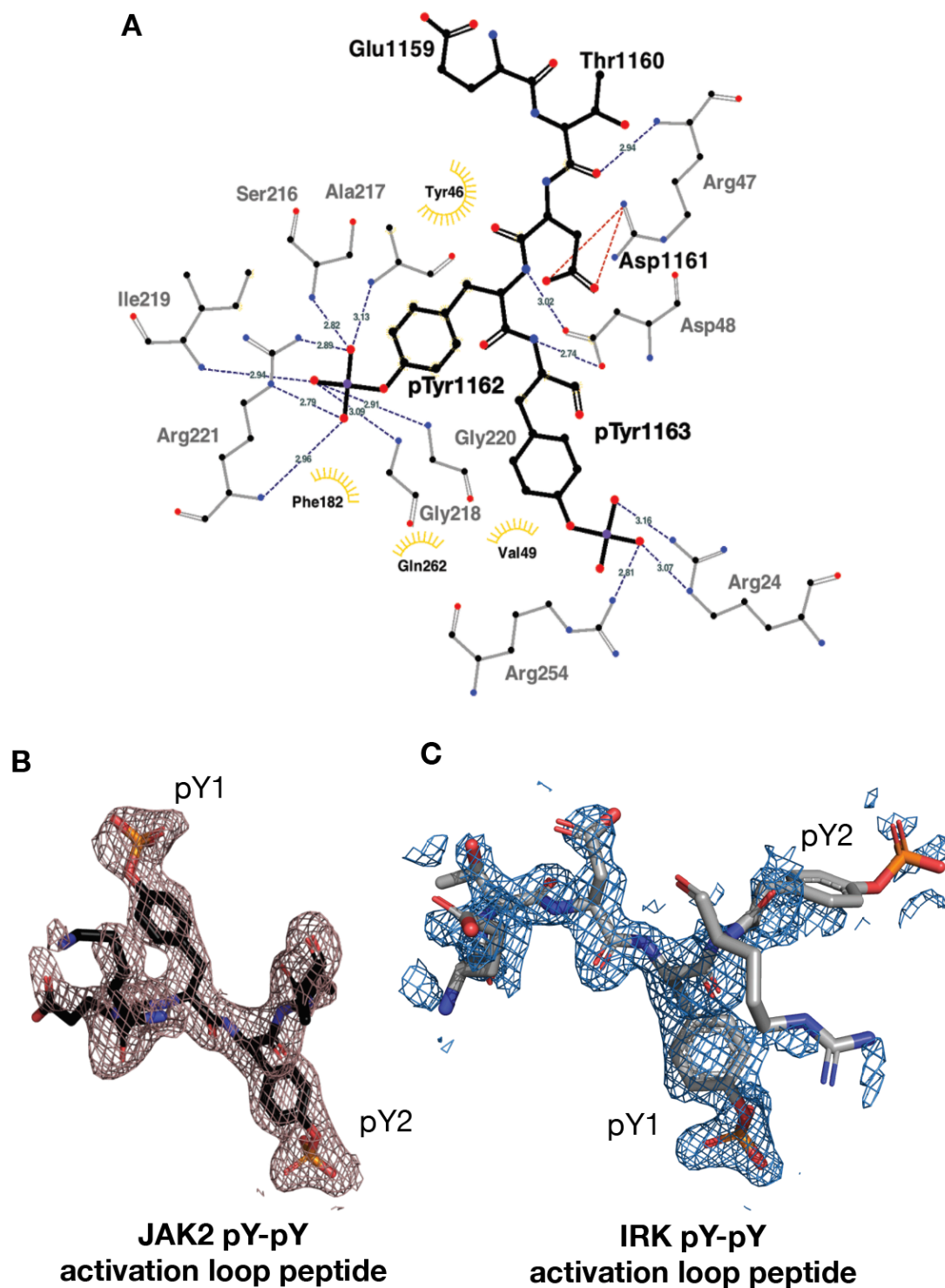


Figure 3.15 IRK and JAK2 activation loop peptide interactions with PTP1B.

A) Schematic diagram representing the interactions between PTP1B and the IRK activation loop peptide. Dotted lines represent hydrogen bonds or electrostatic interactions with distance in angstroms indicated. Yellow dashes crescents represent hydrophobic interactions. Visualized using LigPlot+ (Laskowski and Swindells 2011). **B)** Simulated annealing 2F_o-F_c composite omit map, contoured to 1 σ for the JAK2 pYpY activation loop peptide. **C)** Simulated annealing 2F_o-F_c composite omit map, contoured to 1 σ for the IRK pYpY activation loop peptide.

3.8 PTP1B interactions with singly phosphorylated JAK activation loop peptides

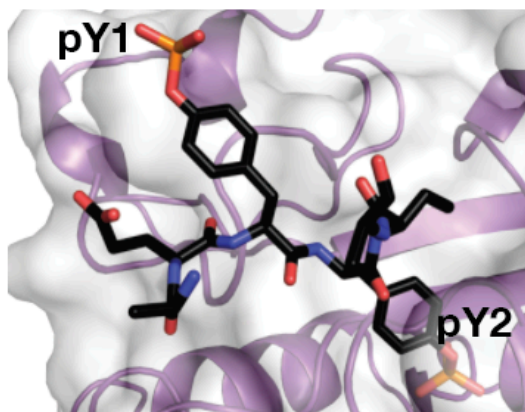
Given the structures of PTP1B DQC in complex with the JAK activation loop peptides show the second phosphotyrosine occupying the catalytic pocket, it was important to investigate whether both the first and second phosphotyrosine residues were capable of binding the catalytic pocket. To test this, crystal trays were set up with singly phosphorylated activation loop peptides of JAK2, henceforth called pYY (first tyrosine phosphorylated only) or YpY (second tyrosine phosphorylated only). Crystal trays were set up in house by hanging drop vapour diffusion by mixing 1 μ L of protein with 1 μ L of precipitant over a 450 μ L reservoir volume. Conditions from previous DQ and DQC structures were screened. Crystals were obtained for PTP1B DQ and DQC for both the pYY and YpY peptides in 12-14% PEG 8K, 150-200 mM MgAc, and 100 mM MES (pH 6.5). The initial crystals of the PTP1B DQC + JAK2 YpY activation loop peptide diffracted, however a solution could not be found using the data collected. To generate new crystals two 96-well screens were set up robotically at the C3 CSIRO as in section 1.5 Crystals were obtained for the DQC + JAK2 YpY peptide in a condition containing 25% w/v PEG 3350, 200 mM NaCl, and 100 mM Tris Cl (pH 8.5). All crystals were cryo-protected in mother liquor containing 20% (v/v) ethylene glycol and flash-frozen in liquid nitrogen.

Diffraction data were collected on the MX2 beamline at the Australian Synchrotron. Data were processed in XDS (Kabsch 2010) to a resolution cut off of 2.4 Å for the DQ + JAK2 pYY, 2.3 Å for the DQ + JAK2 YpY, 2.35 Å for the DQC + JAK2 pYY, and 3.1 Å for the DQC + JAK2 YpY. All datasets had an overall completeness of more than 97%. PTP1B phosphatase domain (PDB ID: 1SUG for DQ pYY and DQC YpY, 4ZRT for DQ YpY and DQC pYY) was used as a search model in PHASER (McCoy et al. 2007). A solution was found for the DQ mutants in the space group $P 4_3 2_1 2$, with one copy in the asymmetric unit, in $P 3_1 2 1$ for the DQC pYY structure with one copy in the asymmetric unit, and in $P 1 2_1 1$ for the DQC YpY mutant with three copies in the asymmetric unit. Complete statistics for all structures are listed in **Table 3.3**.

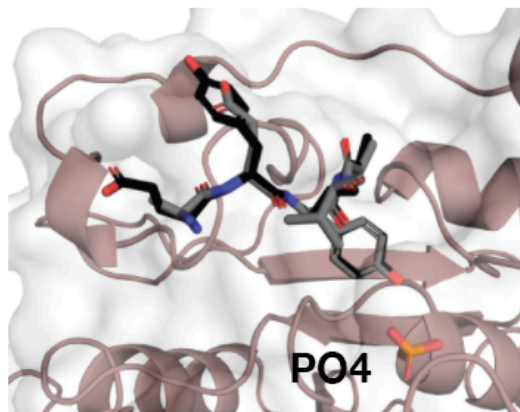
In all four PTP1B-JAK2 single pY activation loop peptide structures, the phosphorylated tyrosine occupied the catalytic pocket. Similar to the PTP1B DQ pYpY structures, the DQ mutant retained catalytic activity and the phosphate had been removed from the tyrosine residue, whilst for the DQC mutant, the phosphotyrosine remained intact **(Figure 3.16)**. omit maps for the single phosphotyrosine peptides can be found in appendix II.

Structures of PTP1B mutants (DQ or DQC) with the JAK2 pYY peptide revealed that the single phosphotyrosine occupies the catalytic pocket, and the +1 tyrosine in the +1 position **(Figure 3.16C and D)**, similar to the structure of the IRK activation loop peptide previously mentioned **(Figure 3.14)**. In both PTP1B mutants with the YpY peptide, the single phosphotyrosine occupies the catalytic pocket, and the -1 tyrosine in the -1 position **(Figure 3.16E and F)**. These structures confirm either phosphotyrosine is able to occupy the catalytic pocket of PTP1B, but there appears to be a preference for the second phosphotyrosine when both tyrosine residues are phosphorylated. The interaction between PTP1B DQC and the single phosphotyrosine peptides is discussed further in the following sections.

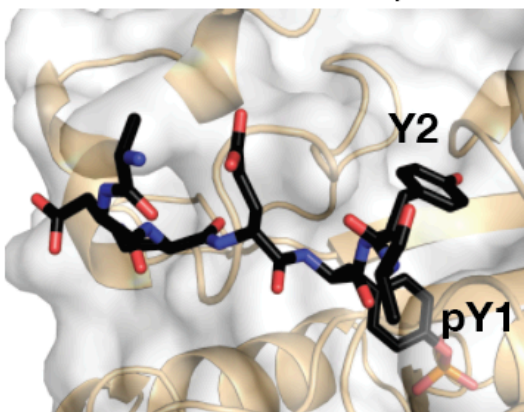
A PTP1B DQC + JAK2 pY-pY



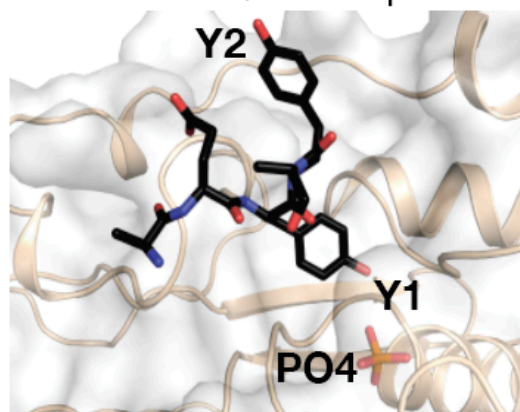
B PTP1B DQ + JAK2 pY-pY



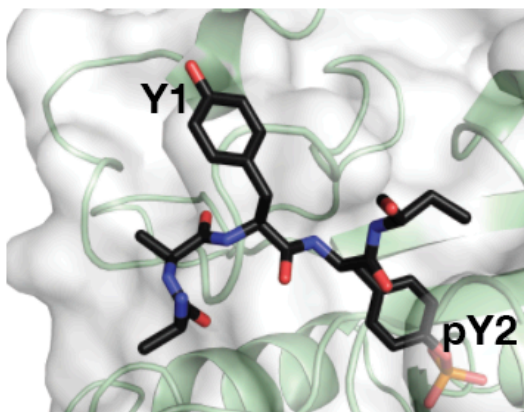
C PTP1B DQC + JAK2 pY-Y



D PTP1B DQ + JAK2 pY-Y



E PTP1B DQC + JAK2 Y-pY



F PTP1B DQ + JAK2 Y-pY

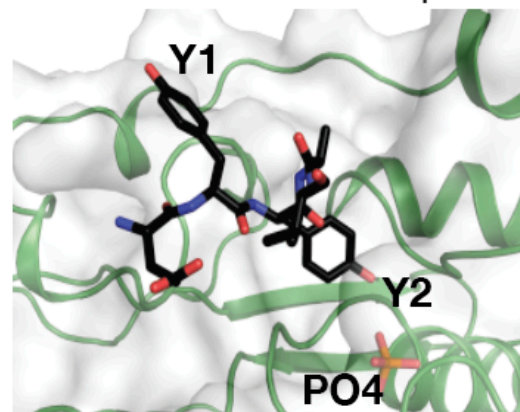


Figure 3.16 Crystal structures of PTP1B DQ and DQC 2-321 phosphatase domain with JAK2 activation loop peptide variants bound.

A) JAK2 pYpY activation loop peptide bound to PTP1B DQC, second phosphotyrosine in the catalytic pocket, first phosphotyrosine in the -1 position. **B)** JAK2 pYpY activation loop peptide bound to PTP1B DQ, a mixture of both tyrosines in catalytic pocket. Both tyrosine residues have been dephosphorylated and a phosphate molecule is present in the catalytic pocket. **C)** JAK2 pYY activation loop peptide bound to PTP1B DQC, first phosphotyrosine in the catalytic pocket. **D)** JAK2 pYY activation loop peptide bound to PTP1B DQ, first tyrosine in the catalytic pocket. The first tyrosine residue has been dephosphorylated and a phosphate molecule is present in the catalytic pocket. **E)** JAK2 YpY activation loop peptide bound to PTP1B DQC, second phosphotyrosine in the catalytic pocket. **F)** JAK2 YpY activation loop peptide bound to PTP1B DQ, second tyrosine in the catalytic pocket. The second tyrosine residue has been dephosphorylated and a phosphate molecule is present in the catalytic pocket.

Table 3.3 Data collection and refinement statistics for PTP1B D181A/Q262A and D181A/Q262A /C215A 2-321 with singly phosphorylated JAK2 Activation Loop Peptides.

	PTP1B DQ + JAK2 pYY	PTP1B DQ + JAK2 YpY	PTP1B DQC + JAK2 pYY	PTP1B DQC + JAK2 YpY
Data Collection				
Space group	P 4 ₃ 2 ₁ 2	P 4 ₃ 2 ₁ 2	P 3 ₁ 2 1	P 2 ₁
Cell dimensions				
a, b, c (Å)	88.9 88.9 131.45	88.67 88.67 131.33	88.38 88.38 190.33	88.22 176.23 43.79
α, β, γ (°)	90 90 90	90 90 90	90 90 120	90 90.29 90
R _{meas} (%)	7.16 (73.09)	5.91 (84.2)	6.01 (74.27)	8.86 (79.95)
I / sigma(I)	15.59 (2.10)	23.36 (2.67)	18.94 (2.51)	12.86 (1.69)
CC _{1/2} (%)	99.9 (85)	100 (86)	99.9 (77)	99.8 (74)
Completeness (%)	99.68 (97.70)	99.92 (99.74)	99.63 (99.25)	99.05 (95.38)
Redundancy	6.5 (6.5)	9.5 (9.1)	5.6 (5.6)	3.5 (3.4)
Refinement				
Resolution (Å)	42.11 - 2.4 (2.48 - 2.4)	42.01 - 2.29 (2.38 - 2.29)	48.85 - 2.34 (2.43 - 2.34)	42.79 - 3.09 (3.20 - 3.09)
No. reflections	21208 (2039)	23991 (2333)	36591 (3580)	24126 (2290)
R-work	19.56 (27.63)	19.94 (28.1)	31.41 (37.83)	25.59 (33.46)
R-free	25.60 (33.53)	23.05 (30.20)	30.70 (35.21)	28.72 (38.25)
No. atoms				
protein	2389	2413	4792	6873
ligand	13	16	16	24
solvent	50	72	118	37
B factors				
protein	66.28	61.20	58.77	62.73
ligand	78.44	66.45	66.10	76.59
solvent	60.64	59.71	53.37	60.63
r.m.s. deviations				
RMS(bonds)	0.008	0.006	0.007	0.009
RMS(angles)	0.81	0.75	0.81	1.27
Ramachandran favored (%)	94.30	95.65	96.61	94.43
Ramachandran allowed (%)	4.70	3.34	2.71	4.44
Ramachandran outliers (%)	1.01	1.00	0.68	1.14
Rotamer outliers (%)	3.59	2.33	1.14	8.58

Values in parentheses are for highest-resolution shell.

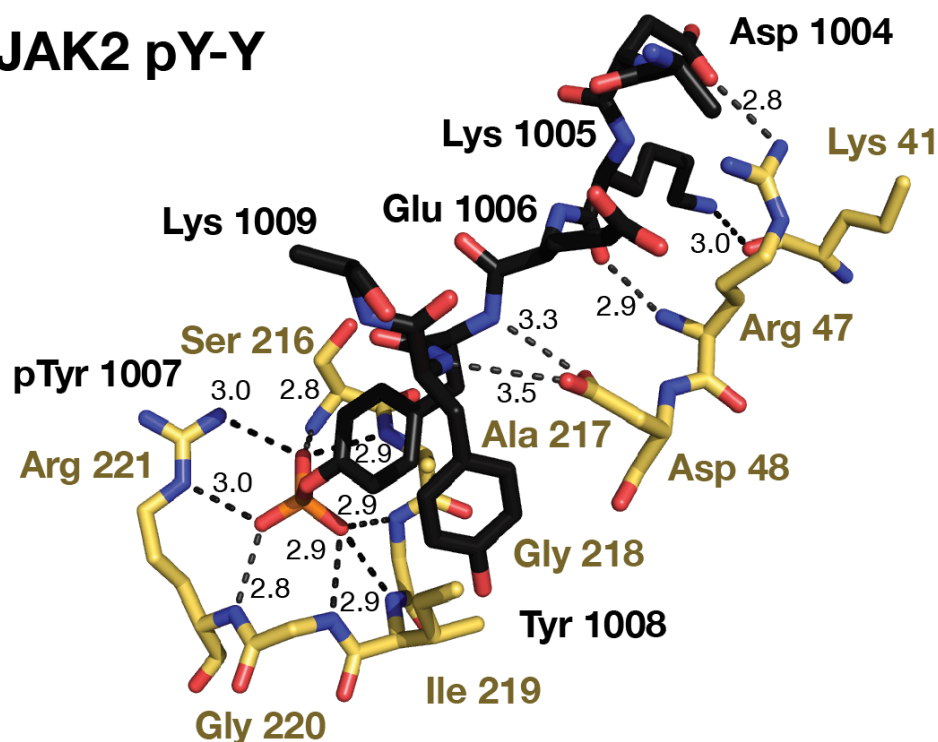
3.8.1 Comparison of the interaction between PTP1B DQC with JAK2 pYY and YpY

For both the JAK2 pYY and YpY activation loop peptides, the phosphotyrosine residue occupying the catalytic pocket is coordinated by amide groups of Ser 216, Ala 217, Gly 218, Ile 219, Gly 220 and Arg 221, and the side chain of Arg 221 (**Figure 3.17**). Asp 48 of PTP1B forms bidentate hydrogen bonds with the amide backbone of the phosphotyrosine occupying the catalytic pocket (pTyr 1007 for pYY and pTyr 1008 for YpY) and the +1 residue (Tyr 1008 for pYY and Lys 1009 for YpY). These interactions were also described for JAK2 and TYK2 pYpY activation loop peptides bound to PTP1B in section 1.6.2.

The PTP1B DQC + JAK2 YpY structure is most similar to the DQC JAK2 and TYK2 pYpY structures, and bares resemblance to a previously published structure of C215S PTP1B in complex with a consensus peptide comprising the sequence ELEFpYMDYE (Sarmiento et al. 2000). This peptide containing a Phe in the -1 position was shown to be a potent PTP1B substrate. These results suggest that both negatively charged and aromatic residues are well tolerated in the -1 position.

Two copies of the PTP1B and peptide are present in the asymmetric unit for the PTP1B DQC + JAK2 pYY structure, with each copy of PTP1B forming different contacts with the JAK2 pYY peptide (**Figures 3.17 and 3.18**). In one copy, Arg 47 of PTP1B interacts with the -3 Asp of the peptide, and the carbonyl oxygen of Lys 41 in PTP1B interacts with the -2 Lys of the peptide (**Figure 3.18A**). In the other copy, Arg 47 interacts with the -2 Glu (1006) in the peptide (**Figure 3.18B**). The differences in contacts between PTP1B and the peptide between the two structures is likely due to the crystal contacts, as in the first copy, the crystal contact likely holds the peptide in place. In both structures, the unphosphorylated tyrosine (Tyr 1008) interacts with Arg 24 and Arg 254 of PTP1B via waters in the second aryl binding site.

JAK2 pY-Y



JAK2 Y-pY

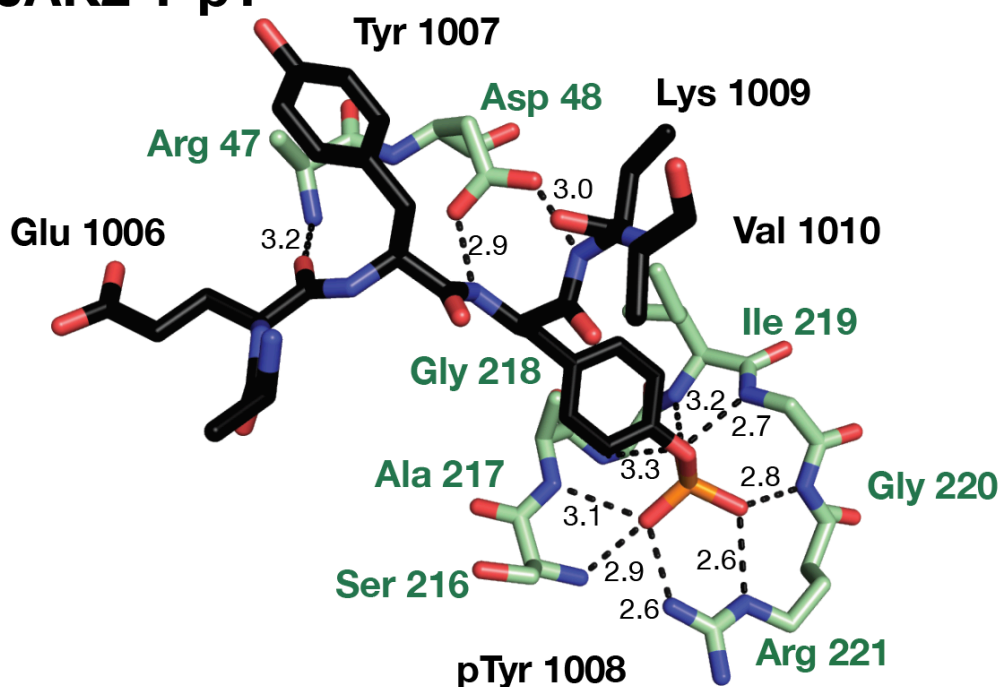


Figure 3.17 Interactions between PTP1B DQC in complex with JAK2 pYY and YpY activation loop peptides.

Top) Structure of the PTP1B DQC 2-321 mutant with the JAK2 pYY activation loop peptide bound, specifically highlighting the interactions between the activation loop peptide and the PTP1B phosphatase domain. **Bottom)** Structure of the PTP1B DQC 2-321 mutant with the JAK2 YpY activation loop peptide bound, specifically highlighting the interactions between the activation loop peptide and the PTP1B phosphatase domain. Dotted lines represent hydrogen bonds or electrostatic interactions, bond distance is indicated in angstroms.

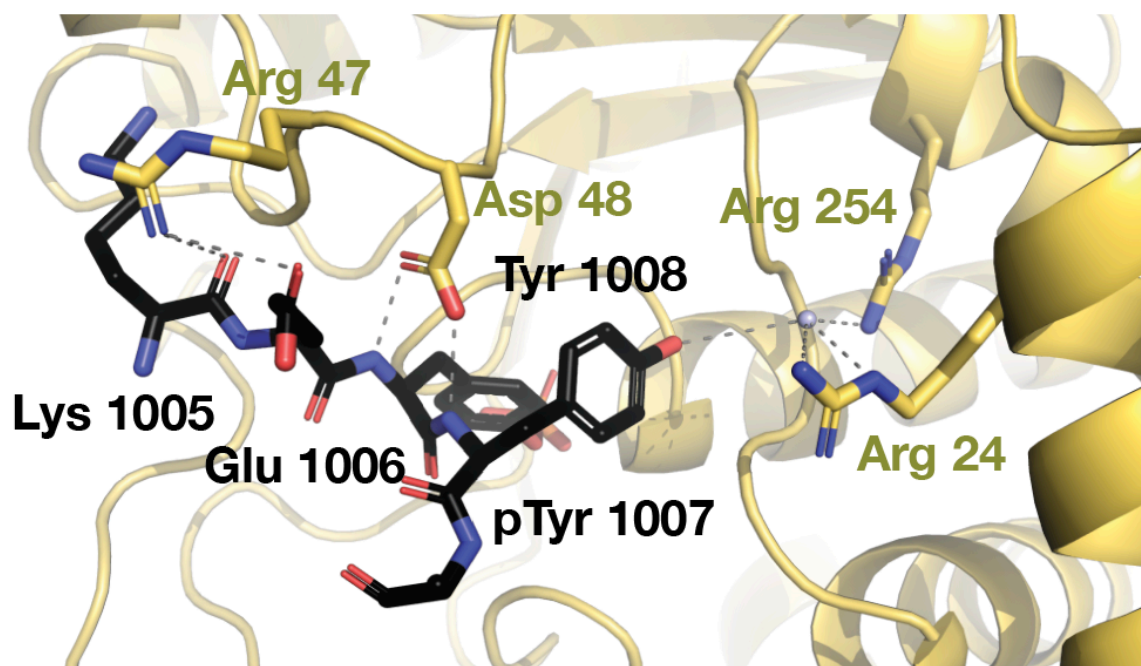
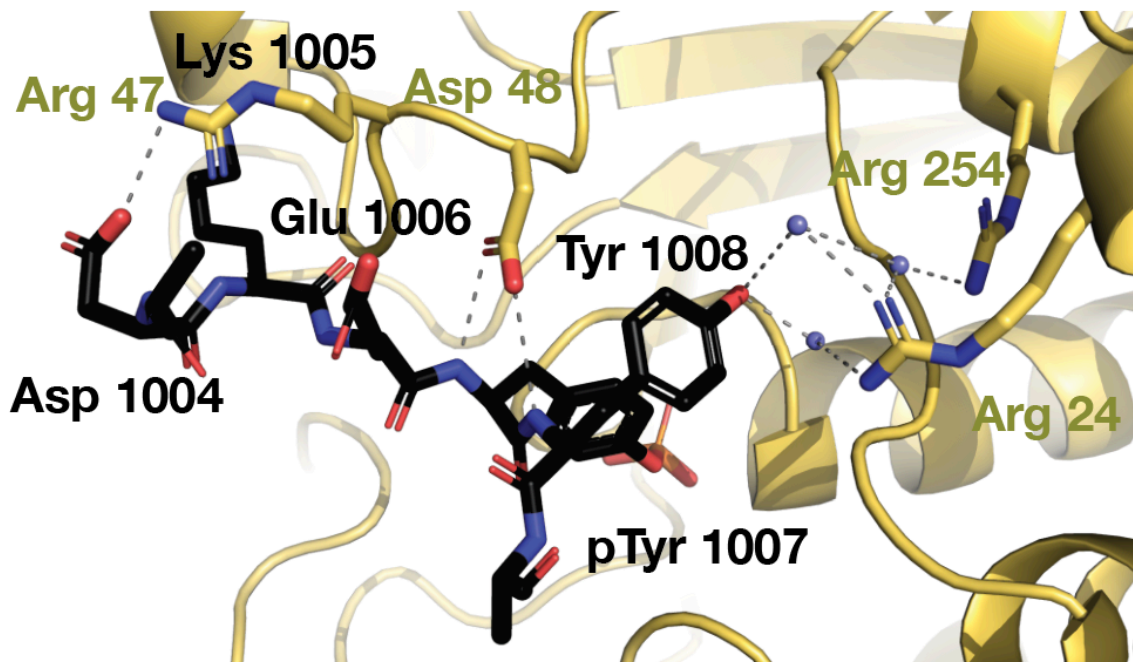


Figure 3.18 JAK2 pYY activation loop peptide interactions in the second aryl binding site of PTP1B DQC.

PTP1B DQC 2-321 mutant with the JAK2 pYY activation loop peptide bound, specifically highlighting the interactions between the activation loop peptide and the PTP1B phosphatase domain. (Top) Copy 1 of PTP1B DQC plus JAK2 pYY (Bottom) Copy 2 of PTP1B DQC plus JAK2 pYY.

3.9 Comparison of the apo and JAK2 activation loop bound PTP1B phosphatase domain

Given that three arginine residues have been identified as potentially important for substrate binding (Arg 47, 24 and 254), crystal trays were set up to capture an apo structure of the PTP1B DQ or DQC 2-321 mutants to compare the positions of these residues in the absence of a substrate specifically for these mutant proteins. 10 mg/mL of PTP1B was used to set up crystal trays in-house by hanging drop vapour diffusion by mixing 1 μ L of protein with 1 μ L of mother liquor over a 450 μ L reservoir volume. Crystals of the DQ mutant grew in 12% PEG 8000, 0.15 M magnesium acetate and 0.1 M MES (pH 6.5). Crystals were flash-frozen in liquid nitrogen, in mother liquor with an additional 20% (v/v) ethylene glycol as cryoprotectant. Diffraction data were collected on the MX2 beamline at the Australian Synchrotron. Data were processed in XDS (Kabsch 2010) to a resolution cut off of 1.6 Å, with a completeness of 99.40%. PTP1B phosphatase domain (PDB ID: 4ZRT) as a search model in PHASER (McCoy et al. 2007). A solution was found in the space group $P 3_1 2 1$ with one copy in the asymmetric unit. Complete statistics for all structures are listed in **Table 3.2**.

Comparison of the apo PTP1B DQ structure with PTP1B DQ structure with a pYpY peptide bound structures revealed that few global changes occur, including closure of the WPD loop in the apo structure despite no substrate being bound as previously seen in other apo structures (Pedersen et al. 2004) (**Figure 3.19B**). The overall RMSD for our apo versus bound structures was 0.4 Å over 298 residues (DALI(Holm and Sander 1995)). In the apo PTP1B structure, the side chain of Arg 47 is visible, and occupies the space where the phosphate group of the first phosphotyrosine residue would sit when bound (**Figure 3.19C and F**). The Asp 48 residue is split between two positions in the apo structure. One conformation is identical to that of the peptide bound structure, and the other conformation allows interactions with the Arg 47 residue (**Figure 3.19F**). Comparison of the Arg 24 and Arg 254 residues in the second aryl binding site between the two structures reveals that in both, Arg 245 is in the same position, however there is a small change in the position of Arg 24 (**Figure 3.19G**). In both structures Arg 24 coordinates a solvent molecule, in the apo structure Arg 254 also participates in this interaction.

Apo PTP1B DQ
PTP1B DQC + JAK2 ALP

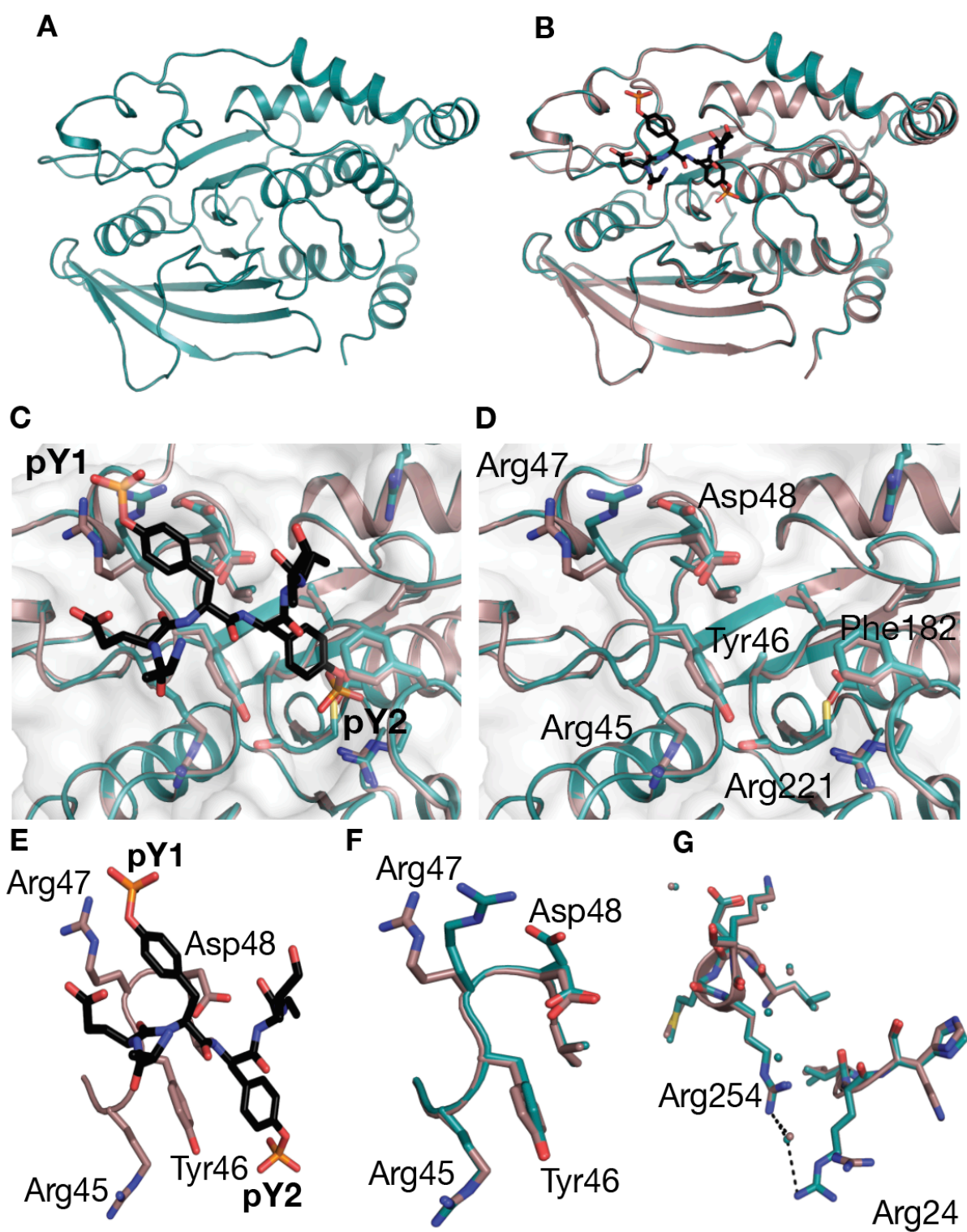


Figure 3.19 Comparison of Apo PTP1B DQ and JAK2 activation loop peptide bound PTP1B DQC.

A) Structure of the Apo PTP1B DQ substrate trapping mutant. **B)** Aligned structures of the Apo and JAK2 activation loop peptide bound DQC mutants. RMSD of 0.4. **C)** Zoomed in view of the substrate-binding site of PTP1B for aligned structures of the Apo and JAK2 activation loop peptide bound DQC mutants. **D)** Zoomed in view of substrate-binding site of PTP1B for both structures of the Apo and JAK2 activation loop peptide bound DQC mutants aligned with the peptide removed and residues highlighted. **E)** JAK2 activation loop peptide and nearby residues in PTP1B. **F)** Arg 45, Tyr 46, Arg 47 and Asp 48 from both structures aligned. **G)** Arg 24 and Arg 254 of the Apo and JAK2 bound structures. In both, Arg 254 coordinates a water molecule, and in the Apo structure, Arg 24 also participates in this coordination.

3.10 Dephosphorylation of the JAK2 activation loop

The previous sections showed that the second phosphotyrosine of the JAK2 activation loop was preferentially bound in the active site. To investigate this enzymatically, two assays were performed to investigate the products of the dephosphorylation reaction; free phosphate and a dephosphorylated tyrosine. The malachite green assay measures the amount of free phosphate in solution. Solution nuclear magnetic resonance (NMR) spectroscopy tracks the emergence of proton signals arising from dephosphorylated tyrosine residues.

To measure the absolute amount of phosphate released from the pYpY or single pY phosphorylated peptides over time, 2 nM PTP1B was added to 400 μ M of either JAK2 pYY, YpY or pYpY peptides. Malachite Green was then added at 0, 15 minutes, and 30 minutes to stop the reactions and quantify the amount of free phosphate by measuring the absorbance of each sample at 620 nM relative to a standard curve. Molybdate in the malachite green reagent forms a complex with orthophosphate in solution allowing free phosphate to be measured using a spectrophotometer (Geladopoulos, Sotiroidis, and Evangelopoulos 1991). Measurement of total phosphate production after 15 and 30 minutes showed that more free phosphate is released into solution from the pYpY peptide than either of the single phosphorylated peptides, while the YpY peptide produced slightly more free phosphate in solution than the pYY peptide (**Figure 3.20A**).

To determine substrate affinity, 2 nM PTP1B was added to varying concentrations of the JAK2 peptides and incubated for 20 minutes at 27°C. Malachite green was then added to stop the reactions and quantify the amount of free phosphate (**Figure 3.20B**). By fitting the raw data to the Michaelis-Menten equation, the maximum velocity of the reaction (V_{max}) and the substrate concentration at which half the maximum velocity is achieved (K_m) can be determined (**Figure 3.20C-E**). The K_m for each reaction gives an indication of the affinity of PTP1B towards the substrate, with lower K_m values representing a higher affinity of PTP1B towards the peptide. These assays revealed a preference for doubly phosphorylated peptides over singly phosphorylated peptides with the K_m of the pYpY JAK2 peptide being approximately 25 μ M. Comparing the two singly phosphorylated

peptides, there was a preference for the second phosphotyrosine YpY over pYY, with the K_m of YpY being approximately 80 μM compared to approximately 700 μM for pYY.

A phosphatase reaction produces two substrates, free phosphate and the dephosphorylated peptide. Therefore, NMR spectroscopy was used to follow the course of the reaction by measuring the product peptide. One-dimensional proton (^1H) NMR spectra of JAK2 activation loop peptide pYpY, pYY, and YpY were recorded as reference spectra (**Figure 3.20F**). Since each of these peptides produced distinct spectra, the conversion of pYpY to pYY or YpY in the presence of PTP1B could be distinguished.

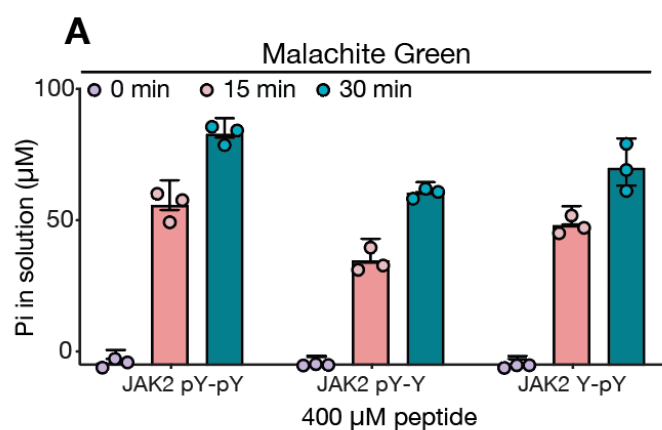
To determine the turnover rate for each peptide, 7 nM PTP1B was added directly to 475 μM of each peptide and the rate of phosphotyrosine signal loss for each peptide was measured by recording ^1H spectra every five and a half minutes (**Figure 3.20G**). The concentration of each species (pYpY, pYY, or YpY) at each time the reaction was calculated by integrating the area under each peak representing each peptide species and normalising this value to the area under the peak determined for the same peptide prior to the addition of enzyme which was at a known concentration. This revealed that the tandemly phosphorylated pYpY peptide and the YpY peptide had similar dephosphorylation rates of 9.3 s^{-1} and 10.0 s^{-1} respectively. The dephosphorylation rate for the pYY peptide was 3.8 s^{-1} .

While these experiments and the malachite green assays are in agreement with the crystallographic data in that they suggest a preference for dephosphorylation of the second phosphotyrosine, they are performed using three separate peptides do not provide conclusive information about which pTyr is most rapidly dephosphorylated within a tandemly-phosphorylated peptide. Therefore, NMR spectroscopy was used to track the dephosphorylation of each of the two phosphotyrosines within the JAK2 activation loop. One-dimensional proton (^1H) NMR spectra of JAK2 activation loop peptide pYpY, pYY, and YpY were recorded as reference spectra (**Figure 3.21F**). Since each of these peptides produced distinct spectra, the conversion of pYpY to pYY or YpY in the presence of PTP1B could be distinguished.

7 nM PTP1B was added to 475 μ M of JAK2 pYpY activation loop peptide and incubated for 100 minutes at room temperature, with ^1H spectra recorded every five and a half minutes. The concentration of each species of activation loop peptide (pYpY, pYY, YpY or YY) at each time point was calculated by integrating the area under the peaks (**Figure 3.20F**) and normalising these values to the area under the peak for the reference peptide spectra (pYpY), whose initial concentration was known prior to addition of enzyme. The data was then plotted as a function of time to generate **Figure 3.20H**. As the reaction progresses, the concentration of the double phosphorylated peptide (pYpY,) decreases. This corresponds to a sharp increase in the concentration of pYY, but a slower increase in the YpY concentration. By 40 minutes, the amount of peptide remaining is mostly pYY, indicating that dephosphorylation of the second pTyr occurs most rapidly. Over time, to the major species becomes 0pY (both phosphotyrosine residues dephosphorylated). Raw data for these reactions can be found in Appendix III.

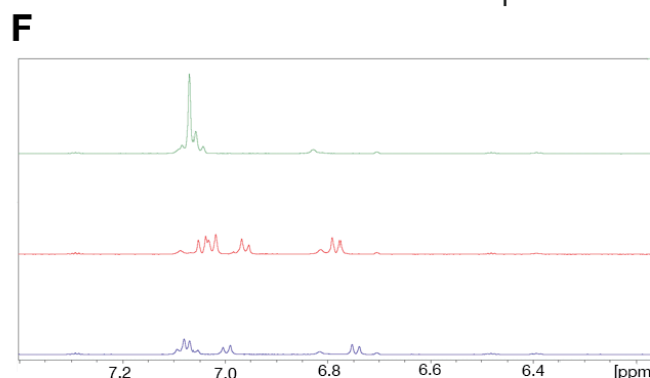
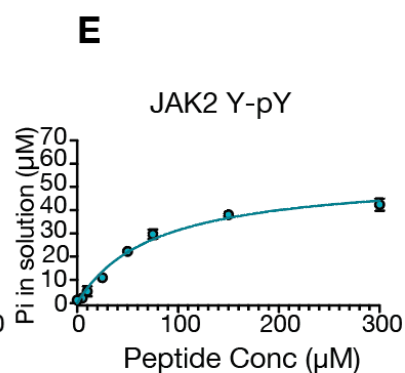
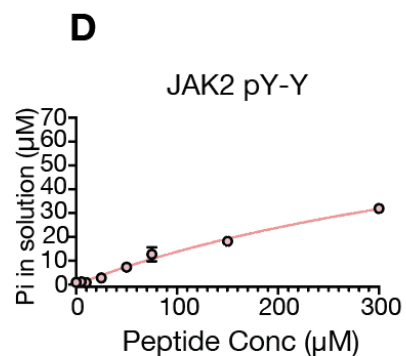
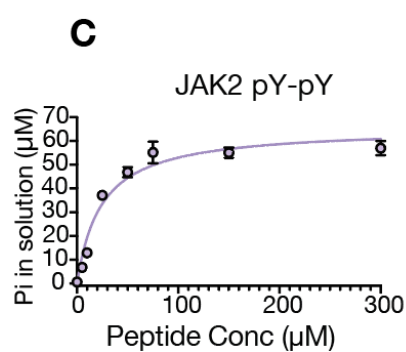
These data support the notion that the second phosphotyrosine is the preferred substrate. An analysis of the initial rates of appearance of the two dephosphorylated species shows that the second pTyr is dephosphorylated four-fold faster than the first with the YpY peptide being dephosphorylated at 5.7 s^{-1} in comparison to pYY at 1.4 s^{-1} .

Combined, these data indicate that in the case of the JAK2 activation loop peptide, the second phosphotyrosine is preferentially dephosphorylated over the first. This contrasts previous published work on the IRK activation loop peptide, where it was suggested that the first phosphotyrosine is the preferred substrate (Salmeen et al. 2000).



B

Peptide	K_m (μ M) $\pm$ SEM
JAK2 pY-pY	24 ± 1.5
JAK2 pY-Y	722 ± 262
JAK2 Y-pY	77 ± 8.0



JAK2 pY-pY: QDKE [pY] [pY] KVKE

JAK2 pY-Y: QDKE [pY] YKVKE

JAK2 Y-pY: QDKEY [pY] KVKE

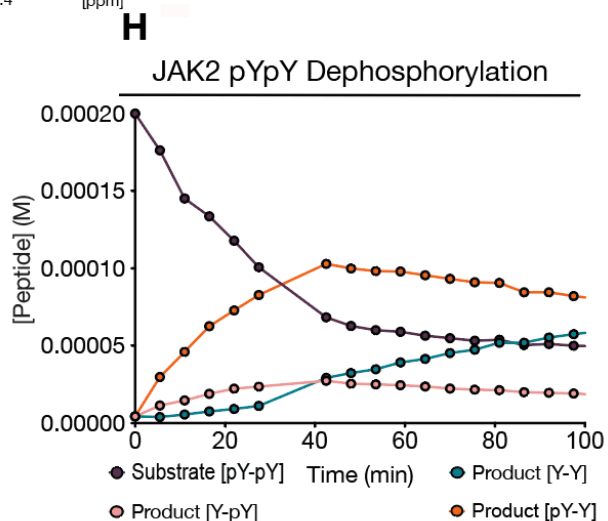
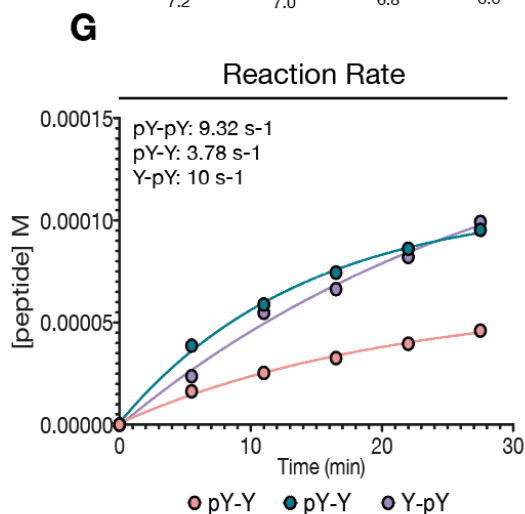


Figure 3.20 Dephosphorylation of the JAK2 activation loop.

A) Comparison of dephosphorylation of 400 μ M JAK2 pYpY, pYY and YpY by PTP1B after 15 and 30 minutes. JAK2 pYpY is the preferred substrate, followed by JAK2 YpY and then JAK2 pYY. $N=3$, error bars are SD. **B)** K_m values calculated from Michaelis-Menten curves in C-E. $N=3$, error bars are SEM. **C-E)** Michaelis-Menten curves for dephosphorylation of JAK2 pYpY, JAK2 pYY and JAK2 YpY. $N=3$, error bars are SEM. **F)** 1D ^1H solution NMR spectra of tyrosine residues for JAK2 pYpY (green) peaks measured between 7.03-7.10 ppm, pYY (red) peaks measured 6.93- 6.97 and 6.97-7.01 ppm and YpY (blue) peaks measured between 7.01-6.97 ppm, and sequences of each peptide. **G)** Dephosphorylation of all 3 peptides over time by 1D ^1H NMR to determine reaction rate. **H)** Dephosphorylation of JAK2 pYpY activation loop peptide showing production of pYY and YpY over time as concentration of each peptide (M). Dephosphorylation of the second phosphotyrosine is preferred over dephosphorylation of the first, but both are fully dephosphorylated over time.

3.11 Dephosphorylation of the IRK activation loop

To determine which phosphotyrosine of the IRK activation loop is preferentially dephosphorylated, the same experiments used for the JAK2 activation loop were performed. The Malachite Green assay revealed that similarly to the JAK2 activation loop, the IRK pYpY peptide was dephosphorylated more than either single pY peptide (**Figure 3.21A-E**). However, there was no discernible difference between the dephosphorylation rate of the pYY and YpY peptides. As shown in (**Figure 3.21B-E**) The K_m for the pYpY IRK peptide was approximately 21 μM , which is very similar to the JAK2 pYpY peptide. The K_m for the pYY and YpY peptides were approximately 41 and 35 μM respectively. These results again suggest there is little difference in affinity between the two singly phosphorylated IRK peptides, but a slight preference for the double phosphorylated peptide. Raw data for these reactions can be found in Appendix III.

When NMR was used to track dephosphorylation of an excess of peptide, the pYpY IRK peptide was shown to be dephosphorylated at 10 s⁻¹, the pYY peptide at 8.5 s⁻¹ and the YpY peptide at 5.1 s⁻¹. Dephosphorylation of the pYpY peptide showed that the pY1 and pY2 residues are dephosphorylated at a similar rate, with a slight (~30%) preference for pY1 (**Figure 3.21G and H**).

Taken together, these results suggest that, unlike JAK2, the two phosphotyrosine residues within the IRK activation loop peptide are dephosphorylated at a similar rate, with a slight preference for the first phosphotyrosine over the second, however this is not as pronounced as the residue preference in the JAK2 activation loop.

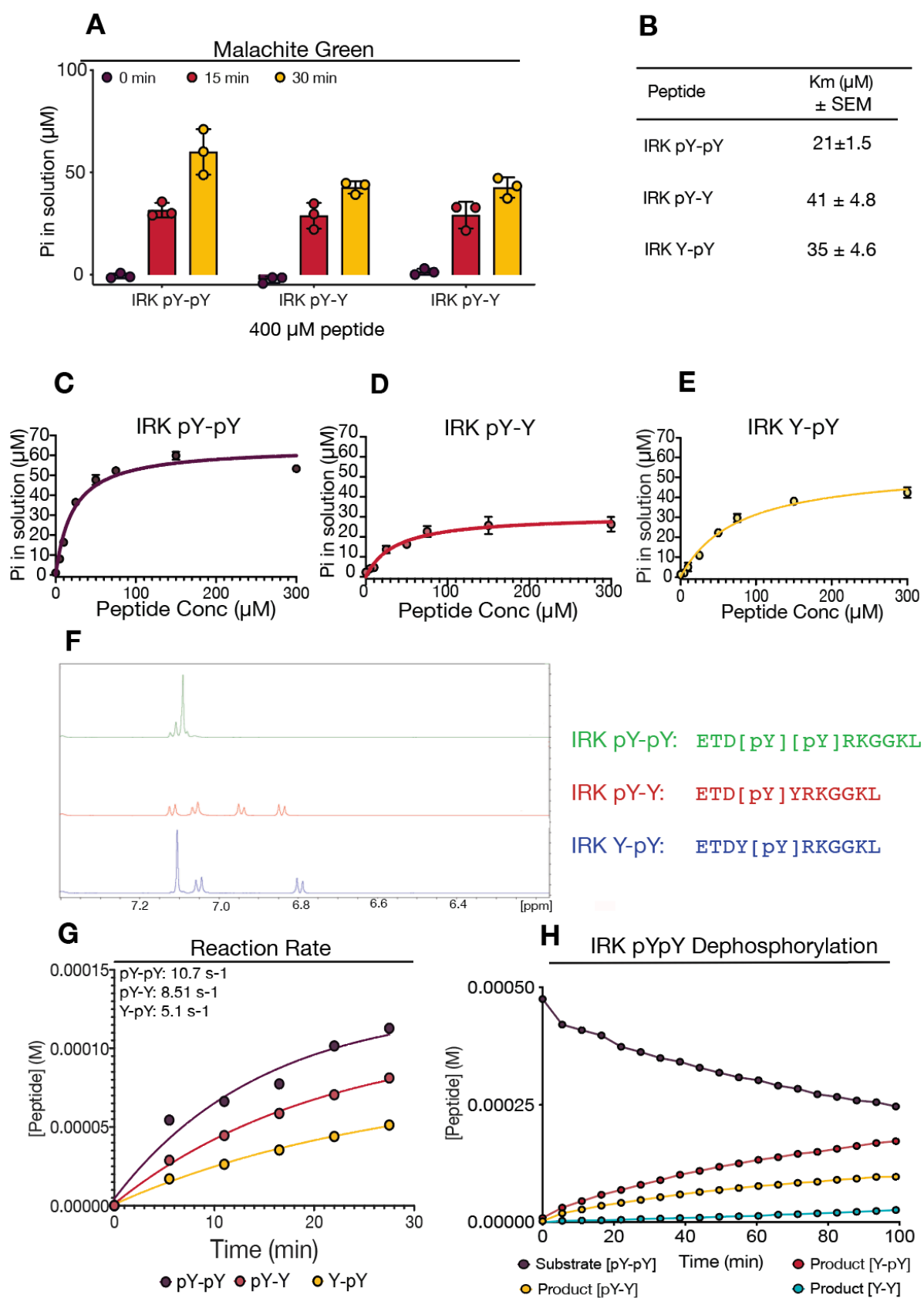


Figure 3.21 Dephosphorylation of the IRK activation loop peptide.

A) Comparison of dephosphorylation of 400 μ M IRK pYpY, pYY and YpY by PTP1B after 15 and 30 minutes. IRK pYpY is the preferred substrate, followed by IRK YpY and IRK pYY being dephosphorylated to a similar level. N=3, error bars are SD. **B)** Km values calculated from Michaelis-Menten curves in C. N=3, error bars are SEM. **C-E)** Michaelis-Menten curves for dephosphorylation of IRK pYpY, IRK, and IRK YpY. N=3, error bars are SEM. **F)** Spectra of tyrosine residues for IRK pYpY (green) peaks measured between 7.07-7.11 ppm, pYY (red) peaks measured between 6.92- 6.96 ppm and YpY (blue) peaks measured between 6.78- 6.81 ppm and sequences of each peptide. **G)** Dephosphorylation of all 3 peptides over time to give reaction rate. **H)** Dephosphorylation of IRK pYpY activation loop peptide showing production of pYY and YpY over time. The second phosphotyrosine is preferred over dephosphorylation of the first phosphotyrosine, but both are fully dephosphorylated over time.

3.12 Selectivity of PTP1B for the JAK activation loop

It was previously shown that all four of the JAK kinase domains can be dephosphorylated by PTP1B (section 3.3). To determine whether differences in the sequences of the JAK activation loop peptide confer PTP1B specificity, the peptides from these activation loops were used as substrates and phosphorylation rate compared using the Malachite Green assay. As shown in **Figure 3.22A**, there was little difference in dephosphorylation rate although, similar to what was seen with the dephosphorylation of the full kinase domains (**Figure 3.3**), the JAK1 activation loop peptide was the poorest substrate.

The structures of PTP1B in complex with peptide show that only a small region surrounding the phosphotyrosine make direct contacts. Given that all four JAK activation loops are highly similar in this region, to determine whether small sequence variations in this region affect substrate selectivity, five artificial variants of the JAK2 YpY peptide were generated (**Figure 3.22B**). The YpY peptide was used as the template and the first tyrosine was left unphosphorylated, as this peptide was the preferred singly phosphorylated substrate. Mutations were made that swapped the charges of the +1, -2 and -3 residues or mutated the valine in the +2 position to an alanine. Dephosphorylation rate and K_m was determined using the Malachite Green assay (**Figure 3.22C and D**). There were some measurable differences in K_m , particularly the Val to Ala mutation at -2 which showed a 2-fold decrease whilst reversing the charge at the +1 position resulted in a 50% increase in K_m . However, most mutations had little, if any, effect on rate or K_m (**Figure 3.22C**). The dephosphorylation rate was the similar for all mutants except for the E to R mutation (1N) which showed a slight reduction, but not as significant as seen for the pYY peptide (**Figure 3.22D**). All previous dephosphorylation assays were performed on peptides of JAK2 with two tandem tyrosines (one phosphorylated). To compare dephosphorylation of a tandem tyrosine peptide to a single tyrosine peptide, the first tyrosine was replaced with an alanine. This peptide was dephosphorylated at half the rate of the parent peptide, implying that PTP1B prefers a substrate with tandem tyrosines (**Figure 3.22E**). Together, these results suggest that single amino acid substitutions of the residues adjacent to the Y-pY moiety do not drive selectivity, however tandem tyrosine residues appear to be increase selectivity to some degree.

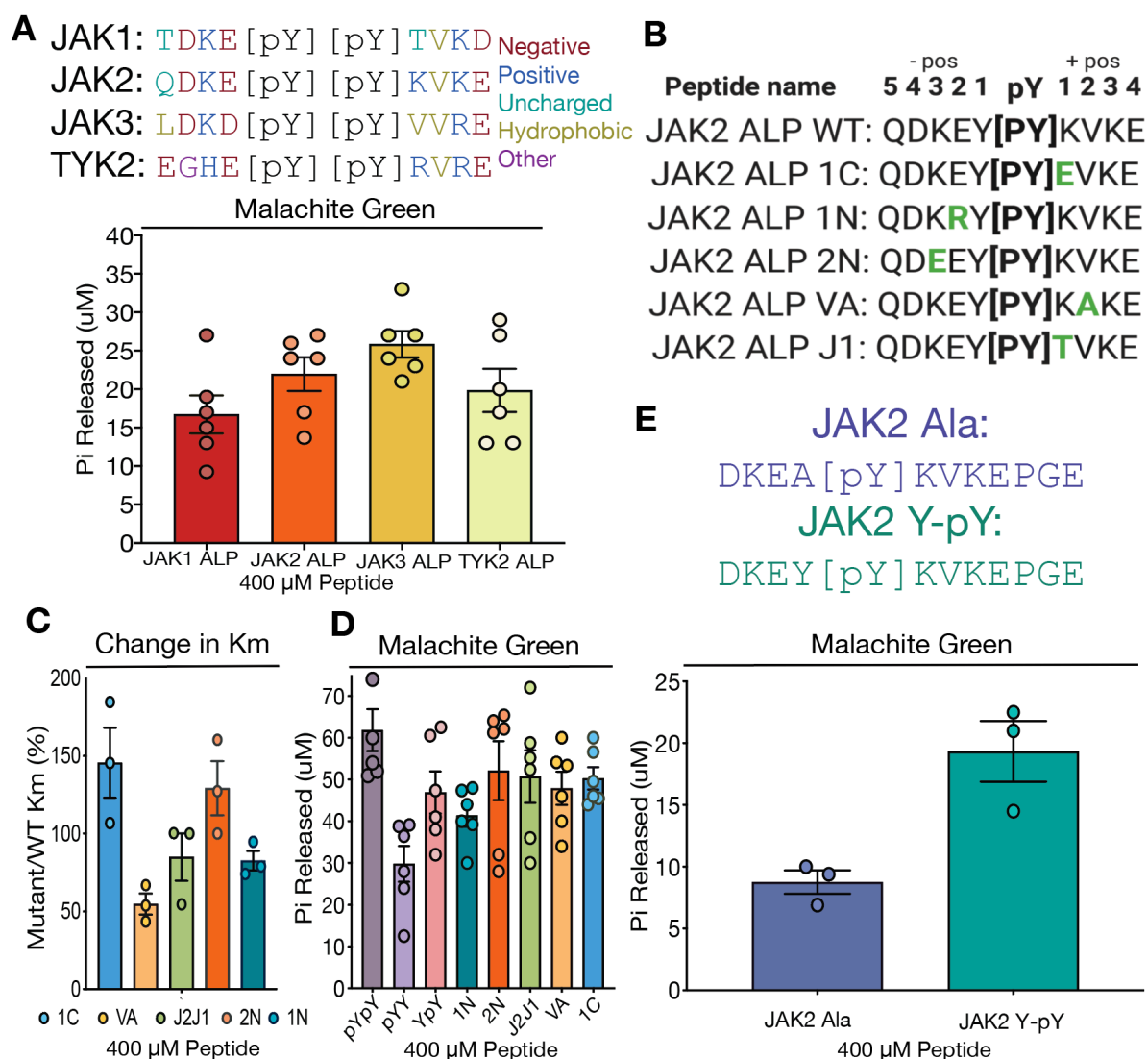


Figure 3.22 Malachite green dephosphorylation assay of modified JAK2 activation loop peptides.

A) (Top) Sequences of the four JAK activation loops with the charge of the residues indicated in colour. (Bottom) Release of phosphate from the JAK activation loop peptides measured by Malachite Green assay after 30 minutes. $N=6$, error bars are SEM. **B)** Sequences of the YpY mutant activation loop peptides (ALP) used in C and D. Mutated residues are indicated in green. **C)** Ratio of mutant JAK2 activation loop peptide K_m to WT JAK2 activation loop peptide K_m . $N=3$, error bars are SEM. **D)** Total amount of phosphate released from each peptide as measured by malachite green assay after 30 minutes. $N=6$ error bars are SEM. **E)** (Top) Sequences of the JAK2 YpY peptide and JAK2 Ala peptide where first tyrosine is replaced by an alanine. (Bottom) Release of phosphate from the JAK2 YpY and Ala activation loop peptides measured by Malachite Green assay after 30 minutes. $N=3$, error bars are SEM.

Dephosphorylation of other phosphotyrosine residues

Having characterized the mechanism of PTP1B dephosphorylation of the JAK activation loop, I returned to investigating the broader role of PTP1B in regulating the IL-6 signalling pathway. Apart from JAK, there are two other phosphorylated proteins essential to the IL-6 signalling cascade; the receptor (IL6ST) and the STATs (STAT1, STAT3). Phosphopeptides representing the 5 phosphotyrosine residues in the IL6ST cytoplasmic tail and the TAD sequences of STAT1 and STAT3 were generated (**Figure 3.23D and E**).

As shown in **Figure 3.23**, PTP1B could dephosphorylate all peptides from IL6ST, although none of them were as efficient as substrates as the doubly phosphorylated JAK2 activation loop. The dephosphorylation of STAT1 at pY701 and STAT3 at pY705 was observable but was significantly reduced compared to other substrates. Although these studies do not prove which proteins are targets of PTP1B *in vivo*, the poor dephosphorylation rate of the STAT1 and STAT3 sequences suggests they are unlikely to be targets.

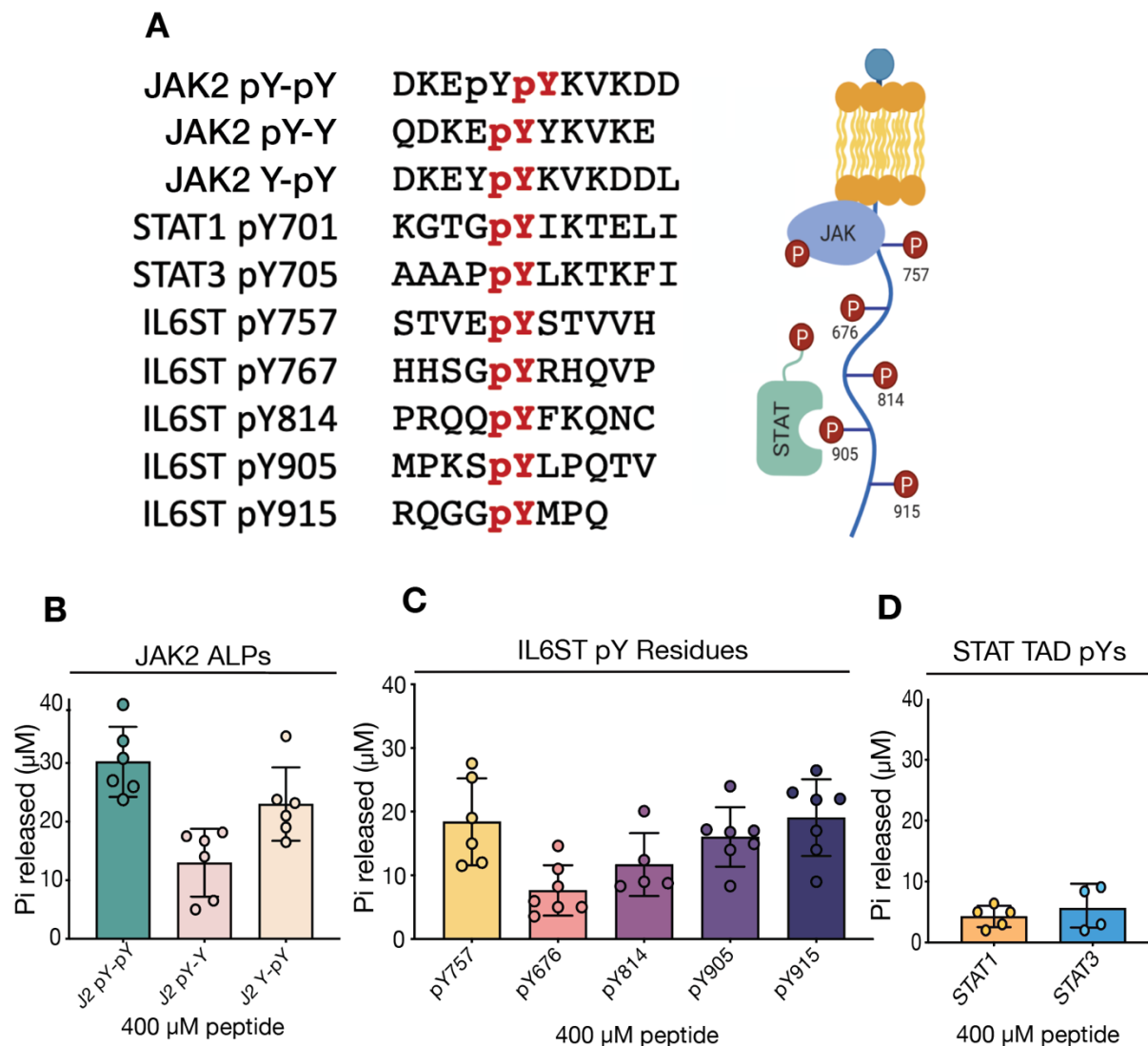


Figure 3.23 PTP1B-mediated dephosphorylation of other phosphotyrosine residues in the IL-6-JAK-STAT pathway.

A) Sequences of 7 main residues in phosphopeptides for all experiments in A-D and Schematic diagram of phosphotyrosine residues. **B-D)** Total phosphate release by PTP1B dephosphorylation of JAK2 activation loop peptides, IL6ST cytoplasmic tail peptides, and STAT1 and STAT3 TAD phosphopeptides (Py701 and pY705 respectively). N=4-6, error bars are SD.

3.13 Accessibility of the JAK activation loop to PTP1B

The activation loop of kinases is known to be a dynamic region. Structural studies of the insulin-like growth factor 1 receptor kinase have demonstrated that the activation loop is able to take on at least three conformations. The first is the inactive conformation where neither tyrosine is phosphorylated and the activation loop is tucked away into the protein (Munshi et al. 2003). The second is the conformation it takes whilst in the process of being transphosphorylated (Wu et al. 2008). The third is the active conformation, where both phosphotyrosine residues are phosphorylated (Favelyukis et al. 2001) (**Figure 3.24A**).

In previous sections of this thesis, the dephosphorylation turnover rate was determined for the activation loop peptides using solution NMR spectroscopy. This technique cannot be used for the intact kinase domain. However, quantification of dephosphorylation by Western blot shows a vastly slower rate than for any linear peptide in solution (**Figures 3.24B and C**). The turnover rate for the JAK2 pYpY peptide is approximately 10 events per second whereas for the intact kinase domain it is nearly 500 times slower (0.023 per second). Although there are many caveats with this analysis, for example the intact kinase domain is not at saturating concentration, there is clearly a large difference in dephosphorylation rate between the kinase domain and the isolated peptide.

I hypothesize that this is due to the steric hindrance by the core of the kinase domain and that it is possible that in cells there may be a change in the position of the activation loop that allows PTP1B access. This section investigates conditions that artificially enhance the accessibility of the JAK activation loop to investigate whether it increases the rate of PTP1B-catalysed dephosphorylation with the aim of exploiting such conformations to generate a PTP1B-JAK complex for further structural studies.

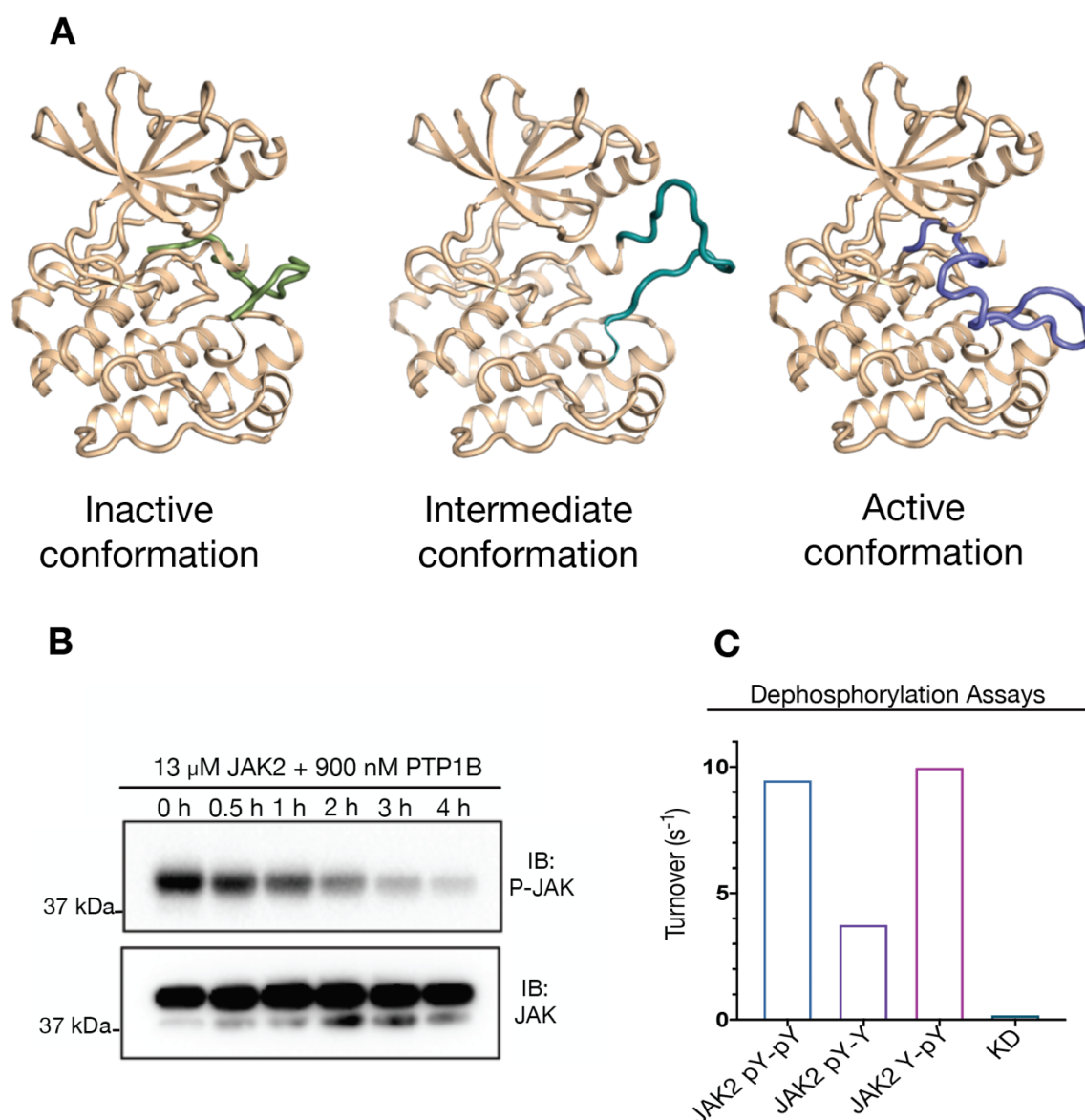


Figure 3.24 Activation loop conformation and accessibility.

A) Conformations of the insulin-like growth factor 1 kinase domain activation loop (PDB IDs, inactive: 1P40 (Munshi et al. 2003) Intermediate: 3D94 (Wu et al. 2008), Active 1K3A (Favelyukis et al. 2001)). Inactive conformation where the activation loop is tucked into the kinase domain (left). (Middle) Intermediate conformation between the active and inactive positions (middle). Active conformation where the activation loop has both phosphotyrosine residues phosphorylated and is positioned out away from the kinase domain (right). **B)** Dephosphorylation of the JAK2 kinase domain by PTP1B. Top panel indicates dephosphorylation over a 4-hour time period as visualised by western blot for P-JAK. Bottom panel shows total JAK2 present in the sample as visualised by western blot. **C)** PTP1B dephosphorylation rates in turnover per second for various JAK2 peptides (pYpY, pYY and YpY) and the JAK2 kinase domain.

3.13.1 Some conformations of the activation loop are more accessible to PTP1B

Kinase inhibitors can be grouped together based on how they bind kinase domains. Type I inhibitors, such as itacitinib, bind and block the ATP-binding site, thereby locking the activation loop in its active conformation (Tvorogov et al. 2018). Type II inhibitors, such as CHZ868 and BBT595, bind at a hydrophobic pocket adjacent to the ATP binding site and cause the activation loop to become more flexible and solvent exposed, potentially similar to the intermediate conformation as seen for the insulin-like growth factor 1 kinase (Wu et al. 2008; Tvorogov et al. 2018).

PTP1B dephosphorylation of the activation loop in different conformations was examined using a Type I inhibitor (itacitinib), a type II inhibitor (CHZ868 and BBT595) or no inhibitor (DMSO control). 5 mM EDTA was added to each reaction to ensure re-phosphorylation of the JAK activation loop would not occur in case there was low concentrations of ATP and magnesium in the reaction buffer. Samples were taken up to four hours and a no PTP1B sample was included to show that dephosphorylation did not change over the four-hour time course in the absence of enzyme. Samples were subsequently separated by SDS-PAGE gel, transferred to a membrane and probed with phosphorylated-JAK antibody to determine phosphorylation levels at the activation loop.

Dephosphorylation of JAK in the presence of Type I inhibitor (itacitinib) was similar to DMSO control. In this conformation, the activation loop is forced into a rigid active position which may be even less accessible to phosphatases than when in the apo state, **(Figure 3.25A and B)**. Interestingly, in the presence of the type II inhibitors (CHZ868 and BBT595), the activation loop was rapidly dephosphorylated by PTP1B. This suggests that the activation loop adopts a more flexible position, allowing phosphatases to interact with the kinase domain and dephosphorylate the activation loop **(Figure 3.25C and D)**.

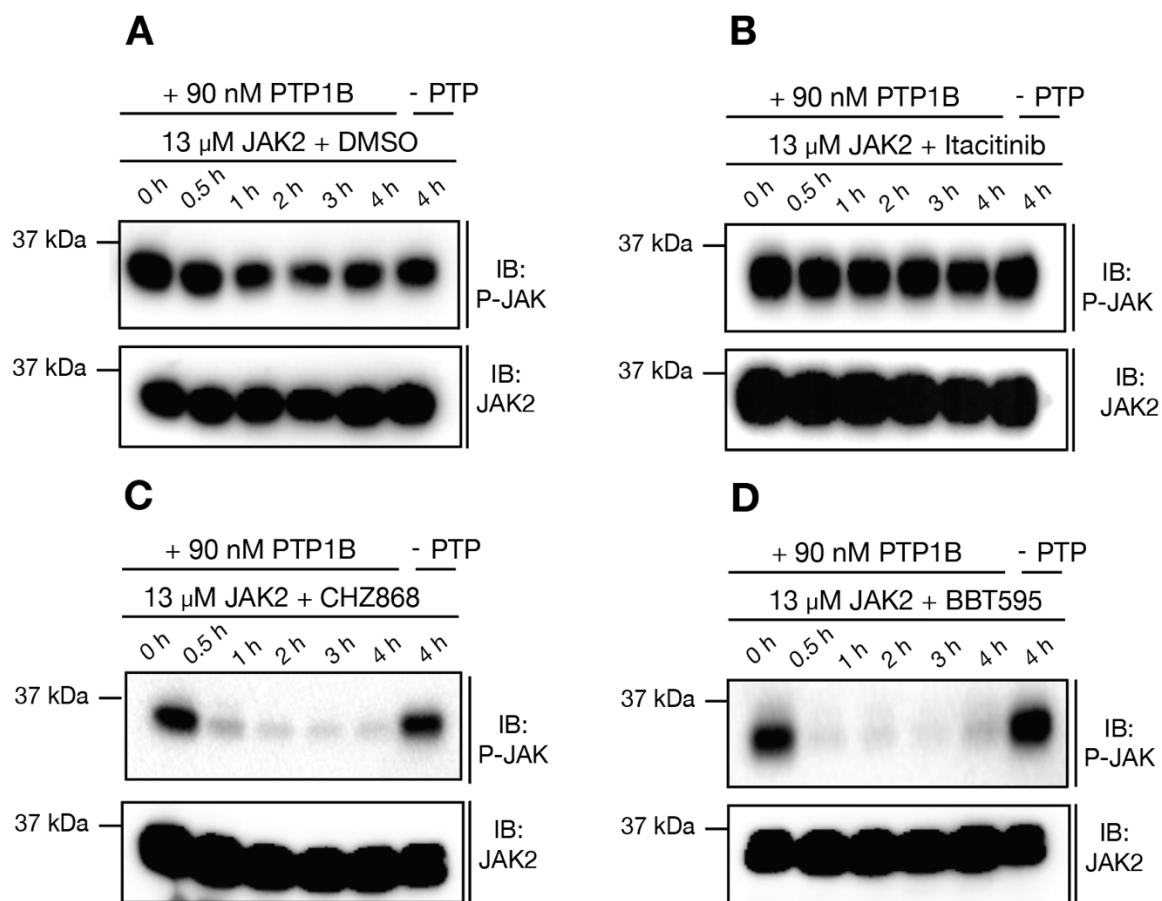


Figure 3.25 Dephosphorylation of the JAK activation loop in the presence of kinase inhibitors.

*Dephosphorylation of the JAK kinase domain by PTP1B. (Top) Immunoblot probing for phosphorylated-JAK at the activation loop. (Bottom) Immunoblot probing for total JAK2 indicating equal loading of samples. JAK kinase domain treated with DMSO **A**) and type I inhibitor Itacitinib **B**) were not dephosphorylated over a 4-hour incubation period. JAK2 kinase domains incubated with the type II inhibitors CHZ868 **C**) and BBT595 **D**) were rapidly dephosphorylated within half an hour, suggesting that the more flexible activation loop was more accessible to PTP1B. Blots are representative of N=3.*

A second approach was also developed to change the accessibility of the activation loop. A construct of JAK1 with a second, artificial linear activation loop at the flexible N-terminus of the domain was used to compare dephosphorylation. This linear activation loop should be more accessible than the native activation loop and most similar to a peptide. The WT JAK1 kinase domain with a type II inhibitor was also used as a positive control, as the previous assay showed rapid dephosphorylation (**Figure 3.26A**). Western blot analysis indicated that the linear activation loop was dephosphorylated far more rapidly than the native activation loop (**Figure 3.26B-D**). Additionally, the linear activation loop is separated from the kinase domain by a protease cleavage site. Addition of precision protease, which cleaves the linear activation loop, revealed that the majority of the phosphorylation signal for the JAK kinase domain + activation loop peptide sample is from the linear activation loop, as the signal is significantly decreased once removed (**Figure 3.29B + precision**). In samples where precision protease was added, no PTP1B was present in the sample. These results suggest that accessibility is a major determinant of dephosphorylation rate for the JAK activation loop.

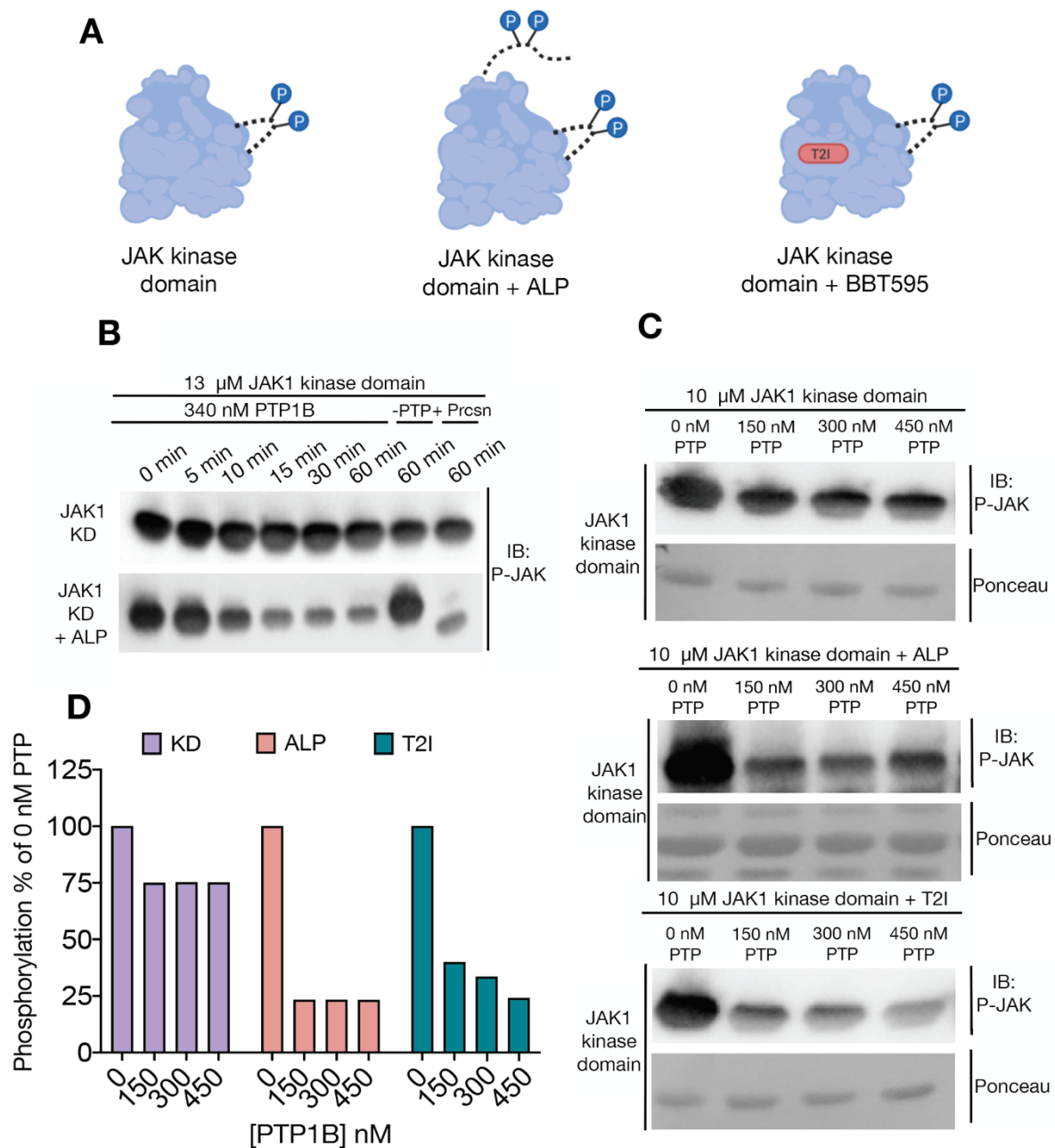


Figure 3.26 Accessibility of the JAK activation loop is important for dephosphorylation.

A) Schematic diagram of the three JAK kinase domain states. From left to right, WT JAK1 kinase domain, JAK1 kinase domain with an extra linear activation loop, and the JAK1 kinase domain plus a type II inhibitor. **B)** Dephosphorylation assay time course of WT JAK1 kinase domain and the JAK1 kinase domain + activation loop peptide. **C)** Dephosphorylation assay with increasing concentrations of PTP1B for three proteins. The WT JAK1 kinase domain is dephosphorylated to a lesser extent than either JAK1 kinase domain + activation loop peptide or the JAK1 kinase domain with a type II inhibitor, BBT595. Ponceau stain indicates equal loading of proteins across lanes. **D)** Quantification of western blots from C as normalised to the sample with - PTP1B (0 nM PTP1B).

Given that the type II inhibitor appeared to enhance the interaction between JAK1 and PTP1B, crystal trays were set up with inactive PTP1B DQC mutant and JAK2 kinase domain pre-incubated with 5 µg/µL of BBT595, a type II inhibitor. Proteins were mixed together at 5 mg/mL each and set up in sitting drop, vapour diffusion crystal screens at C3 CSIRO with 150 nL protein solution and 150 nL precipitant over a 50 µL reservoir volume. Drops were also set up with the same PTP1B DQC and JAK2 kinase domain mix, minus BBT595. Crystals grew in 5/480 conditions tested and of these, three were large enough (~100 µm) to analyze for diffraction at the Australian Synchrotron MX2 beamline. Crystals were flash-frozen in liquid nitrogen, with paratone as cryoprotectant. Three of the four crystals taken to the synchrotron diffracted, with two diffracting to less than 4 Å (**Figure 3.27**), however no data was collected as each crystal was clearly not single. Several attempts have been made to further optimise conditions, however no new crystals have grown in these conditions to date. Additionally, the contents of the crystals could not be verified due to limited sample.

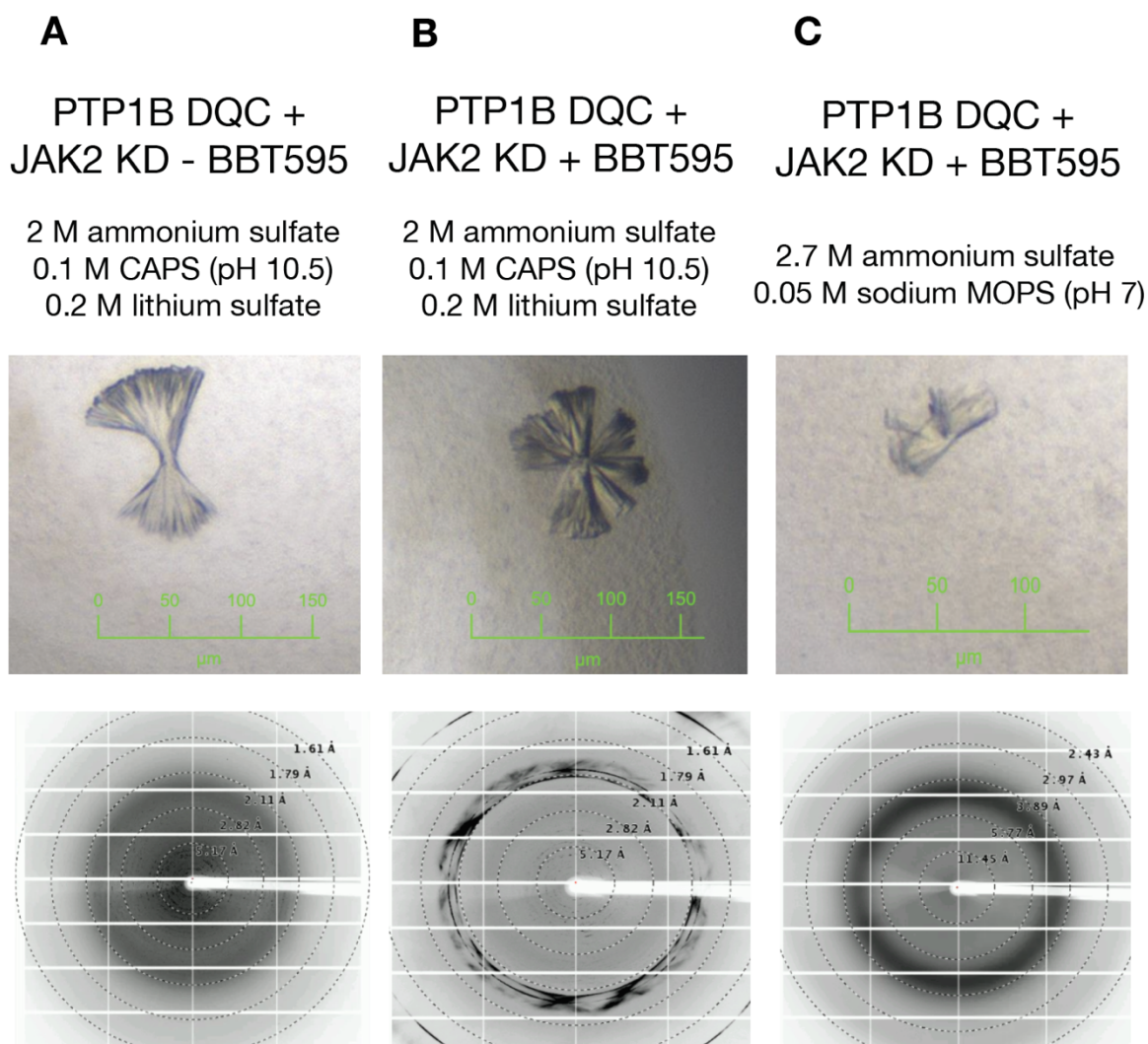


Figure 1.3.27 PTP1B and JAK2 kinase domain plus BBT595 crystals.

Crystals grown in C3 screens with PTP1B DQC and the JAK2 kinase domain A) PTP1B DQC plus JAK2 kinase domain without an inhibitor. B) PTP1B DQC + JAK2 kinase domain with a type II inhibitor (BBT595). C) PTP1B DQC + JAK2 kinase domain with a type II inhibitor (BBT595).

3.14 Complexes between PTP1B and JAK

The results from section 1.17.1 demonstrated how JAK activation loop accessibility determines their dephosphorylation rate by PTP1B. To determine whether other domains in JAK regulate dephosphorylation (perhaps via altering activation loop accessibility), dephosphorylation of the JAK1 kinase domain was compared to the full length JAK1 protein (**Figure 3.28**). This revealed little to no difference in PTP1B dephosphorylation of the activation loop in JAK1 kinase domain or full length construct (**Figure 3.28A**). This suggests equivalent accessibility of the activation loop to PTP1B in the presence or absence of the other JAK domains, therefore an alternate mechanism must regulate accessibility in the cell.

Size exclusion gel filtration chromatography experiments were also performed with the kinase domain or full length JAK construct mixed with the PTP1B DQC mutant to investigate whether the proteins could form a complex. No complex could be generated between the PTP1B DQC and the JAK kinase domain (data not shown). However, gel filtration assays with full length JAK1 and PTP1B revealed that there was some association (**Figure 3.28B, C and D**). Full length JAK1 elutes from a 10/30 column after approximately 12 mL, and PTP1B DQC alone after 16 mL. A series of additional broad peaks were observed when the two proteins were mixed that eluted earlier than either protein alone. SDS PAGE analysis showed that both JAK1 and PTP1B were present in these peaks. Probing for phosphorylated JAK and PTP1B revealed that these peaks were enriched for phosphorylated JAK1 (**Figure 3.28B and E**) as might be expected if the phosphatase is targeting the phosphorylated activation loop. Although this is clearly a highly heterogeneous complex and it is unclear whether this is oligomerisation or simply aggregation, it is the first time I observed evidence of an association that could be partially trapped by gel filtration. Although purifying large amounts of the full-length kinase is difficult, current efforts lie in this direction in order to allow us to study this potential interaction further.

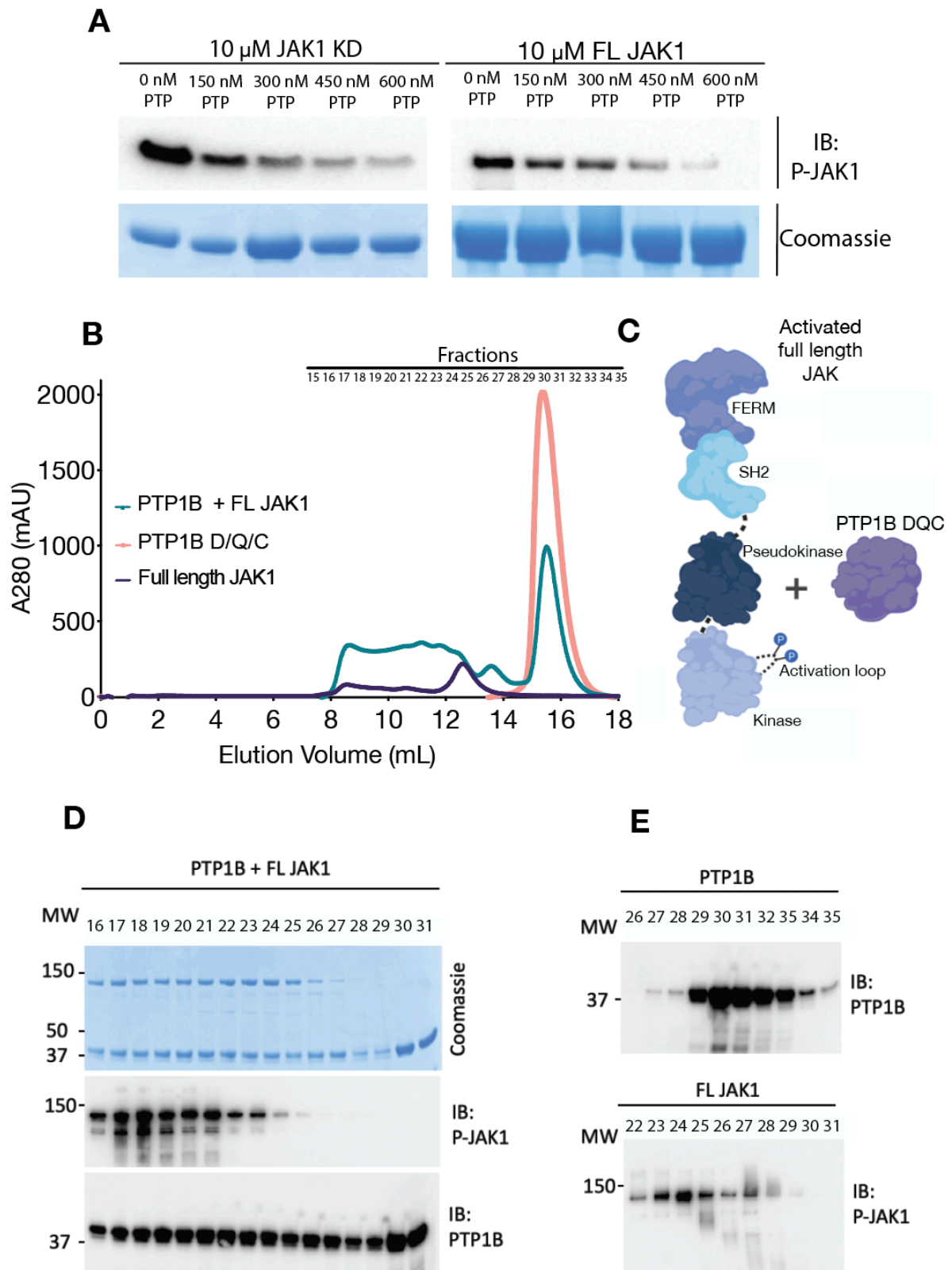


Figure 3.28 Interaction of PTP1B with full length JAK.

A) Dephosphorylation assay of JAK1 kinase domain (KD) and full length (FL) JAK1 by PTP1B. (Top) immunoblot for phospho-JAK1. (Bottom) Coomassie stained gel showing equal loading. **B)** Gel filtration chromatographs from 10/30 column. PTP1B alone (pink) which elutes at approximately 14-17 mLs. Full length JAK1 alone (purple) elutes between approximately 12 and 14 mLs. PTP1B and full length JAK1 pre-incubated together (green) elute 8-17 mLs with multiple peaks. Fractions are indicated. Experiment is representative of N=3. **C)** Schematic of full length JAK and PTP1B. **D)** Fractions from PTP1B and FL JAK1 together. The proteins begin eluting after 8 mLs, with the fractions prior to elution of the singular proteins being from 16-23. (Top) Coomassie stained gel showing both PTP1B DQC (37 kDa) and full length JAK1 (130 kDa) eluting over these fractions. (Middle) Immunoblot for phosphorylated JAK1 (P-JAK1 antibody specific for activation loop) indicating that the majority of phosphorylated JAK1 elutes in fractions 16-23. (Bottom) Immunoblot for PTP1B, which elutes in all the fractions when incubated with FL JAK1. **E)** (Top) Immunoblot for PTP1B for fractions from PTP1B run alone, indicating that the majority of PTP1B elutes in fractions 29-34. (Bottom) Immunoblot for P-JAK1 for fractions of full length JAK1 alone, which shows that the majority of phosphorylated FL JAK1 elutes in fractions 22-27.

3.15 Discussion

Previous studies have highlighted the role of PTP1B in regulating leptin, IL-4 and IL-10 signalling (Zabolotny et al. 2002; Pike et al. 2014; Lu et al. 2008). In this chapter it was shown that PTP1B can also inhibit signalling by IL-6 (**Figure 3.2**), a cytokine that PTP1B has not been previously implicated in controlling. The dominant paradigm of how PTP1B regulates cytokine signalling is that it dephosphorylates the JAK kinases that initiate the intracellular signalling cascade, causing them to transition back into an inactive state, (Myers et al. 2001). However, it was notable that this hypothesis had never been validated using intact, purified PTP1B and JAK proteins. Here it was explicitly demonstrated that PTP1B can indeed dephosphorylate the activation loop of the kinase domain for all four members of the JAK family (**Figure 3.3**).

PTP1B is considered the prototypical protein tyrosine phosphatase, and extensive research has revealed much about its dephosphorylation mechanism, however less is known about its substrate specificity. Since the development of substrate trapping mutants in 1997, it has been possible to determine many of the physiological substrates for PTP1B (Flint et al. 1997), however it has not been possible to solve the structures of phosphatases bound to their (intact) protein targets. Having confirmed that JAK is a substrate of PTP1B my major goal was to use novel substrate-trapping mutants of PTP1B to overcome this problem and generate stable complexes between PTP1B and JAK for structural studies. Despite extensive experimentation it was not possible to generate a stable complex that was amenable to crystallography, at least to date. Therefore, I took an approach similar to that taken by previous phosphatase researchers (Jia 1997; Salmeen et al. 2000). This was to study the dephosphorylation site on JAK as a peptide. In this manner I aimed to thoroughly interrogate this minimalist experimental system in regard to substrate preference, mode-of-interaction, enzyme kinetics and structure. As a short peptide is not an accurate representation of the activation loop of a kinase when it is part of an intact protein domain, many of the findings here can only be extrapolated to the two intact proteins with caution. It is with that caveat in mind that the results will now be discussed.

3.15.1 The PTP1B/JAK activation loop complex

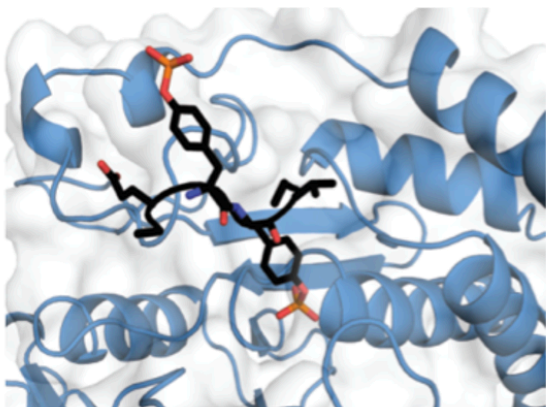
The three key catalytic residues of the PTP1B phosphatase domain are Gln 262, Asp 181 and Cys 215. Two mutants were used in this study lacking two (DQ mutant) or all three (DQC mutant) of these residues. These proteins were used in crystallographic studies with the addition of peptides representing the activation loops of JAK1, JAK2, JAK3 and TYK2. As PTP1B was capable of dephosphorylating the kinase domains of all four JAK proteins (**Figures 3.3**), structures of the activation loops of these kinases in complex with PTP1B were solved (**Figures 3.9, 3.12 and 3.13**). All four of these peptides contain tandem phosphotyrosines and it was expected that the first of these would occupy the active site, considering the established notion that PTP1B uses a second aryl binding site to interact with tandemly phosphorylated tyrosine residues (Puius et al. 1997; Salmeen et al. 2000).

Despite the DQ substrate trapping mutant having reduced catalytic activity (**Figure 3.5**), there was sufficient residual activity such that structures showed dephosphorylation of all phosphotyrosines contained within the peptides used (**Figure 3.9**). It is not completely clear whether hydrolysis of these phosphates occurred within the crystal or prior to crystal formation. However, the presence of a trapped phosphate group in the active site suggests that at least one dephosphorylation event occurred within the crystal lattice. Additionally, given that the catalytic cycle involves release of the dephosphorylated tyrosine prior to hydrolysis of the cysteinyl phosphate intermediate, it was unexpected to see both the peptide and free PO₄ bound to PTP1B. This may indicate that either the Asp 181 or Gln 262 residues, or both, are somehow involved in release of the dephosphorylated substrate from the catalytic site.

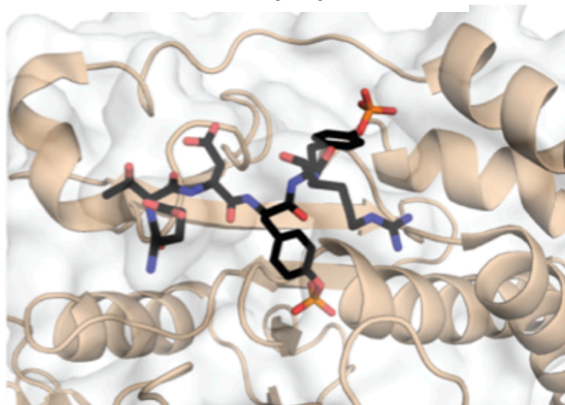
The problem of the residual activity of the PTP1B DQ mutant was circumvented by mutating all three catalytic residues to alanine (Q262, D181 and C215, the DQC mutant) (**Figure 3.10**). This proved to be completely inactive as in structures with this mutant no dephosphorylation was observed. Structures of the DQC mutant with JAK peptides with tandemly phosphorylated activation loops revealed that the second phosphotyrosine occupied the catalytic pocket while the first phosphotyrosine interacted with another positively charged surface of the protein, centered about Arg47 (**Figure 3.12 and 3.13**). The positioning of these two phosphotyrosines was unexpected however, similar

observations have been made previously in structures of PTP1B with bidentate inhibitors (Groves et al. 1998; Asante-Appiah et al. 2002). These studies also noted that the Arg 47 residue is important for coordinating the negatively charged moiety (Asante-Appiah et al. 2002; Groves et al. 1998)(**Figure 3.29 right**). It would be interesting to perform mutagenesis studies on Arg 24 (in the second aryl binding site) and Arg 47 to compare whether mutation of either could force a particular binding orientation (ie. IRK or JAK2-like binding (**Figure 3.29**)).

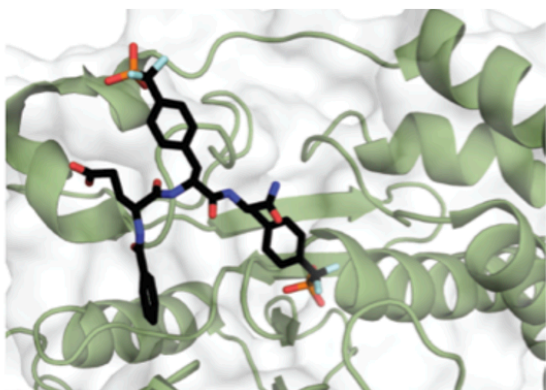
JAK2 pYpY ALP



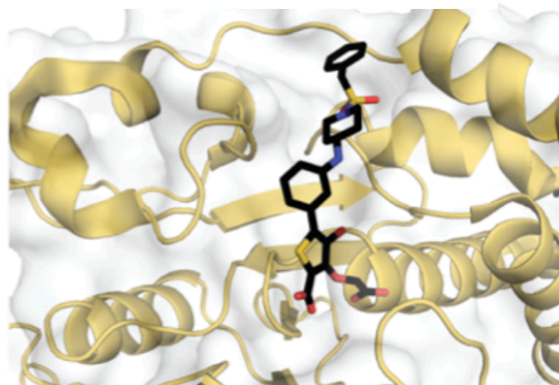
1G1F: IRK pYpY ALP



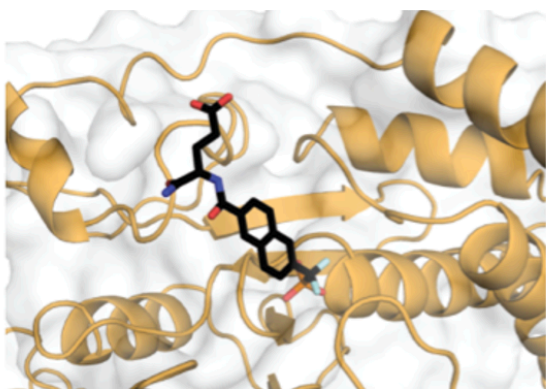
1LQF: bisphosphonate inhibitor



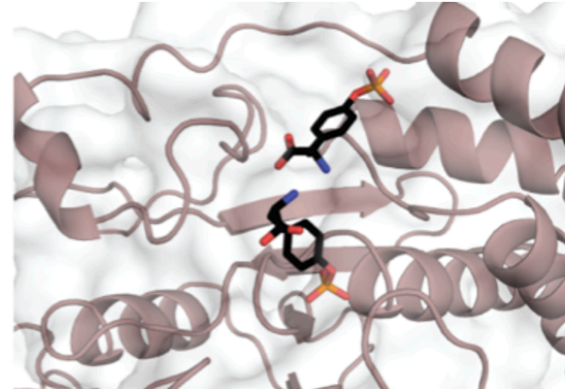
2QBP: PTP inhibitor



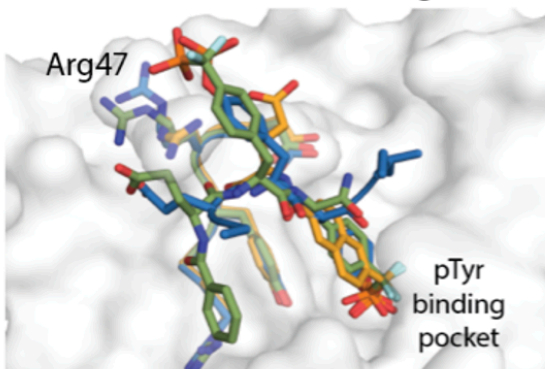
1BZC: PTP inhibitor



1PTY: Phosphotyrosine molecules



JAK2-like binding



IRK-like binding

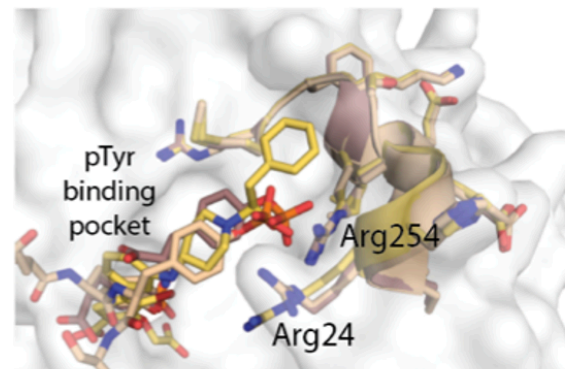


Figure 3.29 Two modes of interaction with PTP1B.

*Structures of PTP1B in complex with molecules that bind the phosphatase domain separated on the basis of how they interact with PTP1B. **Left)** Structures of molecules that bind in a similar orientation to the JAK2 activation loop peptide. From top to bottom: The JAK2 pYpY activation loop peptide structure presented in this thesis, structure of PTP1B with a peptidic bisphosphonate inhibitor bound, PDB ID: 1LQF (Asante-Appiah et al. 2002), with a phosphotyrosine mimetic bound, PDB ID: 1BZC (Groves et al. 1998), and all three structures aligned, with the Arg 47 residue highlighted for each structure (JAK2 in blue, 1LQF in green and 1BZC in yellow). **Right)** Structures of molecules that bind in a similar orientation to the IRK activation loop peptide. From top to bottom: The structure of PTP1B with the IRK activation loop peptide bound, PDB ID: 1G1F (Salmeen et al. 2000), with a PTP inhibitor bound, PDB ID: 2QBP (Wilson et al. 2007), with two phosphotyrosine molecules bound, PDB ID: 1PTY (Puius et al. 1997), and all three structures aligned, with the Arg 24 and Arg 254 residues highlighted for each structure (1G1F in salmon, 2QBP in yellow and 1PTY in pink).*

As the positioning of the phosphotyrosines in the tandemly phosphorylated peptides was unexpected, a thorough enzymatic study was performed to determine whether the second of the phosphotyrosines was indeed the preferred substrate for JAK2. It was observed conclusively that that is the case. For JAK2, a peptide with the second tyrosine phosphorylated is a better substrate than the first and when both are phosphorylated the second is hydrolysed most rapidly (**Figure 3.20**). This was not the case for IRK. Here the analysis showed that there is a slight preference for the first phosphotyrosine in the catalytic pocket (**Figure 3.21**), consistent with previous structures of PTP1B with the IRK activation loop peptide (Salmeen et al. 2000). It is now clear that PTP1B can bind substrates with tandem tyrosine residues with either phosphotyrosine in the catalytic pocket, however, some substrates have a bias to form higher affinity interaction in one or the other conformation.

Despite the fact that PTP1B displayed a preference for binding the second of the JAK2 phosphotyrosines in its active site, structures of complexes between the enzyme and peptides where only the first tyrosine was phosphorylated showed clearly that this could also be accommodated (**Figures 3.16 and 3.18**). Likewise, enzymatic studies indicated that they could be dephosphorylated, albeit at a slower rate (**Figure 3.20**). Therefore, PTP1B is capable, at least in vitro, of dephosphorylating JAK no matter which tyrosine is phosphorylated or whether both are (**Figure 3.30**).

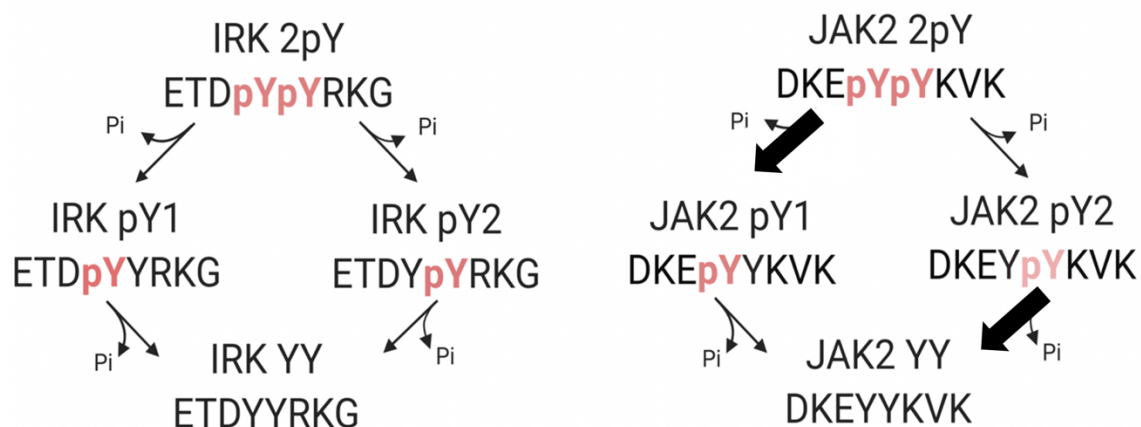


Figure 3.30 Dephosphorylation of the IRK and JAK2 activation loops by PTP1B

PTP1B dephosphorylates both the first and second phosphotyrosine residues of the activation loop in IRK (left) and JAK2 (right) at similar rates. For JAK2, PTP1B dephosphorylates the second phosphotyrosine residue of the activation loop much more rapidly than the first phosphotyrosine residue.

Similar to these studies, complete dephosphorylation of the IRK activation loop has previously been demonstrated for another phosphatase called LAR (Lee et al. 1992). This study revealed that a third phosphotyrosine (pTyr 1158) of the IRK activation loop is dephosphorylated first, followed by either the first or second phosphotyrosine (pTyr 1162 and pTyr 1163) as demonstrated in this chapter (**Figure 3.21**). Likewise, in the case of the JAK proteins, a study of SHP1 in complex with the JAK1 activation loop also showed that SHP1, another phosphatase with a second aryl binding site, binds the second phosphotyrosine in the catalytic pocket (Alicia-Velázquez, Jakoncic, and Boggon 2013). It is interesting that both phosphatases that target the JAK proteins have a preference for the second phosphotyrosine. Previous studies of the activity of the JAKs has revealed that the first tyrosine of the JAK2 activation loop is essential for activation of the kinase, whereas the second phosphotyrosine is dispensable (Feng et al. 1997). This raises interesting questions as to why the two phosphatases that target JAK show a preference for the second phosphotyrosine over the first. Further studies of JAK activity revealed that JAK2 is most active when both tyrosine residues are phosphorylated (Hall et al. 2010). Given that the results presented in this thesis show that the tandemly phosphorylated activation loop has the highest affinity for PTP1B (**Figures 3.20B-E**) it is

possible that PTP1B is only recruited to dephosphorylate JAK when a threshold of activation is reached, ie. when both tyrosine residues become phosphorylated.

The study of SHP1 bound to JAK1 (Alicia-Velázquez, Jakoncic, and Boggon 2013) also suggested that the category one phosphatases be split into two groups; those that interact with tandemly phosphorylated substrates using the second aryl binding site, and those that don't, even if their surface would allow it. The results presented in this chapter suggest that phosphatases with a second aryl binding site can interact with their substrates in both orientations. Most importantly, many inhibitors of PTP1B have been designed to selectively target the active site and second aryl binding site (Puius et al. 1997). With the findings presented that the interactions with Arg 47 and PTP1B are also important, it may be interesting to consider inhibitors that can interact with residues in all three positions. Moreover, this can be extended to drugs targeting other phosphatases such as SHP1 and TC-PTP which are structurally similar to PTP1B. Given that many of these phosphatases are potential therapeutic targets for diseases such as diabetes (Krishnan et al. 2018; Koren and Fantus 2007) and cancer (Julien et al. 2007; Jiang and Zhang 2008), higher affinity and more specific inhibitors will be of interest in the field.

3.15.1.1 JAK and IRK enzyme/substrate complexes

Because both the IRK and JAK2 activation loop peptides have residue property similarities (ie. two tandem phosphorylated tyrosine residues, a negatively charged residue prior to the first phosphotyrosine, and a positively charged residue proceeding the second) (**Figure 3.14**), it is unclear where differences in dephosphorylation stem from. Looking at adjacent residues on either side may provide the answers. In the JAK2 sequence there is a positively charged lysine residue whilst in the IRK activation loop there is an uncharged threonine residue two residues prior to the first tyrosine. It is possible that a lysine in this position is not well tolerated if in the -2 position, whereas an uncharged threonine poses no issue. To test this hypothesis, a version of the JAK2 peptide where this lysine has been swapped to a threonine residue as in the IRK peptide has been generated and will be tested. It is also possible that this threonine residue could be modified by phosphorylation in a cellular context which may alter binding and/or register of the IRK activation loop.

Previous studies examining PTP1B selectivity for various substrates have shown formation of higher affinity interactions with small peptide sequences in comparison to phosphotyrosine alone (Zhang et al. 1994). Catalysis is also improved with the addition of two flanking residues, indicating that the residues adjacent to the phosphotyrosine contribute to binding affinity (Zhang et al. 1994). Moreover, PTP1B appears to have preference for peptides that contain acidic, aromatic, or aliphatic (but not basic) residues in the -1 position (Zhang et al. 1996). In all the structures presented in this thesis, this holds true as the residue in the -1 position is either a tyrosine, a phosphotyrosine, or a negatively charged residue. Consistent with other studies (Sarmiento et al. 2000), the guanidinium group of Arg 47 in PTP1B often formed electrostatic interactions, with both the phosphate moiety in structures with a -1 position phosphotyrosine (**Figures 3.12 and 3.13**), and with negatively charged residues proceeding the phosphotyrosine in structures with only a single phosphorylated tyrosine residue (**Figures 3.16, 3.17 and 3.18**). Data showing that single amino acid substitutions in the YpY JAK2 peptide had little effect on K_m (**Figure 3.22**) is consistent with both the idea that PTP1B is able to dephosphorylate a wide array of peptide substrates, and that Arg 47 can take on multiple conformations to interact with residues proceeding the phosphotyrosine. The guanidinium headgroup of Arg 47 is able to interact with residues in the -1, -2 and -3 positions for many of the structures (**Figures 3.12, 3.13, 3.14, 3.17, 3.15 and 3.18**). This flexibility is also seen in the Apo structure where guanidinium group of Arg 47 is able to occupy the space for pY1 in JAK2 and TYK2 phosphate groups (**Figure 3.19**). Together these data support previous studies (Sarmiento et al. 2000) indicating that Arg 47 is important in forming interactions with residues proceeding the phosphotyrosine in PTP1B substrates, particularly negatively charged residues. This is further supported by studies in which mutation of Arg 47 to Ala decreases k_{cat}/K_m (Sarmiento et al. 1998), and mutation to Glu decreases it further (Zhang, Yao, et al. 2000).

3.15.2 Substrate specificity

PTP1B's ability to dephosphorylate a wide array of peptide substrates (**Figure 3.23**), together with the fact that a wide range of amino acid substitutions about the phosphotyrosine resulted in little change in dephosphorylation rate (**Figure 3.22**) suggest that specificity toward particular substrates may be a function of residues

outside the immediate surrounds of the phosphotyrosine. This was proposed after the structure of PTP1B in complex with the IRK was solved and revealed non-catalytic interactions between PTP1B and IRK. These interactions were suggested to be important for phosphatase recruitment to IRK (Li et al. 2005). A Glu at position 1022 in the IRK kinase domain is critical for this interaction with PTP1B along with Glu 1273 and His 1268. While all four JAKs lack the C-terminal extension seen in IRK containing the latter two residues, JAK1, JAK2 and TYK2 contain a glutamate residue at a similar position to 1022 in IRK. Based on structural alignment of IRK and the three JAK kinase domains, a shift in the loop region containing these Glu residues may facilitate a similar interaction. Despite this only IRK, JAK2 and TYK2 contain a GE sequence (**Figure 3.31**), which may constitute important residues for this interaction, however this requires confirmation by mutagenesis studies.

```

IRK E 1022: IIKGEAETR
JAK1 E 896: RYDPEGNDT
JAK2 E 877: DNTGEVVAV
JAK3 no E: PGDNTGALV
TYK2 E 925: DGTGEMVAV

```

Figure 3.31 Glycine and Glutamate residues in IRK, JAK2 and TYK2 at site of potential non-canonical interaction with PTP1B.

Glutamate 1022 in IRK was proposed as a novel PTP1B interacting site. This residue is preceded by a glycine in IRK. JAK1, JAK2 and TYK2 all have a glutamate residue in a similar location of their kinase domain, however only JAK2 and TYK2 have both a glycine and glutamate residue at this position.

It was notable that, although alterations in the sequence about the phosphotyrosine residue led to minor changes in dephosphorylation rate, by far the biggest effect was when the substrate peptide was part of an intact protein (**Figure 3.24**). Thus, in vivo, it is possible that PTP1B substrates are defined entirely by having an accessible phosphotyrosine, rather than the sequence around that phosphotyrosine. It was observed in this chapter that JAK1 incubated with a type II inhibitor was dephosphorylated more rapidly than the WT, which I hypothesize reflects a more

accessible activation loop position (**Figures 3.25 and 3.26**). If this is true it highlights the limited understanding of how the activation state and other factors may influence the position of the activation loop and therefore its susceptibility to dephosphorylation. In similar studies of the interaction between the SH2 domain of the SOCS proteins with the JAK activation loops, SOCS1 was shown to bind the JAK activation loop as a peptide, however a complex could not be formed between the intact protein, similar to what was observed with PTP1B in this chapter. Likewise, the addition of a linear activation loop or type II inhibitor allowed formation of a complex between the JAK and SOCS1 (Liau et al. 2018). This raises the possibility that there may be mechanisms in cells that allow for conformational changes in the JAK activation loop, making it more accessible for regulator binding. Given the incomplete understanding of JAK protein activation upon cytokine binding to receptors, more work is required to understand the mechanisms that control the position of the activation loop.

3.15.3 Towards a PTP1B-JAK complex

Substrate trapping mutants have been instrumental in discovering physiological substrates of PTP1B, and for understanding the PTP1B-substrate interaction using crystallography (Boubekeur et al. 2011; Flint et al. 1997; Li et al. 2005; Myers et al. 2001). Despite this utility, their efficacy depends on the ability of PTP1B to interact with its substrate under experimental conditions. Using gel filtration chromatography, no stable complex could be formed between PTP1B and any of the JAK kinase domains at concentrations adequate for structural studies (**Figures 3.6**). The absence of complex formation suggests that either the proteins were unable to interact, or that the interaction was too weak to survive the gel filtration assay at the concentrations used. Despite this, JAK1 incubated with a type II inhibitor is dephosphorylated more rapidly than the apo JAK1 kinase domain, which may reflect a more accessible activation loop position (**Figures 3.25 and 3.26**). Following from these findings, crystal trays of PTP1B mixed with JAK2 kinase domain plus BBT595, a type II inhibitor were set up with the idea that the high concentrations of the two proteins may drive complex formation. These yielded crystals (**Figure 3.27**), so far however, they have not produced diffraction data amenable to solving a structure and require further optimisation. Results indicating that the position of the activation loop of the JAKs is critical for them to be dephosphorylated

further highlight the limited understanding of how the activation state and other factors may influence the position of the activation loop. While it was not possible to generate a complex with the JAK kinase domain, an interaction between PTP1B and full length JAK1 was observed (**Figure 3.28**). This may indicate that interactions between PTP1B and other domains of the kinase are important for binding. Limited proteolysis could be used to investigate the interface with the kinase domain versus the full-length protein. Moreover, these molecules may be better candidates for protein crystallography or cryo-EM.

3.16 Conclusions

These are the first studies showing explicitly that PTP1B can dephosphorylate the JAK proteins and dissect its interaction with their activation loops. Phosphatases can be grouped into five categories based on their mode of binding to substrates. PTP1B is the classical member of category one, comprising phosphatases containing a second aryl binding site and open gateway region (Barr et al. 2009; Puius et al. 1997). In this chapter it was shown that while PTP1B is indeed able to bind tandemly phosphorylated substrates using this second aryl binding site, this is not the mode of binding for all substrates. PTP1B can bind tandemly phosphorylated tyrosine residues in two orientations, using Arg 47 or Arg 24 to interact with a phosphotyrosine in the -1 or +1 positions respectively. In fact, the second aryl binding site accommodates a diversity of side chains including hydrophobic residues, such as the Leu residue seen in the structure of PTP1B in complex with the EGFR peptide substrate (Milarski et al. 1993), and the phosphotyrosine residue as seen in the structure of PTP1B in complex with the IRK activation loop peptide (Salmeen et al. 2000). The Arg 47 residue of PTP1B interacts with negatively charged residues upstream of the phosphotyrosine in the catalytic pocket as seen in the structures presented in this thesis, and as previously shown, both aromatic and aliphatic residues can also be accommodated in this region. Together, these interaction sites accommodate substrates with varying sequences, while providing some specificity for certain side chains and peptide sequences.

This chapter also presented further evidence that the conformation of the JAK activation loop is imperative for interactions with negative regulators of the JAK proteins, and that

a deeper appreciation is required of the molecular dynamics of the activation loop and the conditions mediating their conformation to understand how and when the negative regulators can interact with JAKs. Together these results provide a survey of PTP1B phosphatase domain interactions with the JAK activation loop to modulate JAK activity.

Chapter 4 LNK, a Negative Regulator of Cytokine Signalling

4.1 Introduction

LNK (SH2B3) is a member of the SH2 domain containing adaptor family of proteins, which also comprises APS (SH2B2) and SH2B (SH2B1) (Velazquez 2012; Hu and Hubbard 2006). All three proteins comprise a dimerization domain, plekstrin homology domain and an SH2 domain and are regulators of various signalling pathways downstream of cytokines and growth factors. LNK is specifically implicated in negatively regulating signalling. Given this, there is interest in studying LNK to understand how, and which pathways LNK is able to control (See section 1.4.2.1).

LNK has previously been shown to negatively regulate JAK2 activity downstream of MPL (Bersenev et al. 2008) and the EPOR (Tong, Zhang, and Lodish 2005), implicating LNK in the regulation of megakaryopoiesis and erythropoiesis. LNK has also been suggested to interact with a suite of other signalling proteins involved in proliferation and differentiation, including c-KIT (Gueller et al. 2008), FLT3 (Lin et al. 2012), c-FMS (Gueller et al. 2010) and PDGFR (Gueller et al. 2011). While these proteins have been identified as potential binding partners for LNK, they have not been validated as such by biochemical studies using purified proteins. The need to identify which biological processes are regulated by LNK is further underscored by the identification of LNK mutants in patients with a range of inflammatory, immune and haematopoietic diseases including cancer (Oh et al. 2010; Wang et al. 2016; Lavrikova et al. 2011). Currently the mechanism by which LNK negatively regulates signalling is poorly understood, and so the role of LNK mutations in these diseases is unclear. Understanding how LNK interacts with its substrates may shed light on how this negative regulation occurs, and why mutations in LNK contribute to disease burden in patients. The PH and SH2 domains of LNK are hotspots for mutations (Hurtado et al. 2011), and both are essential for the function of LNK. Given that the SH2 domain of LNK is the recognition domain for its inhibitory function, studying this domain is of great interest.

This chapter presents the structure of the LNK SH2 domain, the first structure of any LNK domain. The structure sheds light on the basis of the specificity of the SH2 domain for specific peptide sequences. Additionally, binding studies performed with phosphopeptides corresponding to phosphotyrosine residues on suggested LNK targets

confirm potential binding sites for LNK on EPOR, FLT3 and JAK3. Further studies of point mutants of the SH2 domain detected in patients with myeloproliferative neoplasms show how mutations in the SH2 domain may contribute to disease onset or progression by altering the ability of LNK to negatively regulate signalling via the JAKs due to reduced binding affinity to phosphorylated target sequences and potential loss of stability of the SH2 domain.

4.2 Studying the role of LNK in the regulation of TPO signalling

LNK has been characterised as a negative regulator of TPO signalling through its interaction with JAK2 downstream of the TPO receptor (MPL) (Tong and Lodish 2004). Conversely, APS and SH2B have been suggested to increase JAK2 activation in various pathways (Rui and Carter-Su 1999; Kurzer et al. 2006). The ability of APS, SH2B and LNK to regulate TPO signalling *in vitro* was investigated using M1 cells expressing MPL. These cells, termed M1-Cas9 + MPL are able to respond in a dose-dependent manner to TPO (**Figure 4.1B**) by differentiating into macrophages (**Figure 4.1A**). Upon differentiation into macrophages, the cells cease proliferation and undergo cell death. The ability of these proteins to regulate signalling can therefore be determined by comparing cell growth after TPO stimulation.

M1-Cas9 + MPL cells were transduced with doxycycline (Dox) inducible expression vectors containing either full-length APS, SH2B or LNK (all from mouse). Cells were grown for 4 days in DMEM supplemented with either saline, Dox only, 50 ng/mL of TPO only, or Dox and 50 ng/mL of TPO to determine the effect of overexpression of APS, SH2B or LNK on M1 differentiation. After 4 days, cell cultures were mixed with equal volumes of CellTitre-Glo (**Figure 4.1C**), which generates a luminescent signal relative to the amount of ATP present in the sample that is directly proportional to the number of living cells. In this assay. As TPO stimulation drives terminal differentiation, an increase in number of living cells is indicative of less cytokine signalling. Growth of cells in each treatment were normalised to growth of the saline control for each cell line (**Figure 4.1D**).

Growth of the WT M1-Cas9 cells (- MPL) was similar across all conditions consistent with their inability to respond to TPO. Stimulation of the M1-Cas9 + MPL cells with TPO resulted in a 60-65% reduction in growth in comparison to their saline or Dox only treated counterparts. A 35-40% reduction in growth was seen for both the M1-Cas9 + MPL + APS and M1-Cas9 + MPL + SH2B cells stimulated with TPO, with no significant difference observed between TPO only and TPO plus Dox. Conversely, when grown in TPO only, the M1-Cas9 + MPL + LNK cells grew on average 50% as much as the cells treated with a saline, however this reduction in growth partially rescued by overexpression of LNK, with the cells growing on average 80% as much as the saline control treated cells **(Figure 4.1D)**.

These results suggest that overexpression of APS and SH2B has no effect on response to TPO in the M1 cells, however overexpression of LNK negatively regulates TPO signalling, and causes less cellular differentiation, consistent with previous observations (Tong and Lodish 2004; Seita et al. 2007). Although expression of APS and SH2B had no observable effect on response to TPO, this may be due to the biological system used in this experiment or due to a lack of assay sensitivity. We had previously attempted to perform similar experiments in the Baf3 cell line, however as LNK may be a regulator of IL-3 signalling which is required for Baf3 survival these results were difficult to interpret. Moving forward we plan to characterise the change in response to TPO stimulation upon overexpression of LNK, SH2B and APS via the use of titration assays as carried out with IL-6 in Chapter 3 as this allows small changes in response to cytokines to be seen in a reproducible manner.

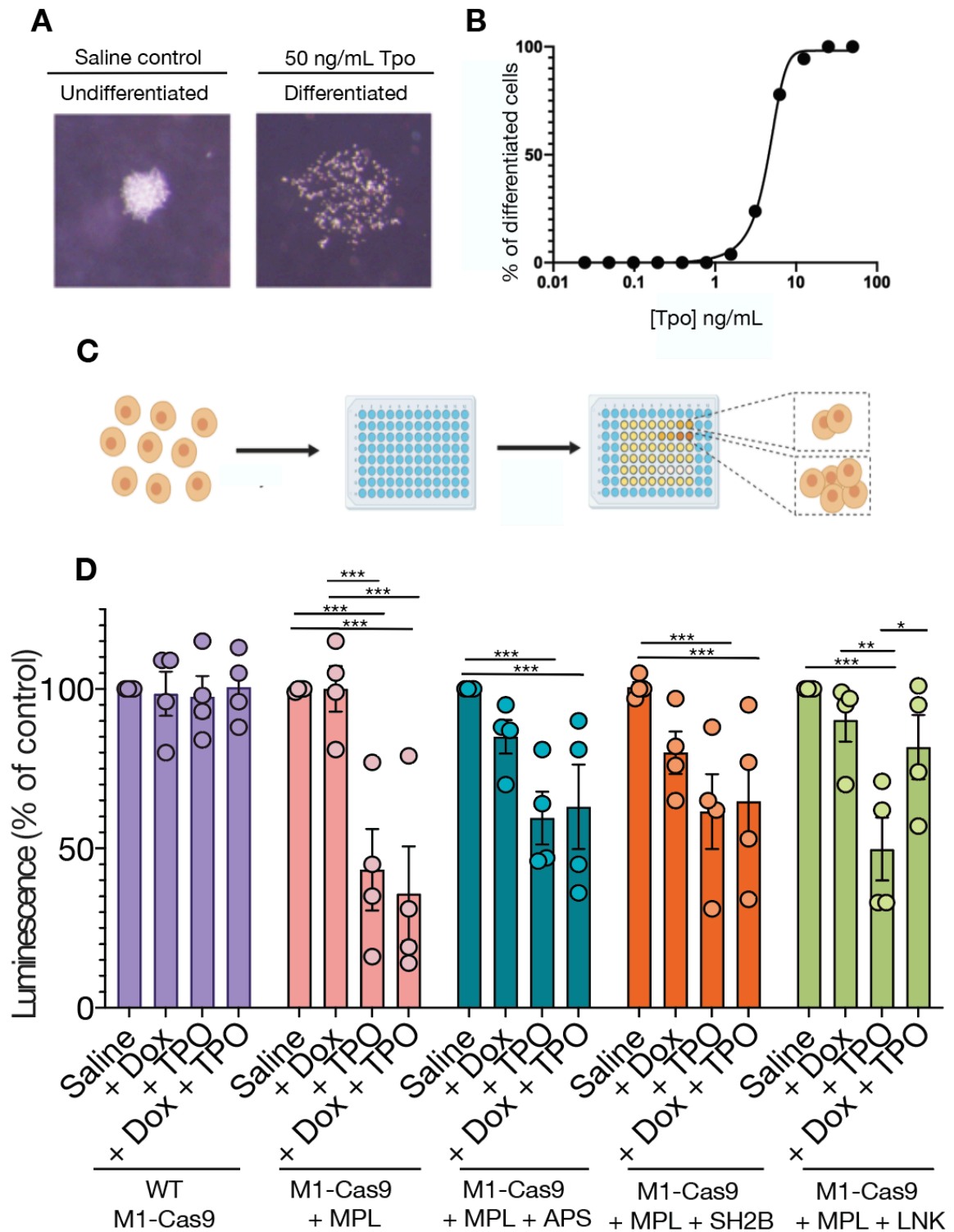


Figure 4.1 LNK negatively regulates TPO signalling in M1 cells.

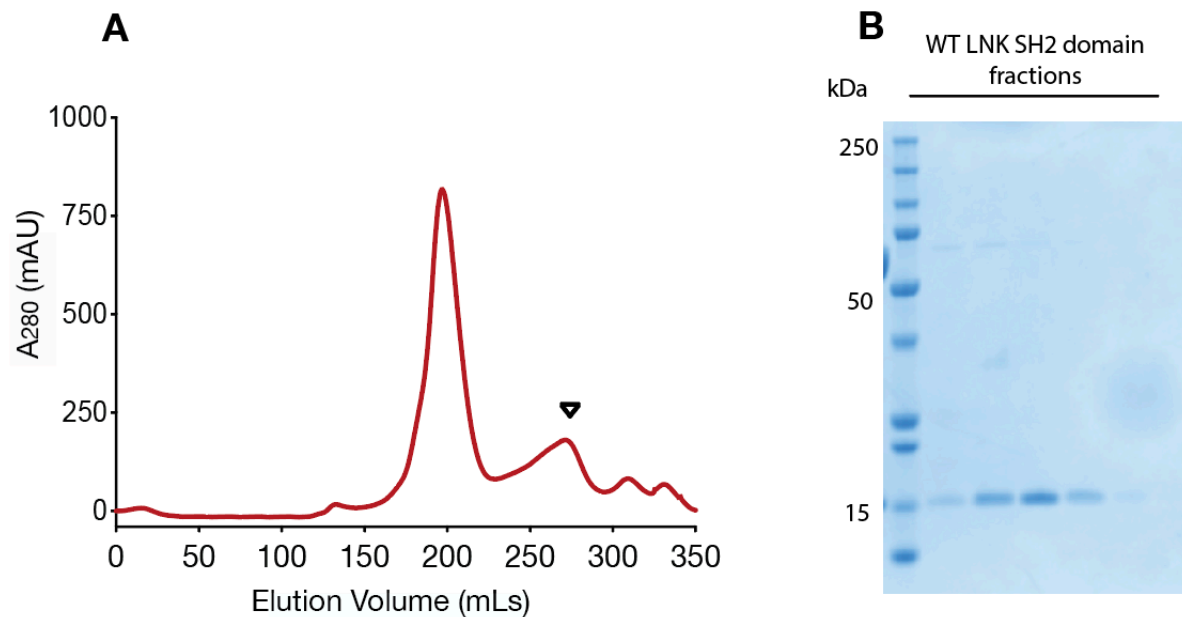
A) Image of M1 cells in semi-solid media assay. Left is untreated cells forming tight bundle colonies. Right is TPO stimulated cells that have differentiated into macrophages and gained the ability to disperse through the culture. **B)** Dose-response curve of M1 Cas9 + MPL cells to varying concentrations of TPO. The EC_{50} is approximately 5 ng/mL of TPO. **C)** Schematic diagram of the TPO-stimulation assay process. Cells transfected with Dox inducible plasmids were plated in a 96-well plate in various conditions and grown for 4 days. At the end of the incubation period, cells were mixed with CellTitre-Glo and transferred to a white opaque plate for quantifying luminescence in a plate reader. **D)** Response of cell lines to TPO stimulation. N= 4 and error bars are SEM. Two-way ANOVA with Tukey's multiple comparisons test; P= *** < 0.0001, ** 0.0068, * 0.00456.

4.3 The LNK SH2 domain binds pY813 of JAK2

In the previous section, it was shown that LNK is able to negatively regulate TPO mediated signalling in M1 cells. Previous studies have suggested that the LNK SH2 domain binds the pY613 and pY813 residues of JAK2 to downregulate activity (Bersenev et al. 2008; Gery, Cao, et al. 2009). To study the interaction between LNK and these phosphotyrosine residues in JAK2, the LNK SH2 domain was recombinantly expressed and tested for its ability to interact with these residues using isothermal titration calorimetry (ITC).

Using a construct containing a NusA fusion (De Marco et al. 2004), the LNK SH2 domain was expressed in BL21 (DE3) *E. coli* (**Figure 4.2**). NusA is a 55 kDa protein that interacts with and stabilises the passenger protein during translation, allowing higher yields of pure protein to be expressed (De Marco 2006). Using this system, the LNK SH2 domain with an N-terminal His tag was purified by nickel affinity. The NusA fusion protein was subsequently cleaved from LNK using tobacco etch virus (TEV) protease. Size exclusion chromatography was performed to yield pure LNK SH2 domain that could be used for structural and biochemical studies (**Figure 4.2A and B**). See section 2.13 of methods for details. A *M. musculus* construct of the LNK SH2 domain was used for expression, sharing 92.9% identity with the *H. sapiens* SH2 domain (**Figure 4.2C**).

Purified WT LNK SH2 domain was tested for its ability to bind to the JAK2 pY613 and pY813 residues using ITC. This technique allows the affinity of the SH2 domain for these peptides to be determined by measuring the heat released or absorbed as a result of binding. Phosphopeptides corresponding to two suggested LNK binding sites on JAK2; pY813 and pY613 were titrated into 10 μ M WT LNK SH2 domain to determine binding affinity (**Figure 4.2D**). The LNK SH2 domain bound to the JAK2 pY813 phosphopeptide with a high affinity of approximately 260 nM. No binding could be detected for the JAK2 pY613 peptide. It should be noted that the stoichiometry from the ITC analysis reproducibly appeared to be less than 1. This is potentially due to precipitation during the course of the experiment which we commonly see with our SH2 domains.



C

H. sapiens WFHGPISRVKAAQLVQLQGPDAHGVFLVRQSETRRGEYVLTFNFQGI AKHLRLSLTERGQ
M. musculus WFHGPISRVRAAQLVQLQGPDAHGVFLVRQSESRRGEYVLTFNLQGRAKHLRLVLTERGQ
 *****:*****:*****:*****:*****

H. sapiens CRVQHLHFPSVVDMLHHFQRSPIPLECGAACDVLSSYV 462
M. musculus CRVQHLHFPSVVDMLRHFQRSPIPLECGAACDVLSGYV 434
 *****:*****:*****:*****:*****

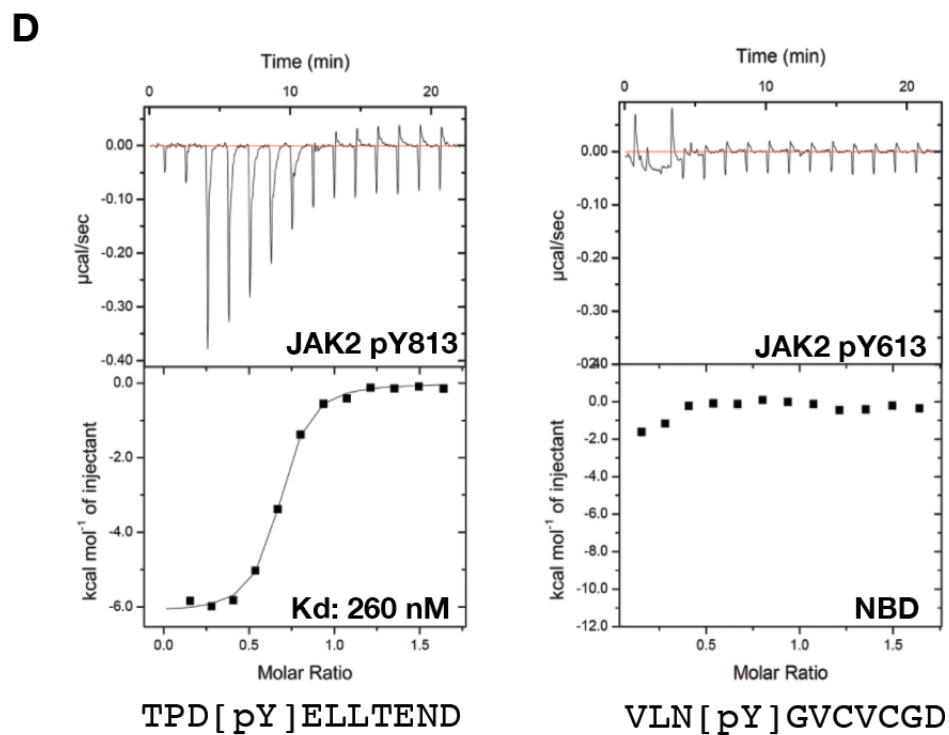


Figure 4.2 Purification of a functional LNK SH2 domain.

A) Representative chromatograph from size exclusion chromatography of the LNK SH2 domain. LNK elutes from the column at approximately 270 mL, to the right (arrow above) of the major peak, which is NusA on an S200 Superdex Hiload 26/600 column. **B)** Coomassie stained SDS-PAGE gel of peak fractions from gel filtration. The LNK SH2 domain is approximately 12 kDa. **C)** Sequence alignment of the mouse and human LNK SH2 domains. There are only 7 differing amino acids between the two proteins, giving them an overall sequence identity of approximately 92.9% **D)** Isothermal titration calorimetry curves. The K_D as determined by fitting to a 1:1 binding model is indicated on the figure. NBD = No binding detected. Experiment is representative of 2 experiments performed.

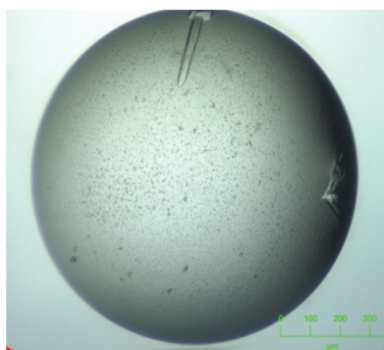
4.4 Studying the interaction between LNK and JAK2

Using purified, recombinant LNK SH2 domain, LNK was shown to bind a peptide based on the pY813 residue of JAK2. To understand this interaction at a molecular level, X-ray crystallography and surface plasmon resonance (SPR) experiments were performed.

4.4.1 Crystal structure of the LNK SH2 domain and the JAK2 pY813 peptide

SH2 domains are structurally conserved domains which bind phosphotyrosine residues with high affinities ranging from approximately 0.2 to 1 μ M (Machida and Mayer 2005). Their specificity for their target proteins is determined primarily by residues near to the target phosphotyrosine (Bradshaw and Waksman 1999). Here, X-ray crystallography was used to determine the structural basis for the interaction between the WT LNK SH2 domain and a JAK2 pY813 peptide. A pre-crystallisation test was performed to determine the appropriate LNK concentration for crystallisation (5 mg/mL). Sitting drop, vapour diffusion crystal trays screening 576 conditions were set up robotically at C3 CSIRO using 5 mg/ml of the LNK SH2 domain with a two-fold molar excess of the JAK2 pY813 phosphopeptide with 150 nL protein solution and 150 nL precipitant over a 50 μ L reservoir volume. Crystals or crystalline material formed in more than 90 conditions. The four largest crystals were cryoprotected with paratone, flash frozen in liquid nitrogen and taken to the MX2 beamline at the Australian Synchrotron. A dataset was collected for a crystal grown in 0.2 M ammonium sulfate, and 20% w/v PEG 3350, and the data was processed to 1.9 Å in XDS (Kabsch 2010) (**Figure 4.3**).

The structure of the LNK SH2 domain was solved by molecular replacement using the apo SH2B SH2 domain (PDB ID: 2HDV) as a model. A solution was found in the space group $P1\ 2_1\ 1$, with a top TFZ score of 12.5, and one copy of the protein in the asymmetric unit. Refinement was performed using PHENIX (Adams et al. 2010) and the model was built in Coot (Emsley and Cowtan 2004). Further details can be found in **Table 4.1**.

A

Condition: 0.2 M ammonium chloride and 20% w/v PEG3350

Unit Cell: 35.06, 37.875, 38.883, 90, 97.023, 90

Space group: 21

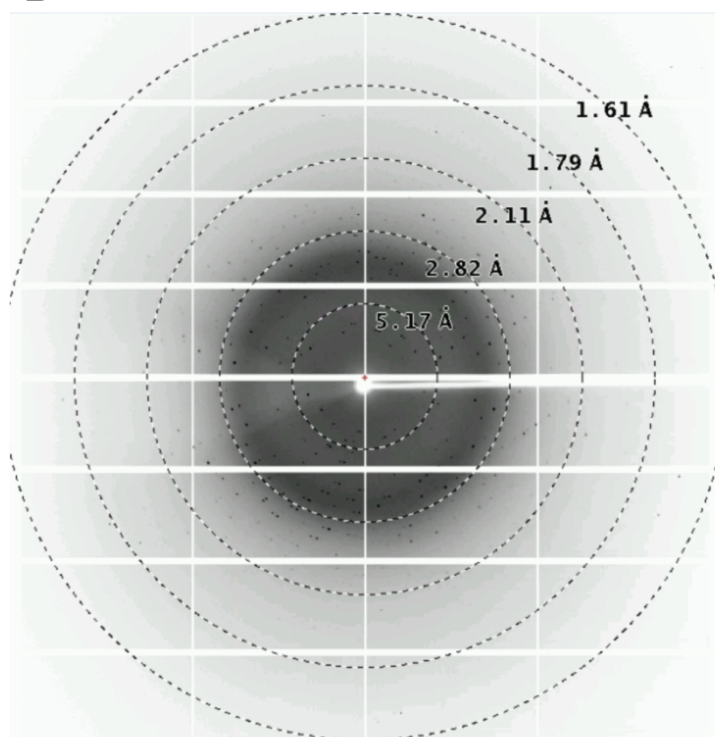
B

Figure 4.3 Crystals of the WT LNK SH2 domain with a JAK2 pY813 peptide.

A) Crystals generated by sitting drop vapour diffusion with crystal conditions, unit cell dimensions and space group listed. Crystal at top of drop is approximately 100 μM in length and 15 μM in width. **B)** Diffraction pattern from this at the MX2 beamline at the Australian synchrotron. Diffraction to 1.88 $\text{\AA}$.

Table 4.1 Data collection and refinement statistics for the LNK SH2 domains with phosphopeptides bound.

	WT LNK SH2 + JAK2 pY813	WT LNK SH2 + EPOR pY454	E400K LNK SH2 + JAK2 pY813
Data Collection			
Space group	P 2 ₁	P 6 ₁	C 2
Cell dimensions			
a, b, c (Å)	35.06 37.87 38.88	86.15 86.15 38.05	75.82 37.61 50.49
α, β, γ (°)	90 97.02 90	90 90 120	90 108.76 90
R _{meas} (%)	4.24 (46.51)	4.65 (53.90)	4.93 (76.49)
I /sigma(I)	18.70 (2.84)	31.00 (2.81)	20.45 (2.49)
CC _{1/2} (%)	99.90 (86.30)	100 (90.40)	99.90 (81.80)
Completeness (%)	99.62 (98.88)	99.77 (99.45)	99.04 (97.32)
Redundancy	3.4 (3.4)	9.6 (6.1)	6.8 (6.9)
Refinement			
Resolution (Å)	34.8 - 1.9 (1.97 - 1.9)	28.2 - 2.30 (2.38 - 2.3)	34.53 - 1.64 (1.70 - 1.64)
No. reflections	27662 (2731)	70442 (4411)	111266 (11000)
R-work	19.2 (22.9)	18.5 (25.8)	17.9 (23.8)
R-free	25.0 (29.2)	22.8 (28.3)	21.4 (26.9)
No. atoms			
protein	898	923	914
solvent	36	28	76
B factors			
protein	38.70	57.00	32.45
solvent	43.08	60.62	43.76
r.m.s. deviations			
RMS(bonds)	0.007	0.005	0.006
RMS(angles)	0.85	0.78	0.85
Ramachandran favored (%)	92.92	91.15	95.41
Ramachandran allowed (%)	6.19	7.08	4.59
Ramachandran outliers (%)	0.88	1.77	0.00
Rotamer outliers (%)	2.27	4.40	2.08

Values in parentheses are for highest-resolution shell.

The structure of the LNK SH2 domain adopts a typical SH2 domain fold comprising three central β -strands flanked by two α -helices (**Figure 4.4A**). Two loops in SH2 domains, termed the BG and EF loops, are often responsible for forming interactions with residues C-terminal to the pTyr of peptide substrates. Both the EF and BG loop of the LNK SH2 domain could be resolved, with residues from both loops forming contacts to residues C-terminal to the pTyr within the JAK2 pY813 peptide (**Figure 4.4B**). The +5 Glu residue was not resolved in the structure. There was additional density close by that could possibly correspond to residues +6 (Gln) and +7 (Asp) but this could not be unambiguously determined and they have not been fitted. The latter two residues, if present, did not make additional contacts with the LNK SH2 domain, but rather were at a crystal contact.

The Phosphotyrosine inserts into the canonical phosphotyrosine binding pocket (**Figure 4.4C**). This pocket is formed by Ser 366, Glu 367, Ser 368, Arg 369, Arg 387, His 385, Arg 343, Arg 364 and Val 374. The phosphate moiety forms hydrogen bonds with the invariant Arg 343 and a highly conserved Arg 364 residues along with the backbone amide nitrogen of Glu 367, and the sidechain of Ser 368 (**Figure 4.5A**). Glu 814 of the JAK2 peptide (P+1) forms a hydrogen bond with Lys 384 of LNK and a water molecule (**Figure 4.5B**). Leu 816 of the peptide (P+3) inserts into a hydrophobic pocket common to SH2 domains that is formed by Leu 386, Val 398, Leu 401, Phe 403, Phe 413, Ile 418 and Leu 420 of LNK (**Figure 4.5C**). Numerous backbone interactions form the remainder of intermolecular contacts. The backbone amide of Glu 421 hydrogen bonds with the +3 Leu carbonyl oxygen and the Pro 419 carbonyl oxygen hydrogen-bonds with the amide nitrogen and side chain of the +4 Thr of the pY813 peptide. Additionally, the backbone amide nitrogen of Arg 387 hydrogen bonds with the carbonyl oxygen of the +1 Glu through a water molecule, and the backbone carbonyl oxygen of His 385 hydrogen bonds with the amide nitrogen of the +1 Glu. Gln 399 forms two hydrogen bonds with the amide nitrogen and carbonyl oxygen of the +3 Leu.

Together, this model suggests that pTyr 813, Glu 814 (+1) and Leu 816 (+3) are the most important peptide residues for interaction with the LNK SH2 domain.

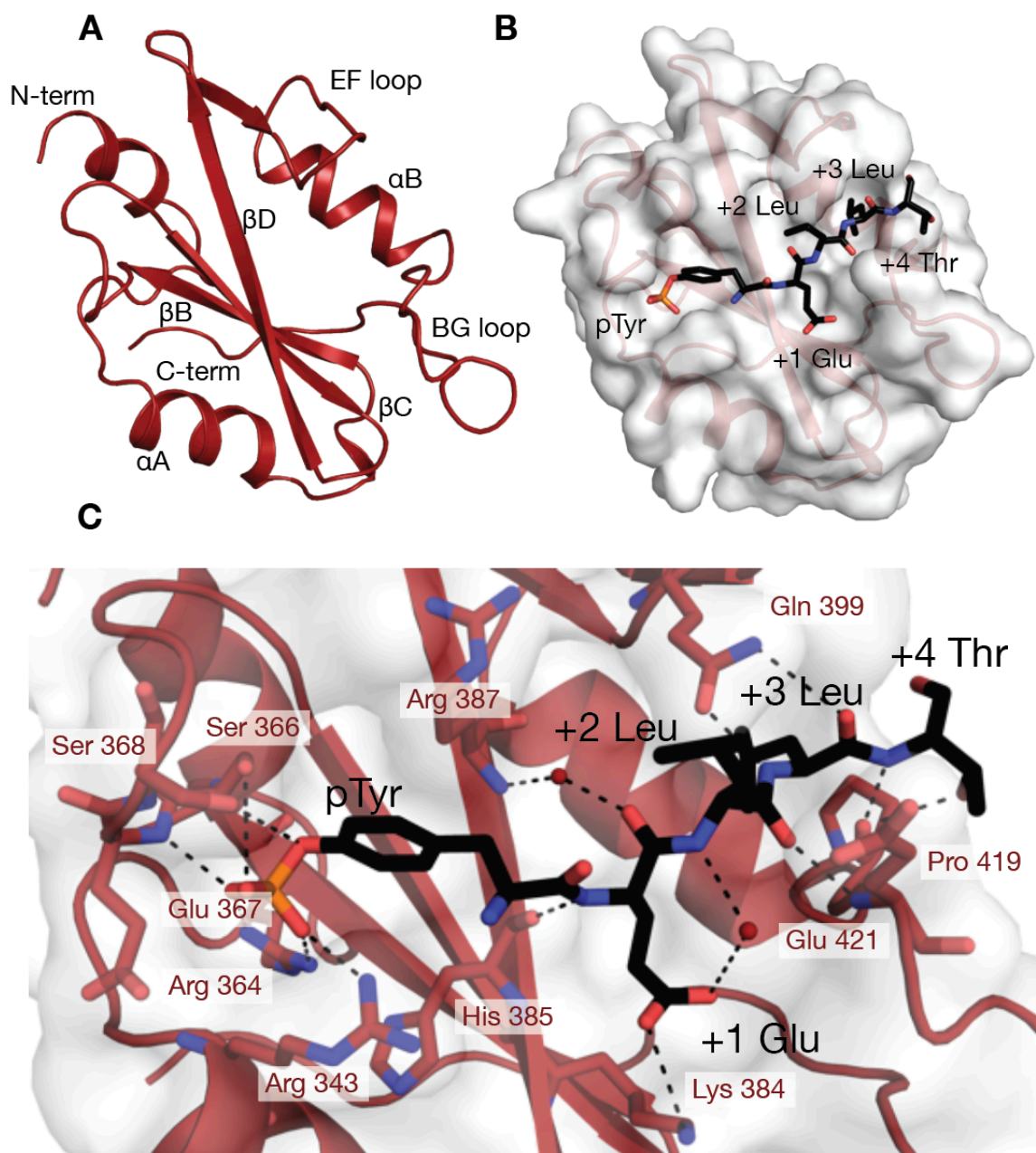


Figure 4.4 Structure of the LNK SH2 domain with a JAK2 pY813 phosphopeptide bound.

A) The backbone of the WT LNK SH2 domain with the features indicated (peptide not shown) is shown in cartoon representation. **B)** LNK SH2 domain structure with surface and peptide shown. Peptide residues are labeled. **C)** Peptide in black with LNK SH2 domain shown as a cartoon (red) and surface (white). The pTyr sits in the phosphotyrosine binding pocket of the SH2 domain, the +1 Glu forms a salt bridge with a Lysine residue in LNK and the +3 Leu sits in a hydrophobic pocket formed in part by the EF and BG loops of LNK.

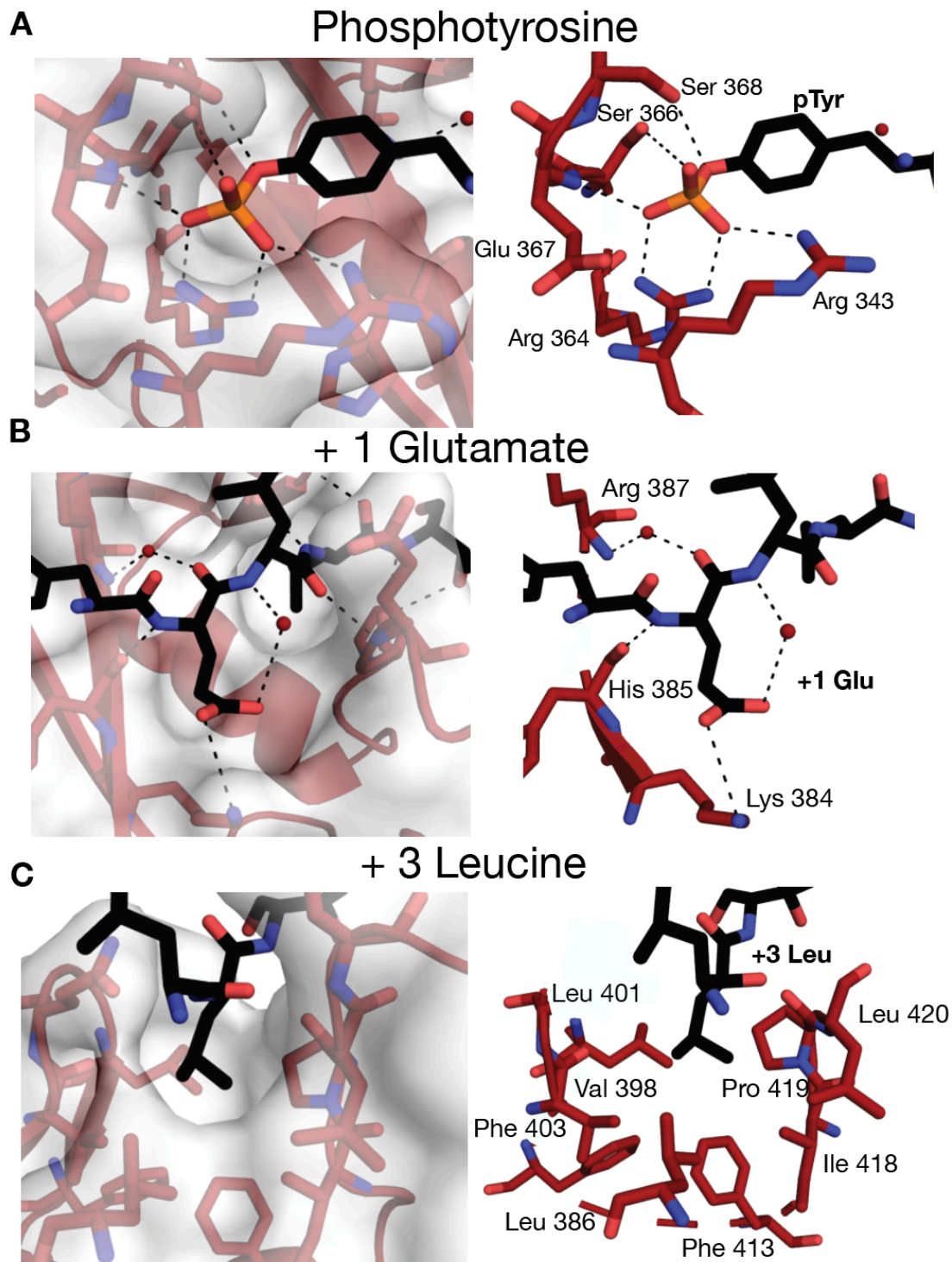


Figure 4.5 JAK2 pY813 peptide interactions with the LNK SH2 domain.

A) The phosphotyrosine residue is coordinated by R364, S368, R343, and the nitrogen of E367 of LNK. **B)** The +1 Glu residue forms a hydrogen bond with Lys of LNK and a water. The carbonyl group of His 383 in LNK also interacts with the amide backbone of the peptide. **C)** Leucine at the +3 position inserts into a hydrophobic pocket in LNK formed by Leu 386, Val 398, Leu 401, Phe 403, Phe 413, Ile 418 and Leu 420. Hydrogen bonds are indicated by dotted lines.

4.4.2 A comparison of LNK with the other SH2B family members

Although the results from the M1 TPO stimulation experiment indicate that APS and SH2B do not play a role in regulating TPO signalling (**Figure 4.1**), both have also been shown to bind to the pY813 residue of JAK2 (Hu and Hubbard 2006; Kurzer et al. 2006). Given that these proteins can all bind JAK2 via the same phosphotyrosine residue, understanding whether they bind JAK2 in a different conformation may provide insights into their opposing roles in regulation. The SH2 domain structures of SH2B and APS were therefore compared to LNK to compare binding modes (**Figure 4.6**).

The SH2 domains of LNK and SH2B share 68.7% sequence identity and alignment of the LNK and SH2B (PDB ID:2HDX) (Hu and Hubbard 2006) SH2 domains reveals that these proteins are almost identical in their fold, with a root-mean-square deviation (RMSD) of 1.3 Å over 103 residues however there are several notable differences (**Figure 4.6A and B**). In both structures, the phosphate group of pY813 occupies the canonical phosphotyrosine binding pocket and is coordinated by the invariant arginine (Arg 364 in LNK) (**Figure 4.6C and D**). In LNK, Arg 369 and 387 are involved in a hydrogen bonding network with Glu 372 and do not interact with the phosphate moiety. This same hydrogen bonding network is recapitulated by SH2B through Arg 560, Glu 563 and Arg 578, however the geometry of the sidechains allows Arg 560 to salt bridge with the phosphate moiety. Additionally, in SH2B the -1 Asp of the pY813 peptide forms a hydrogen bond with Arg 555 of SH2B, however this residue could not be resolved for LNK. The remainder of the interactions between the SH2 domains and peptide are almost identical between both structures. In LNK the +1 Glu interacts with Lys 384 and in SH2B hydrogen bonds with Ser 613 which is likewise hydrogen bonded with Lys 575 and in both structures, the +3 Leu inserts into a hydrophobic pocket between the EF and BG loops. The affinity of SH2B for the pY813 peptide is 500 nM (Hu and Hubbard 2006), which is comparable to the affinity of LNK for the same peptide.

The SH2 domains of APS and LNK share 64.6% sequence identity, and the structures have an overall RMSD of 2.4 Å over 112 residues. While LNK and APS both retain the typical SH2 domain fold, there are several notable differences. The α B helix of APS is longer and

extends behind the SH2 domain and allows it to form homodimers (**Figure 4.6F**). The +3 hydrophobic pocket that is usually a key determinant of peptide binding forms part of the dimer interface and as such the peptide binding surface is significantly altered. The structure of APS bound to this peptide has not been determined, however, there is a structure of the APS SH2 domain in complex with the insulin receptor kinase (PDB ID: 1RQQ)(Hu et al. 2003). For ease of comparison, in **Figure 4.6H** the phosphorylated activation loop of IRK (the binding site of APS) is shown in isolation. While the N-terminal half of LNK and APS both retain the typical SH2 domain fold, there are several notable differences between the C-terminal halves. Following the β D strand in the APS SH2 domain, there are several disordered residues which are followed by the α B helix, which is longer and extends behind the SH2 domain, allowing for dimer formation with a second SH2 domain molecule (**Figure 4.6F**). This α B helix extension and dimerization site changes the peptide binding surface, and, as seen in the alignment between LNK and APS. In the LNK SH2 domain with JAK2 pY813 structure the phosphotyrosine up to the +4 residue spans the surface of LNK, crossing the core beta-strands. In the case of the IRK peptide, pTyr 1158 binds the canonical phosphotyrosine binding pocket, and instead of the peptide crossing the core of the SH2 domain, the majority of the interactions come from the β D strand (**Figures 4.6G, H and I**). It should be noted that the interaction between APS and the IRK kinase domain differs significantly from most SH2 domain target structures as the ligand does not adopt a linear extended conformation. It would therefore be interesting to compare a structure of APS with the JAK2 pY813 peptide bound to understand how APS engages with this motif in comparison to the very similar interaction seen for LNK and SH2B.

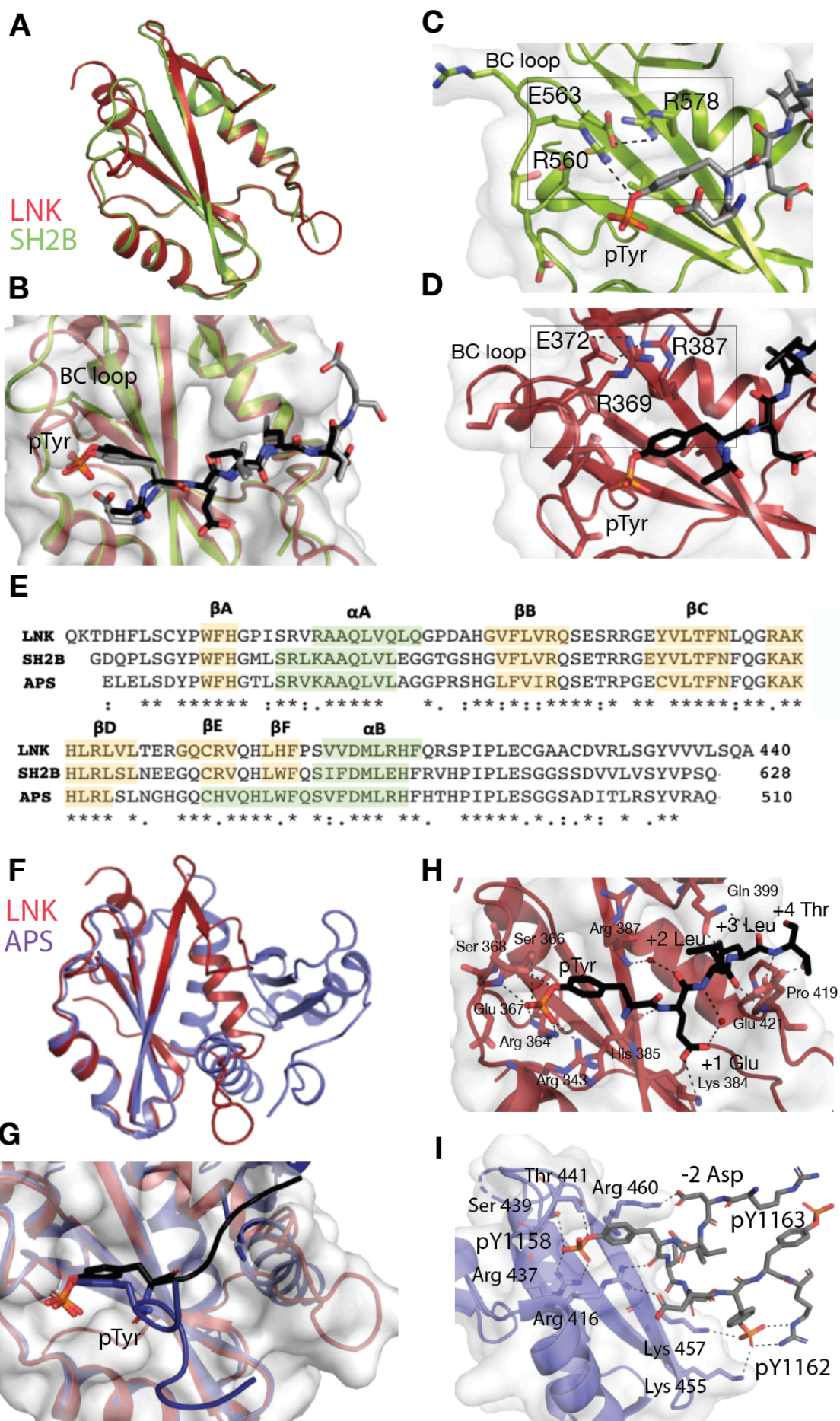


Figure 4.6 Comparison of the LNK, APS and SH2B SH2 domains.

A) Alignment of LNK and SH2B (PDB ID: 2HDX) SH2 domains, RMSD of 1.3 Å. **B)** Alignment of LNK and SH2B structures with JAK2 pY813 peptide. Peptides sit in identical positions in both structures. **C)** Interactions of the Arg560 Arg578 and Glu 563 of SH2B. Arg makes contacts with the pTyr **D)** Interactions of the Arg 369, Arg 387 and Glu 372 of LNK. This interaction may stabilise the BC loop in LNK. **E)** Alignment of APS, SH2B and LNK SH2 domain protein sequences. **F)** Alignment of LNK and APS (PDB ID: 1RQQ), RMSD of 2.4 Å. **G)** Alignment of LNK and APS structures with JAK2 pY813 peptide for LNK and IRK activation loop peptide for APS. Where the pY813 peptide of JAK2 covers the surface of LNK, only the pTyr and -1 Glu in the IRK peptide contact APS. **H)** LNK SH2 domain with JAK2 pY813 peptide sitting across surface of SH2 domain. **I)** APS SH2 domain with IRK activation loop peptide bound. All dotted lines represent H-bonds.

4.4.3 Basis for the recognition of pY813 by the LNK SH2 domain

The crystal structure of the LNK SH2 domain bound to JAK2 pY813 peptide revealed three residues in the pY813 peptide that are important for recognition by LNK; the phosphotyrosine, the +1 Glutamate and the + 3 Leucine **(Figure 4.4)**. Mutagenesis focussed on residues in the -1, +1, +3, +4 and +5 positions which were, in general, mutated to alanine or in some cases to residues with an opposite charge or a more bulky sidechain.

The effect these mutations had on binding was determined by an SPR competition assay **(Figure 4.7)**. LNK SH2 domain was pre-incubated with increasing concentrations of JAK2 pY813 peptides before being passed over a chip coated with immobilized low affinity phosphopeptide (derived from a site on the intracellular domain of the IL-6 receptor, IL6ST). The binding (response measuring how much LNK binds the immobilised peptide) of LNK in the presence of the JAK2 pY813 peptides is normalised to an LNK only control (representing 100% binding). Normalised data is then plotted against the $\log_{10}$ concentration of JAK2 pY813 peptide using a nonlinear regression analysis. The IC_{50} value is the concentration of peptide required to inhibit binding to the chip surface by 50%, the higher the peptide binding affinity, the lower the concentration of peptide required to inhibit binding **(Figure 4.8)**.

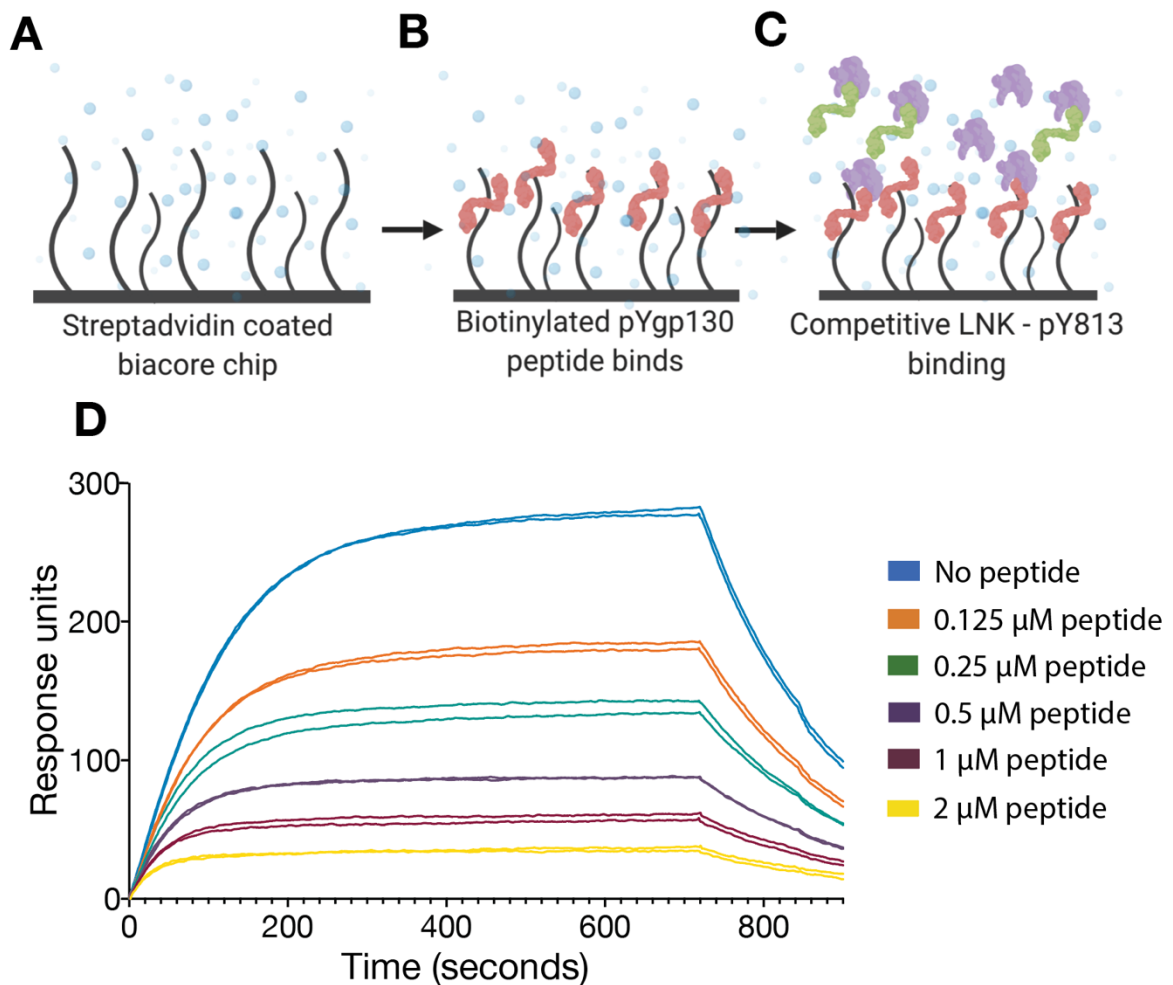


Figure 4.7 SPR competition assay with the LNK SH2 domain.

Schematic diagram detailing the SPR assay. A-B) A biotinylated phosphopeptide is captured onto a streptavidin chip and LNK pre-incubated with receptor peptides at different concentrations is passed over the chip. C) The ability of the receptor peptides to inhibit binding to the phosphorylated peptide is measured to determine affinity. D) Representative binding curves for the WT LNK SH2 domain competition assay with the WT JAK2 pY813 peptide over an IL6ST chip. Curves represent WT LNK SH2 binding to the chip after pre-incubation with peptide. Data shown is from one experiment, with two internal replicates.

Results of these experiments indicated that several residues were important for high-affinity binding. These include the glutamate at +1, which displayed a 7-fold decrease in affinity when mutated to alanine and complete loss of binding when mutated to arginine and the glutamate at +5 which led to a 5-fold drop in affinity when mutated to alanine. The latter result was unexpected as the +5 Glu residue could not be seen in the crystal structure of the protein, however this could form hydrogen bonding interactions with LNK and later experiments (see Figure 4.27) support this hypothesis.

The leucine at +3 was buried in a hydrophobic pocket in the structure and therefore was mutated to the more bulky hydrophobic residue, a phenylalanine. This change in size reduced binding resulting in a 22-fold increase in IC_{50} . Given the depth of the hydrophobic pocket in LNK, it is possible that a number of hydrophobic residues could be tolerated at this position, however it seems that a smaller hydrophobic residue such as a Leu is preferable.

Given that the +2 Leu pointed into solution in the crystal structure, no mutagenesis was performed on this residue. Mutating the aspartate at -1 or the threonine at +4 resulted in only minor (1-5-2.5 fold) decreases in affinity. These results, in combination with the SH2 domain structure, suggest four residues as key binding determinants (Glu 814, Leu 816 and Glu 818 along with pTyr 813). This suggests that the amino acids at the +1, +3 and +5 positions will determine the specificity of the LNK SH2 domain towards its binding partners.

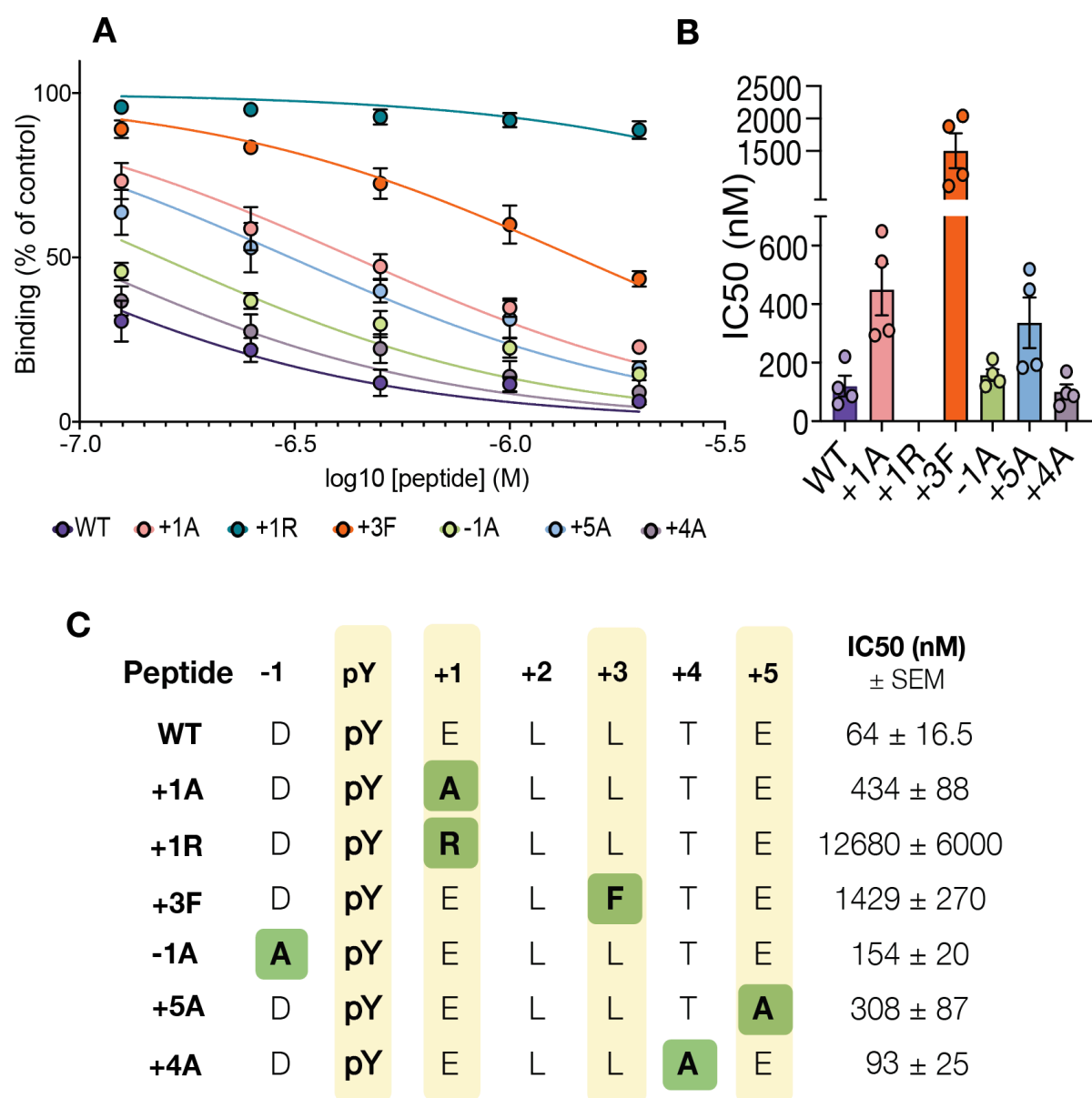


Figure 4.8 Using SPR to determine which residues in the JAK2 pY813 peptide contribute to LNK SH2 domain binding.

A) Percentage of binding (measured by the response at equilibrium) to the IL6st peptide chip after pre-incubation with varying concentrations of peptide. $N=4$, error bars are SEM. **B)** Point at which 50% of binding is inhibited shown as IC_{50} . $N=4$, error bars are SEM. **C)** Peptide sequences and IC_{50} values listed. Residues highlighted in green have been mutated from WT JAK2 pY813 peptide sequence. Positions highlighted in peach were identified as important for binding.

4.5 Investigating other LNK SH2 domain binding partners

The results from the previous section indicated that the pTyr, +1, +3 and +5 residues of the pY813 peptide were important for interactions with LNK. Using this information, the sequences of other phosphotyrosine motifs in proteins can be investigated to identify proteins that may also interact with the LNK SH2 domain.

Through studies in cells many proteins have been suggested to contain phosphotyrosine residues that act as docking sites for the LNK SH2 domain. These include, the JAK2 pY613 residue (Bersenev et al. 2008), intracellular phosphotyrosine residues in the EPO receptor (EPOR) and TPO receptor (MPL) (Tong, Zhang, and Lodish 2005), (Tong and Lodish 2004), various phosphotyrosine residues of JAK3 (Koren-Michowitz et al. 2010), and three intracellular phosphotyrosine residues in FLT3 (Lin et al. 2012) (others are listed in Figure 1.15 of the Introduction). However, none of these targets have been validated via biophysical methods using purified protein. The validation of LNK targets is an important step in understanding its mechanism-of-action. Although biophysical methods do not prove that an interaction exists *in vivo*, they provide a powerful tool to show which interactions *do not* occur. Given that SH2 domains will bind to most phosphorylated sequences to some degree, throughout the course of these studies the assumption is made that if the affinity of the interaction is stronger than ca. 2 μ M then the interaction may occur *in vivo* whereas if it is weaker, it is unlikely to do so. This is in line with the considerable literature on SH2 domains and their targets, see for example (Machida and Mayer 2005)

4.5.1 Binding to kinase activation loop peptides

As APS is known to bind the activation loop of its target kinase (IRK, (Hu et al. 2003)), the ability of the LNK SH2 domain to bind single (YpY and pYY) and double phosphorylated (pYpY) JAK2 activation loop peptides, in addition the IRK activation loop peptide was tested by SPR competition assay as described in section 4.4.3. No binding was observed for any of these peptides (**Figure 4.9**), suggesting they are not targets of the LNK SH2 domain.

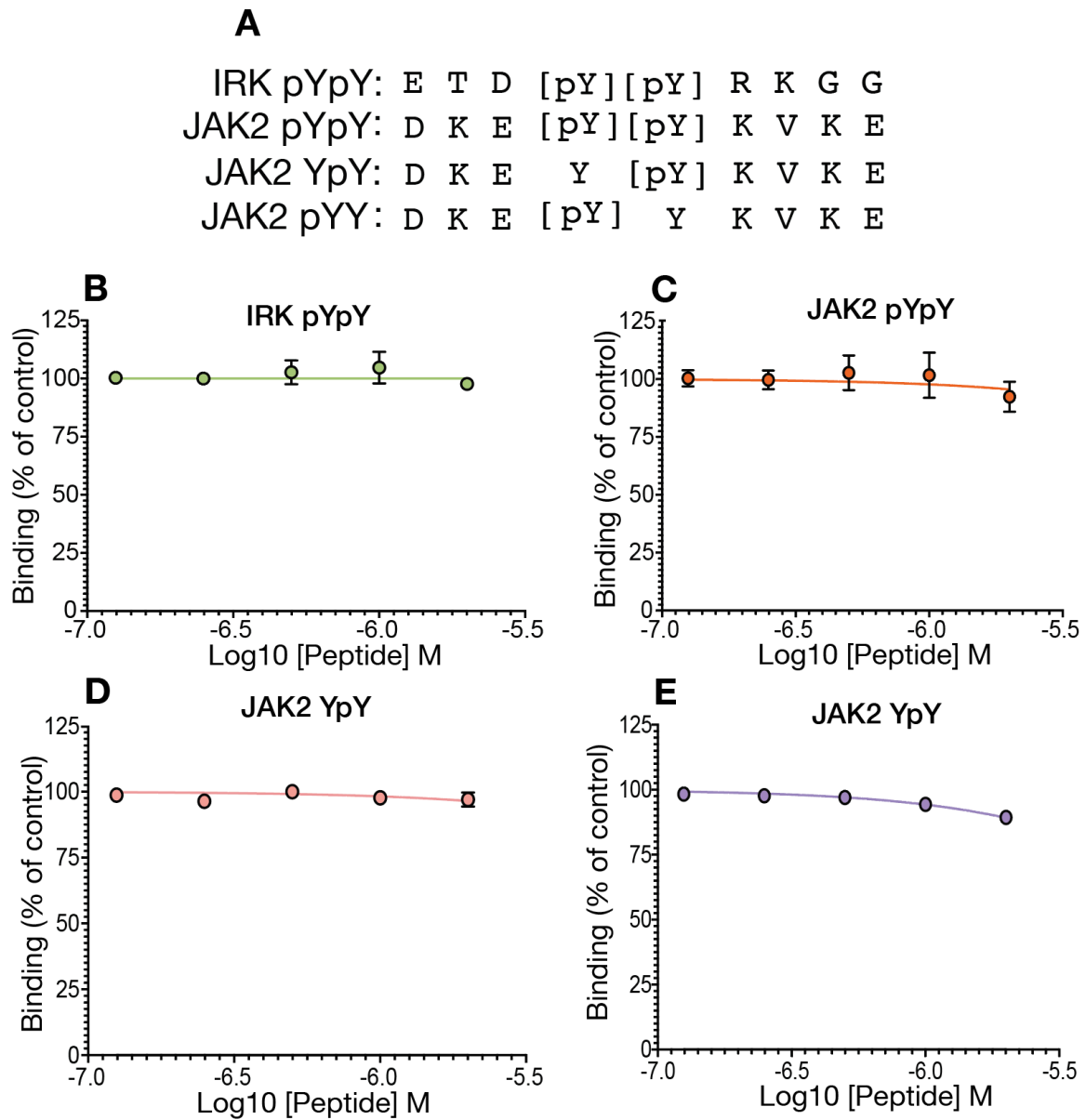


Figure 4.9 Competition assays for candidate activation loop binding partners.

A) Sequences of the phosphopeptides representing suggested LNK binding sites on other proteins. Inhibition curves determined by SPR competition assay with a JAK2 pY813 peptide for **B-E)** Inhibition curves for the IRK pYpY activation loop peptide, JAK2 pYpY, pYY and YpY activation loop peptides. For all experiments $N=2$, error bars are SD.

4.5.1 LNK binds pY785 in JAK3 with similar affinity to pY813 in JAK2

Of the other three JAK proteins, only JAK3 has a sequence similar to the JAK2 pY813 motif between the kinase and pseudokinase domains, at position 785 (both contain a DYELL motif). To determine whether LNK binds this motif an SPR competition assay was performed as per section 4.4.3. As shown in **Figure 4.10C and D** the LNK SH2 domain has a similar affinity for this motif from both JAK2 pY813 and JAK3 pY785, (IC_{50} 170 nM and 300 nM respectively).

To further compare the interaction between LNK and these two peptides a thermostability assay was performed. (**Figure 4.10A**). The LNK SH2 domain was diluted to 100 μ M in TBS + 2 mM TCEP and incubated with a 5-fold molar excess of each peptide. A control experiment using phenyl phosphate (a phosphotyrosine mimetic) was also performed. 10 μ L of each sample was transferred to a capillary and using a nanotemper NT.6, the intrinsic fluorescence of tryptophan and tyrosine residues was detected at both 350 nm and 330 nm as a 30°C/min temperature ramp was applied from 35-95°C. The ratio of fluorescence at each wavelength was plotted against temperature to determine the melting temperature (T_m) (**Figure 5.10B**). The T_m represents the temperature at which half the protein has unfolded, where the higher the T_m , the more thermostable the protein. In general, addition of a ligand to a protein will stabilize the protein.

The apo LNK SH2 domain had a T_m of approximately 60°C, which increased to 66 °C in the presence of 10 mM phenyl phosphate. The increase in stability by phenyl phosphate binding highlights that the SH2 domain is more stable when the phosphotyrosine binding pocket is occupied. Further stability can be achieved in the presence of a peptide which can interact with a larger surface of the protein. In the presence of both the JAK3 pY785 peptide and the JAK2 pY813 peptide, the T_m further increased to 70°C.

Together, the results from both experiments show that the LNK SH2 domain is able to interact with both peptides with a similar affinity, and that both peptides cause a similar increase in the stability of the domain.

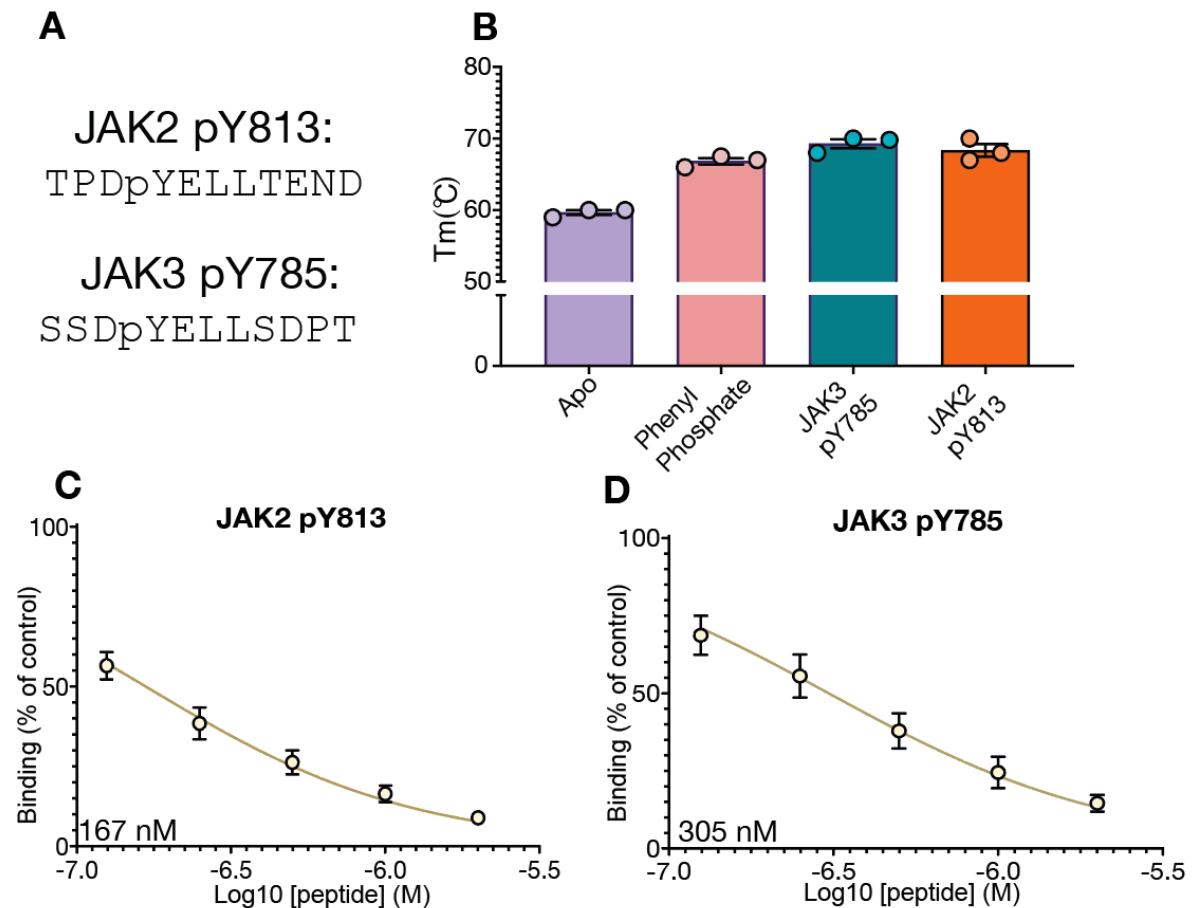


Figure 4.10 The LNK SH2 domain binds JAK3 pY785.

A) Sequence of the JAK2 pY813 and JAK3 pY785 phosphopeptides. **B)** Melting temperature (T_m) for LNK with each peptide or phenyl phosphate. Apo LNK has a T_m of approximately 60°C, with phenyl phosphate approximately 67°C, and with either the JAK2 pY813 or JAK2 pY785 peptides a T_m of approximately 70°C. $N=3$, error bars are standard deviation **C)** Inhibition curves and the IC_{50} values for the JAK2 pY813 peptide competing with an IL6ST pY757 peptide. $N=3$ error bars are SEM. **D)** Inhibition curves and the IC_{50} values for the JAK3 pY785 peptide competing with an IL6ST pY757 peptide. $N=3$ error bars are SEM.

4.5.2 Haematopoietic cytokine receptors as LNK binding partners

As LNK has been shown to regulate signalling downstream of EPO (Tong, Zhang, and Lodish 2005) and TPO (Tong and Lodish 2004), both the EPO and TPO receptors have been suggested to bind to LNK. Both of these receptors have five intracellular phosphotyrosine residues that can potentially act as binding sites for proteins with SH2 domains. Therefore, phosphopeptides corresponding to these positions were tested using the SPR competition assay described in section 4.4.3.

The five TPO receptor phosphotyrosine peptides, pY631, pY591, pY521, pY552, and pY626 (**Figure 4.11A and B**) showed little or no inhibition of LNK binding to the immobilised phosphopeptide on the chip surface, showing that these sequences are not binding targets of the LNK SH2 domain (**Figure 4.11C**). In contrast, three (pY454, pY368 and pY426) of the five EPOR peptides were able to quantifiably inhibit LNK binding to the immobilised phosphopeptide (**Figure 4.11D**). Consistent with their ability to inhibit binding, these three peptides also increased the thermostability of the WT LNK SH2 domain as seen with the JAK2 and JAK3 peptides (**Figure 4.12**).

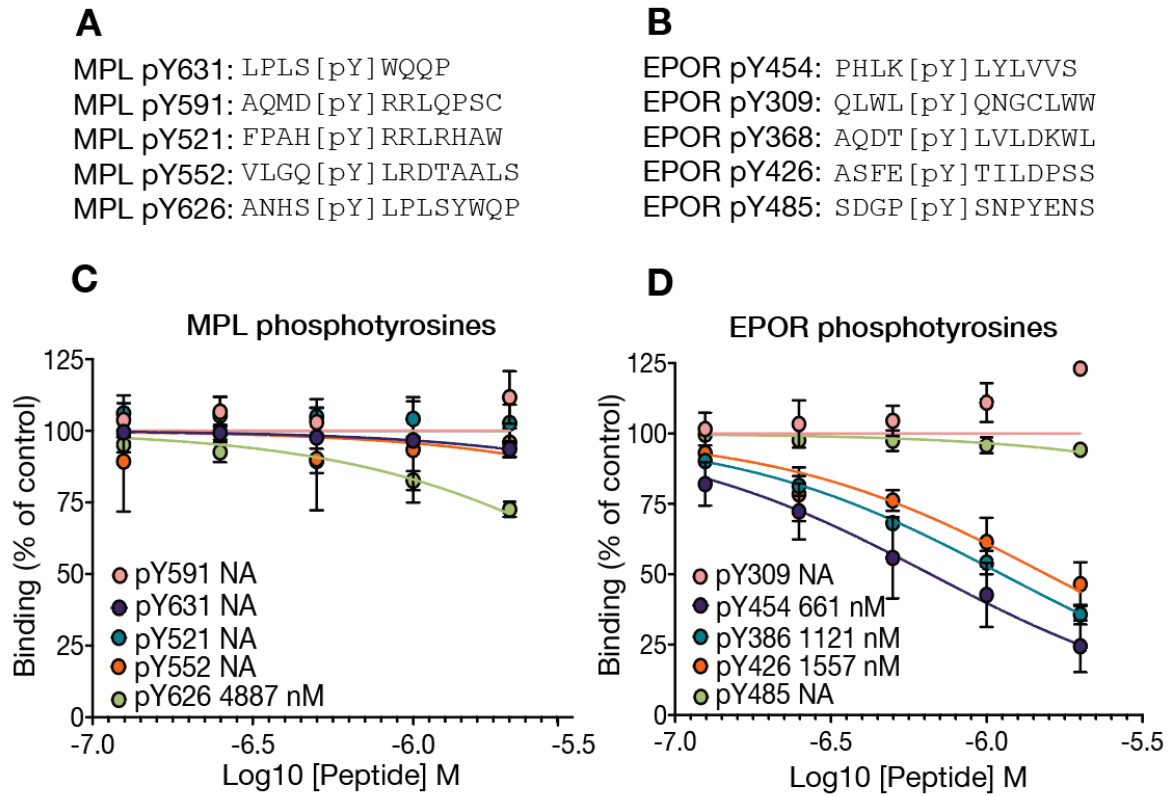


Figure 4.11 LNK SH2 domain binding to phosphotyrosine residues on the EPO and TPO receptors.

A) Sequences of the TPO receptor, MPL peptides. **B)** Sequences of the EPOR peptides. **C)** Inhibition curves and the IC_{50} values for the five MPL receptor phosphopeptides. None of the MPL receptor phosphopeptides were able to significantly inhibit binding to IL6ST peptide. $N=2-3$, error bars are SD. IC_{50} shown beside peptide name. **D)** Inhibition curves and the IC_{50} values for the five EPOR receptor phosphopeptides. The pY454, pY368 and pY426 peptides were able to inhibit binding to the IL6ST receptor peptide. $N=2-3$, error bars are SD. IC_{50} shown beside peptide name.

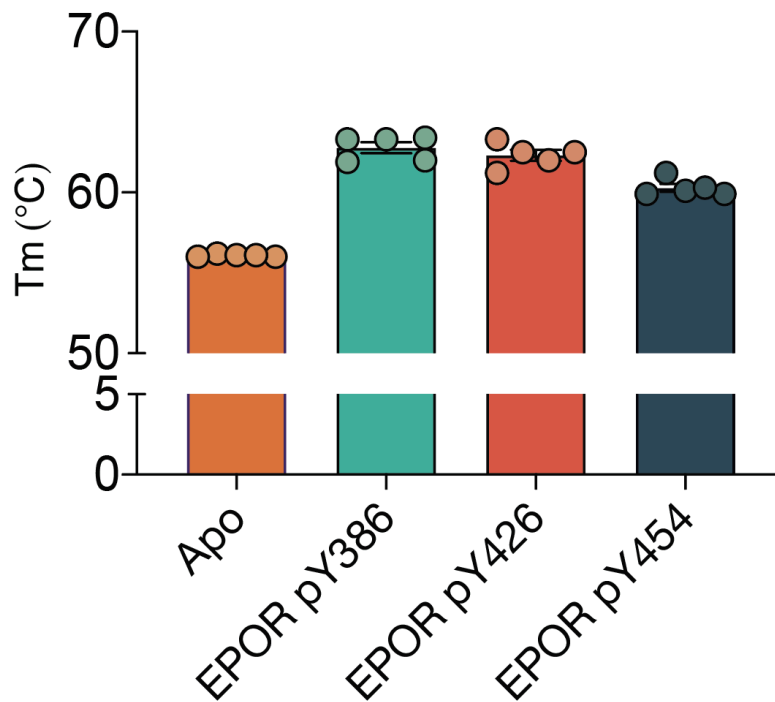


Figure 4.12 LNK SH2 domain binding to three EPOR peptides.

A) Melting temperature (T_m) for LNK with three EPOR peptides. Apo LNK has a T_m of approximately 57°C, with the pY386 peptide approximately 63°C with the pY426 peptide approximately 62°C, and with the pY454 peptide approximately 60 °C. $N=5$, error bars are SEM.

4.5.1 FLT3 as a potential LNK target

Along with the classical haematopoietic cytokine receptors, LNK has also been suggested to negatively regulate normal and leukemic FLT3 (Lin et al. 2012). FLT3 is a receptor tyrosine kinase that binds FLT3 ligand at the cell surface, triggering intracellular signalling cascades important for growth, division and survival of haematopoietic progenitor cells. The ability of LNK to regulate this protein may suggest an essential role for LNK in regulating the differentiation of haematopoietic cells into more mature cell types. Moreover, FLT3 is the most commonly mutated protein in AML (Daver et al. 2019), implicating LNK as a potential tumour suppressor. Of the multiple intracellular phosphotyrosine residues, three were shown to be important for LNK binding; pY572, pY591 and pY919 (Lin et al. 2012) (**Figure 4.13A**). As shown in **Figure 4.13B and C**, LNK bound two sequences from FLT3 with measurable affinity; pY591 and pY919.

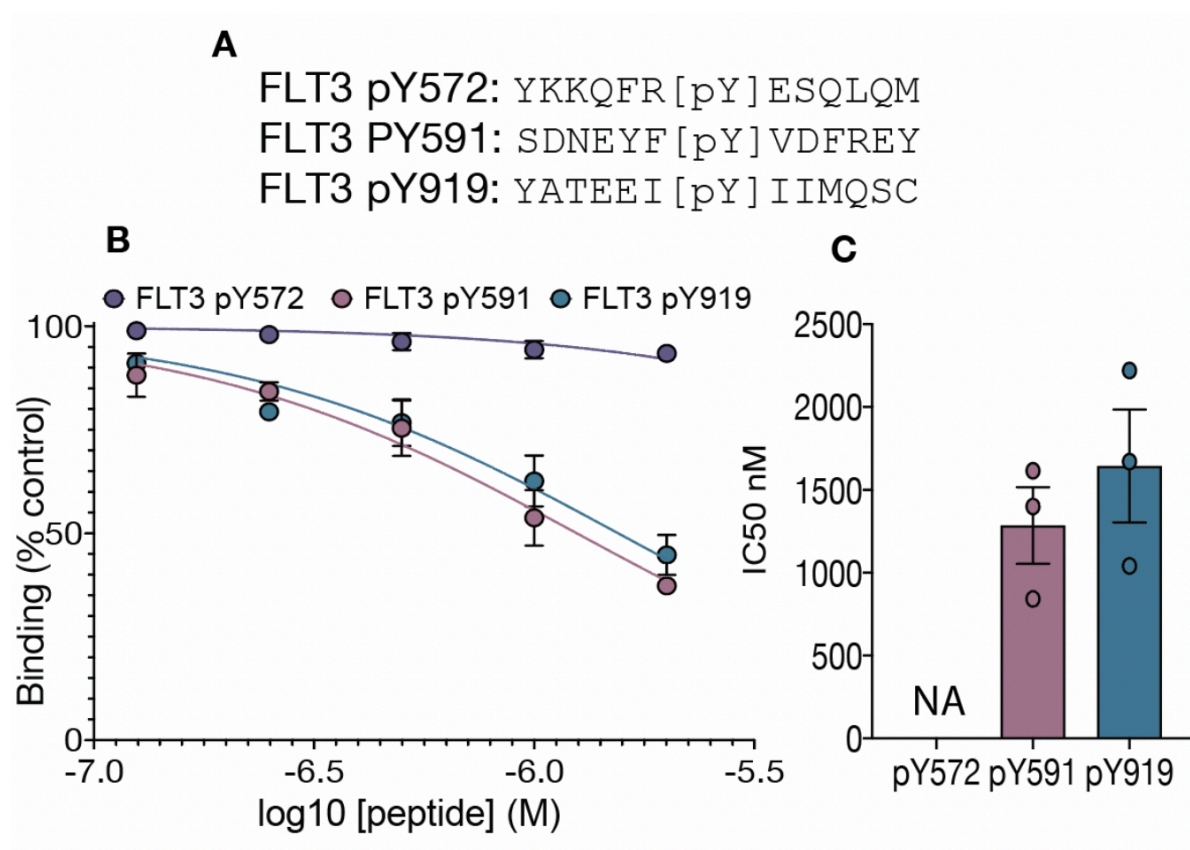


Figure 4.13 LNK SH2 domain interaction with three FLT3 peptides.

A) Sequences of the phosphopeptides representing suggested LNK binding sites on the FLT3 protein. **B)** Inhibition curves for FLT3 peptides pre-incubated with LNK and flowed over an IL6ST pY757 chip. N=3, error bars are SEM. **D)** IC₅₀ values for the FLT3 receptor peptides represented by inhibition curves in C. N=3, error bars are SEM.

To assess the ability of the three FLT3 peptides to increase the stability of the SH2 domain, a thermal shift assay was performed as previously described in section 4.5.1 (**Figure 4.14**). The pY591 peptide increased the stability from a T_m of 60°C for the Apo protein to 67°C in the presence of peptide, significant although slightly less than the increase in stability seen for JAK2 pY813. The pY919 peptide increased thermostability to 63°C whilst the pY752 peptide had no effect.

Taken together, the results from the SPR competition assay and thermal shift assay indicate that the LNK SH2 domain can bind FLT3 pY591 and pY919, raising the possibility that these residues are targets of LNK *in vivo*.

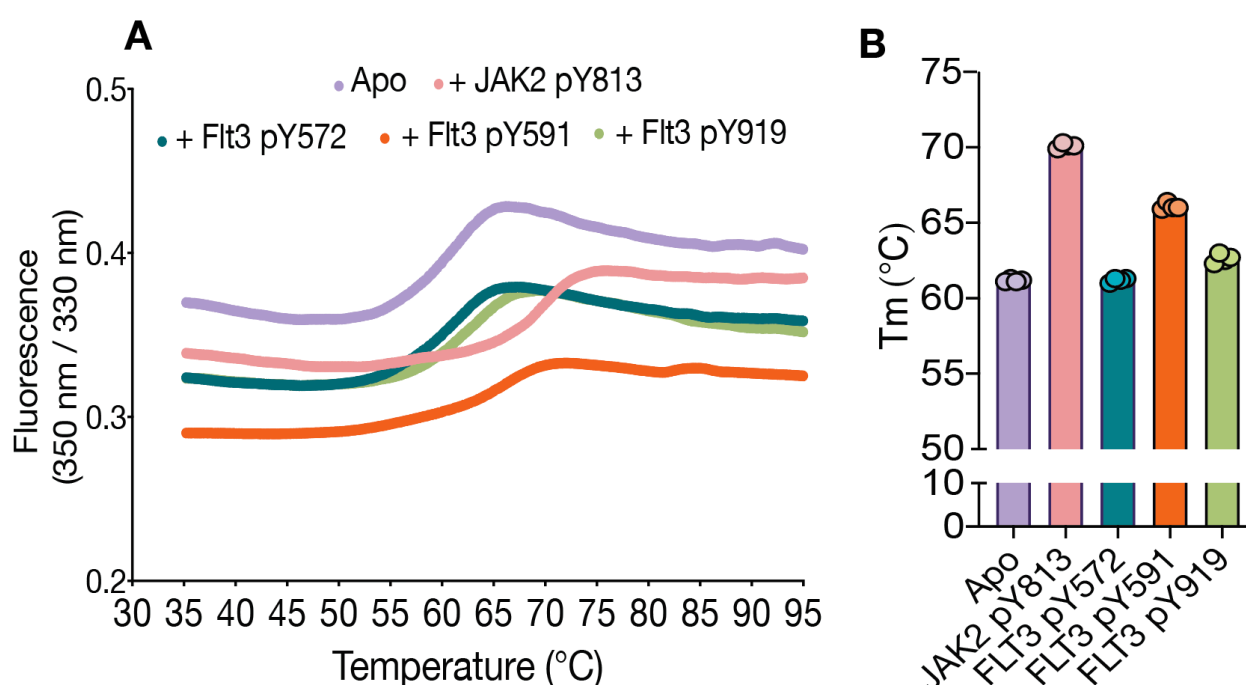


Figure 4.14 Stability of the LNK SH2 domain with three FLT3 peptides.

A) Representative melting curves for apo LNK, and LNK with the JAK2 pY813, FLT3 pY572, pY591 and pY919 peptides. **B)** Melting temperature (T_m) in °C for LNK with each peptide. $N=4$ Error bars are SEM.

4.5.2 Analysis of phosphorylated sequences confirmed to bind LNK

Of all the phosphopeptides tested, six peptides other than the JAK2 pY813 peptide were able to interact with the LNK SH2 domain with an IC_{50} of less than 2000 nM; JAK3 pY785, the three EPOR residues, pY368, pY454 and pY626 and FLT3 pY591 and FLT3 pY919. The most obvious common factor between these peptides other than the phosphotyrosine residue is they all contain a hydrophobic residue at the +3 position, consistent with the notion that a hydrophobic residue at this position is critical for binding to the LNK SH2 domain (**Figures 4.15**). The JAK2 pY813 and JAK3 pY785 peptides have the lowest IC_{50} of approximately 170 nM and 300 nM respectively, indicating tight binding. Of the three EPOR peptides, two contain a Leu at the +1 position, which appears to be well tolerated, and the weakest binder, pY426, contains an uncharged Thr. The FLT3 pY591 and pY919 peptides both contain hydrophobic residues at the +1 and +3 positions, and the pY591 peptide also contains a +5 Glu, which may increase the binding affinity, as seen for the JAK2 and JAK3 peptides. These data would indicate that the +1 and +3 residues are most important for the interaction between LNK and the EPOR peptides, however a negatively charged residue at the +5 position appears to be favorable.

Those peptides with an IC_{50} of more than 2000 nM generally either lacked a hydrophobic residue at the +3 position, or if they did contain a hydrophobic residue, contained an Arg at the +1 position (**Table 4.2**). Given the previous experiments indicating that having an Arg at the +1 position abolished binding (**Figure 4.8**), this is consistent with the inability to bind MPL peptides pY591 and pY521, despite both having a Leu at the +3 position (**Figure 4.11A and C**). Although the MPL pY626 peptide contains a leucine at the +1 and +3 positions, this peptide only weakly inhibited binding to the immobilised phosphopeptide. It is possible that the +2 proline may introduce a kink in the peptide, hindering binding.

From these results the general binding motif can be considered as pY(E/L/I/T)X(L/M/F)X(E/D). However, the residues in the +1 and +3 positions appear to be the most important for determining specificity (**Figure 4.15**).

Table 4.2 Peptide sequences and IC₅₀ values for all WT LNK SH2 domain competition assays.

Protein	Phosphotyrosine	Sequence															IC ₅₀ (nM)
		-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7	
JAK2	pY813				F	T	P	D	pY	E	L	L	T	E	N	D	167
	pY1007/pY1008	A	I	E	T	D	K	E	pY	pY	K	V	K	E	P	G	>10,000
	pY1007	A	I	E	T	D	K	E	pY	Y	K	V	K	E	P	G	>10,000
	pY1008	I	E	T	D	K	E	Y	pY	K	V	K	E	P	G		>10,000
JAK3	pY785				I	S	S	D	pY	E	L	L	S	D	P	T	305
IRK	pY1445/pY1446					E	T	D	pY	pY	R	K	G	G	K	G	>10,000
MPL	pY631				L	P	L	S	pY	W	Q	Q	P				>10,000
	pY626				A	N	J	S	pY	L	P	L	S	Y	W	Q	4887
	pY552				V	L	G	Q	pY	L	R	D	T	A	A	L	>10,000
	pY521				F	P	A	H	pY	R	R	L	R	H	A	W	>10,000
	pY591				A	Q	D	M	pY	R	R	L	Q	P	S	C	>10,000
EPOR	pY426				A	S	F	E	pY	T	I	L	D	P	S	S	1557
	pY368				A	Q	D	T	pY	L	V	L	D	K	W	L	1121
	pY309				Q	L	W	L	pY	Q	N	G	C	L	W	W	>10,000
	pY454				P	H	L	K	pY	L	Y	L	V	V	S		661
	pY485				S	D	G	P	pY	S	N	P	Y	E	N	S	>10,000
FLT3	pY572		Y	K	K	Q	F	R	pY	E	S	Q	L	Q	M		>10,000
	pY591		S	D	N	E	Y	F	pY	V	D	F	R	E	Y		1285
	pY919		Y	A	T	E	E	I	pY	I	I	M	Q	S	C		1644

0-999 nM

1000-1999 nM

2000-10,000 nM

> 10,000 nM

Peptide	-1	pY	+1	+2	+3	+4	+5	IC ₅₀ (nM)
JAK2 pY813	D	pY	E	L	L	T	E	167
JAK3 pY785	D	pY	E	L	L	S	D	305
EPOR pY454	K	pY	L	Y	L	V	V	661
EPOR pY368	T	pY	L	V	L	D	K	1121
FLT3 pY591	F	pY	V	D	F	R	E	1285
EPOR pY426	E	pY	T	I	L	D	K	1557
FLT3 pY919	I	pY	I	I	M	Q	S	1644

Figure 4.15 Binding sites for LNK on other proteins.

Sequences of the five LNK binding partners and their respective IC₅₀ values for competition assays over the IL6ST pY757 peptide. Those residues previously shown to be important or contributing to binding are highlighted in green. At the +1 position, all peptides, contain either a Glu, a hydrophobic residue or a Thr. At the +3 position, all peptides contain a hydrophobic residue. At the +5 position, the amino acids are variable.

4.6 Crystal structure of the LNK SH2 domain with an EPOR pY454 peptide

The LNK SH2 domain was shown to interact with three phosphopeptides corresponding to the EPOR, namely pY368, pY454 and pY426, all of which contain a +3 leucine, but do not contain a negatively charged residue in the +1 or +5 positions. To understand how the LNK SH2 domain interacts with a peptide sequence that contains different residues at the +1 and +5 position, X-ray crystallography was used. Hanging drop vapour diffusion crystal trays were set up by hand with 5 mg/mL of the LNK SH2 domain and a two-fold molar excess of EPOR pY454 peptide, with 1 μ L protein solution and 1 μ L precipitant over a 450 μ L reservoir volume.

The pY454 peptide was chosen as it has the highest affinity of the three peptides. Crystals grew in a condition containing 20% PEG 8000 0.1 M magnesium acetate and 0.1 M Tris pH 8.0 (**Figure 4.16**). Crystals from this drop were cryoprotected in paratone and flash-frozen in liquid nitrogen before being taken to the MX2 beamline at the Australian Synchrotron. Data was processed to 2.3 Å in XDS (Kabsch 2010). The WT LNK SH2 domain structure solved previously with the JAK2 pY813 peptide removed was used for molecular replacement and a solution was found in the space group $P6_1$, with a top TFZ score of 34.4, and one copy of the protein in the asymmetric unit. Refinement was performed using Phenix (Adams et al. 2010) and the model was built in Coot (Emsley and Cowtan 2004). Further details of this structure can be found in **Table 4.1**.

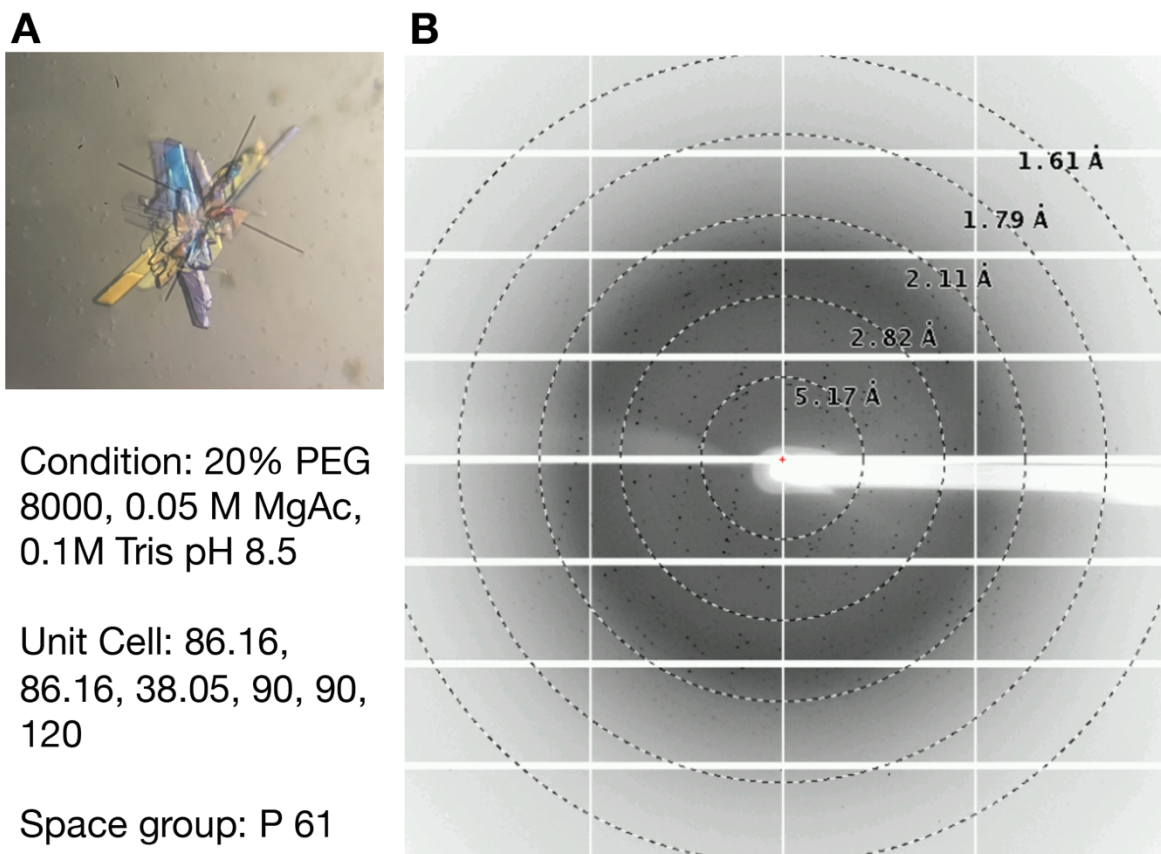


Figure 4.16 Crystals of the WT LNK SH2 domain and an EPOR pY454 peptide

A) Crystals generated by hanging drop vapour diffusion with crystal conditions, unit cell dimensions and space group listed. **B)** Diffraction pattern from this at the MX2 beamline at the Australian synchrotron. Diffraction to 2 Å.

As described for the LNK SH2 domain in complex with JAK2 pY813 (**Figure 4.17A and B red**), the EPOR pY454 phosphopeptide spans the surface of the SH2 domain, and a number of interactions are recapitulated. The phosphotyrosine residue is coordinated by the same residues and the +3 leucine sits in the hydrophobic pocket between the EF and BG loops of LNK (**Figure 4.17C**).

The overall RMSD between these two structures was calculated to be 1.0 Å over 111 residues (DALI(Holm and Sander 1995)). This difference is largely due to a change in the conformation and position of the BG loop. The +1 position in EPOR pY454 is Leucine and therefore hydrophobic, whereas the +1 position in JAK2 pY813 is glutamate and negatively charged. LNK accommodates both of these residues in a similar hydrophobic pocket formed by Phe 377, Leu 375 and Leu 379 of the β C strand along with Ile 418, Leu 420, and Val 428 of the BG loop (**Figure 4.18A**). The +1 Glu residue is bent, such that methylenes sits in the hydrophobic pocket and the carboxylate is pointing away from the pocket and forms a salt bridge with Lys 384. The +1 Leu sits in the hydrophobic pocket and forces Leu 420 on the BG loop to move by ~ 1.5 Å in order for the +1 Leu to be accommodated. This in turn results in a shift of up to 5 Å for some residues in the BG loop, however the BG loop makes different crystal contacts in both crystals which could also be contributing to the differences observed. The +1 Glu in the JAK2 peptide forms a salt bridge with Lys 384 of LNK, which may contribute to the increase in affinity seen for the JAK2 peptide (**Figures 4.18C and 4.19**).

Several backbone and Van der Waals contacts form the remainder of intermolecular interactions. The +4 Val makes Van der Waals contacts with the BG loop of LNK, at 4 Å away from the flat orientation of the peptide bond between residues Leu 240 and Glu 241. The EPOR pY454 +5 Val is stacked over His 400, at Van der Waals distance (~ 4 Å). The sidechain of Arg 343 hydrogen bonds with the carbonyl oxygen of the -1 Asp in the EPOR peptide and the Pro 419 carbonyl oxygen hydrogen-bonds with the amide nitrogen of the +4 Val. Additionally, the backbone carbonyl oxygen of His 385 hydrogen bonds with the amide nitrogen of the +1 Glu and Gln 399 forms two hydrogen bonds with the amide nitrogen of the +2 Tyr and carbonyl oxygen of the +1 Leu.

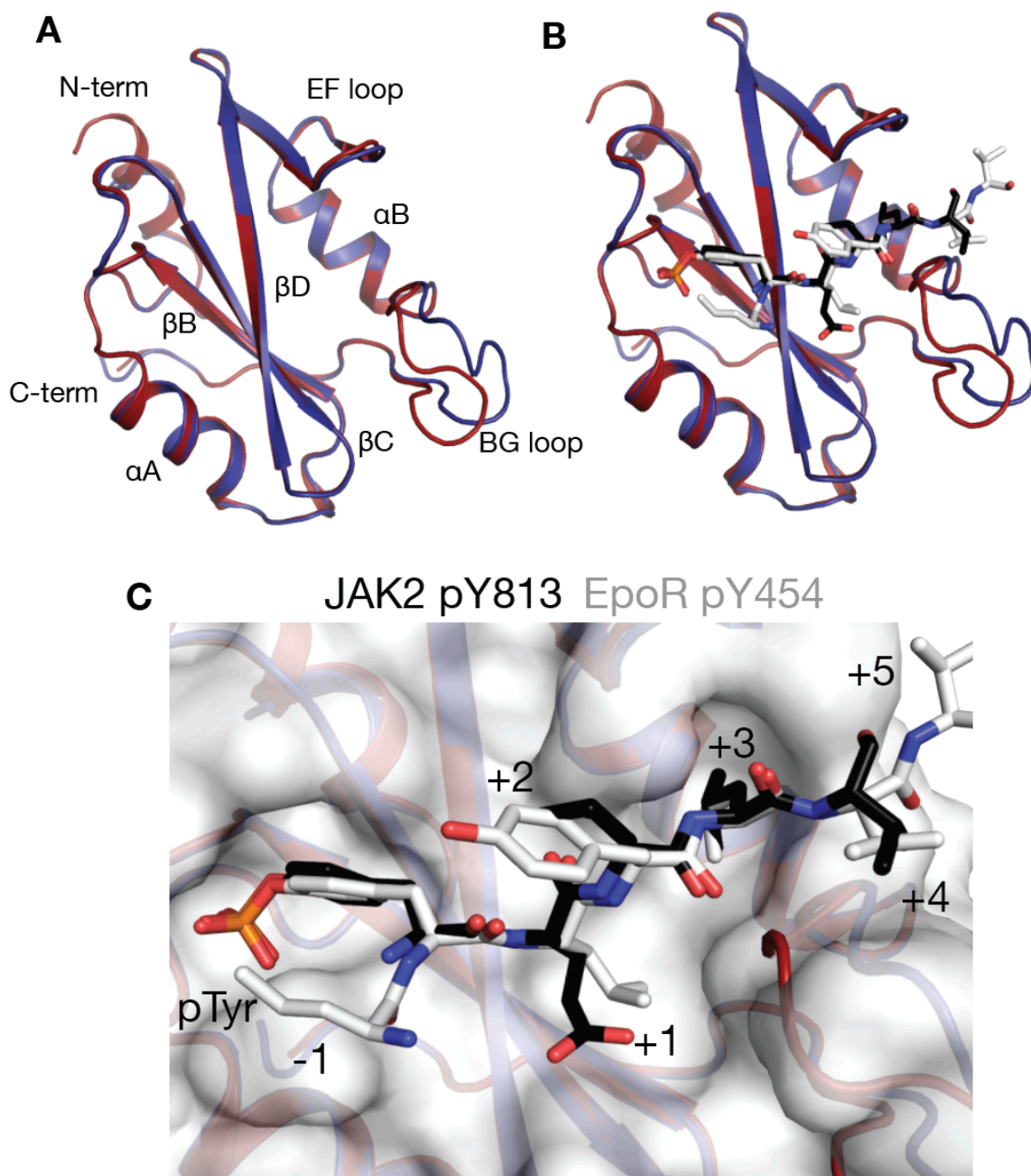


Figure 4.17 Structure of the WT LNK SH2 domain bound to JAK2 pY813 and EPOR pY454

A) Alignment the backbone of the WT LNK SH2 domain from the JAK2 pY813 bound structure (red) and the EPOR pY454 bound structure (purple) with the features indicated. **B)** Alignment of the JAK2 pY813 bound structure (red backbone, black peptide) and the EPOR pY454 bound structure (purple backbone, white peptide) **C)** The pY813 JAK2 (black) and EPOR pY454 (white) peptides bound to the SH2 domain.

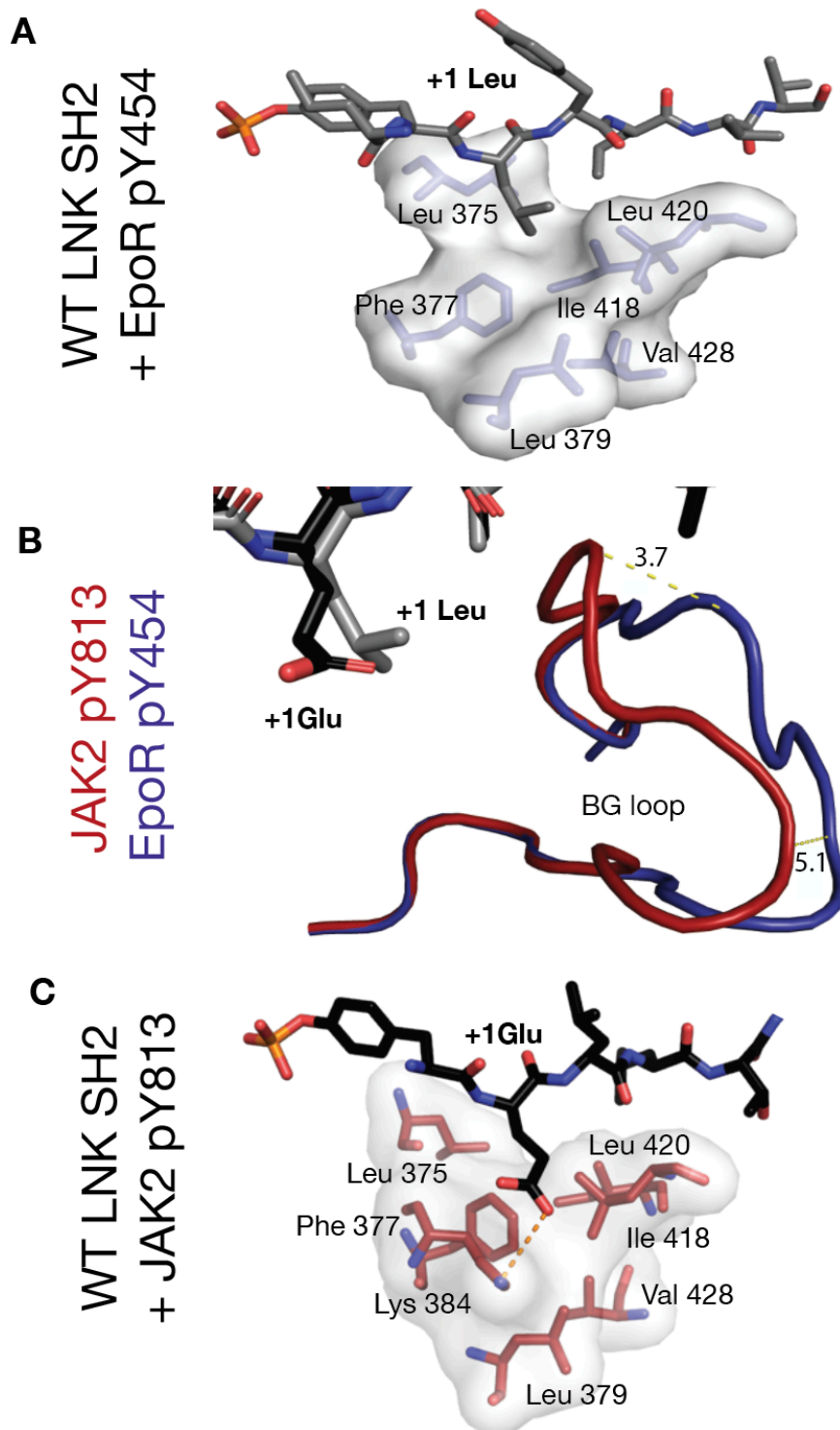


Figure 4.18 Changes to the +1 position in the JAK2 pY813 and EPOR pY454 bound LNK SH2 domain structures.

A) +1 Leu of the EPOR pY454 peptide interacts with a hydrophobic surface in. **B)** The BG loop moves outwards in the EPOR (purple) bound structure by approximately 5 Å to accommodate the Leucine in comparison to the JAK2 pY813 bound structure (red). Dotted lines represent angstroms. **C)** +1 Glu of the JAK2 pY813 peptide interacts with the same hydrophobic surface as the +1 Leu of the EPOR peptide, but the charged headgroup also forms a salt bridge interaction with Lys 384 of LNK.

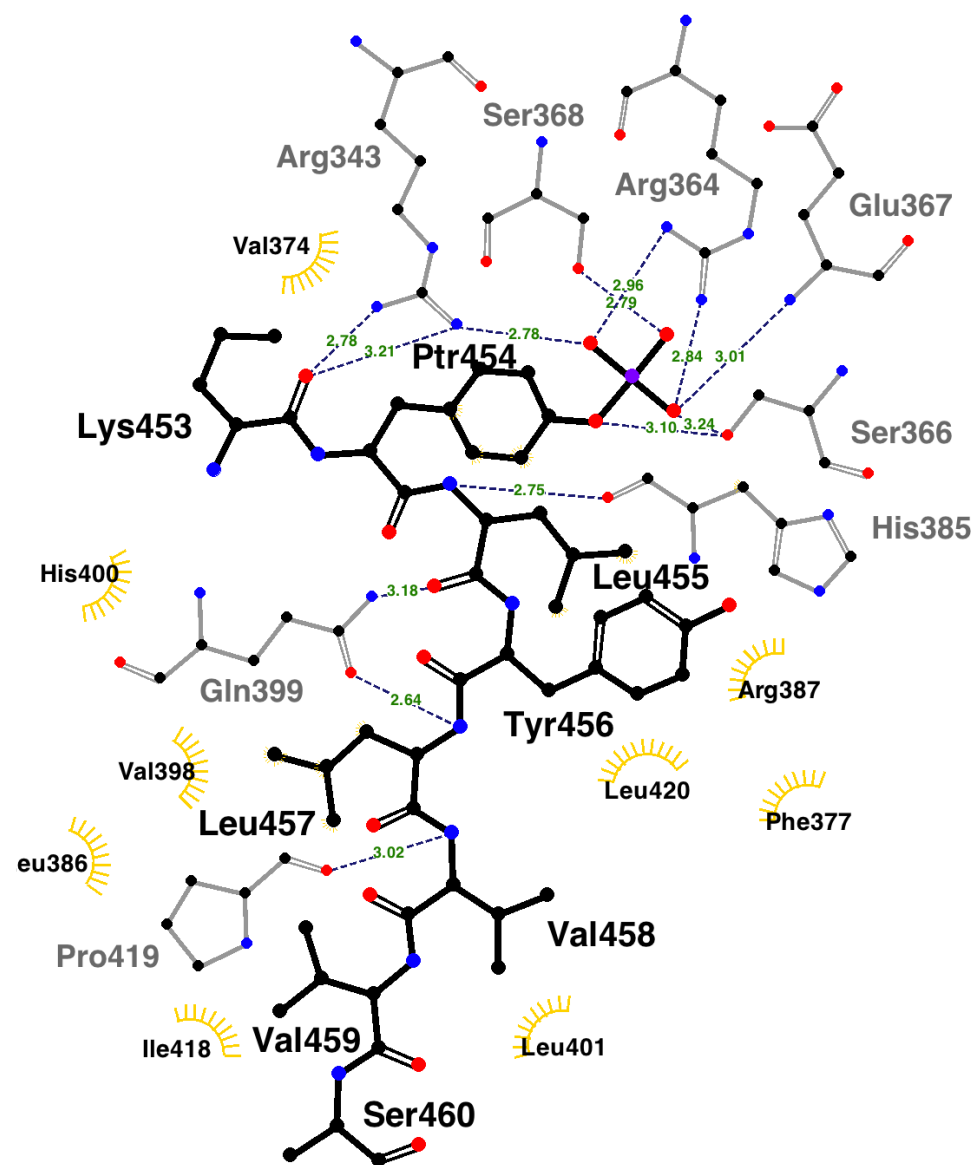
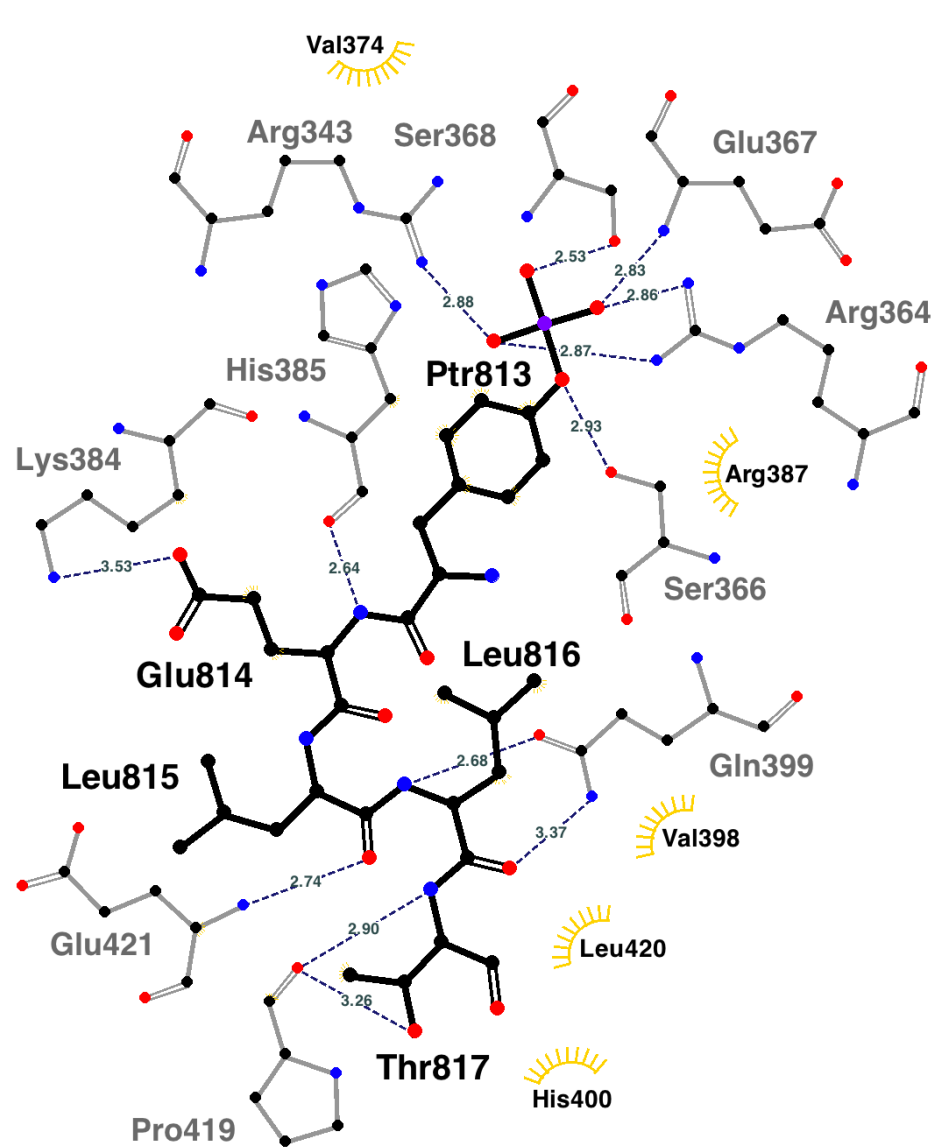


Figure 4.19 Interactions between WT LNK and the JAK2 pY813 and EPOR pY454 peptides.

2D representation of WT LNK with the JAK2 pY813 peptide on the left and EPOR pY454 peptide on the left. Interactions are indicated by blue dotted lines, with the distance between the residues in angstroms indicated. Dotted lines represent hydrogen bonds or electrostatic interactions. Yellow dashes crescents represent hydrophobic interactions. Peptide residues are indicated in black and protein residues in grey. Visualized using LigPlot+ (Laskowski and Swindells 2011).

4.7 Mutations in the LNK SH2 domain

Several mutations in LNK have been linked with disease, with the majority occurring in the PH domain, the domain responsible for interactions with the plasma cell membrane. More recently, mutations located in the LNK SH2 domain have been described in patients with a variety of diseases including the myeloproliferative neoplasms, a group of disease characterized by excessive JAK-STAT signalling resulting in hyperplasia of specific mature blood lineages (Barbui et al. 2018). Using the WT LNK SH2 domain structure as a guide, four mutations in the LNK SH2 domain that had been identified in patients with myeloproliferative neoplasms or a closely related disease termed idiopathic erythrocytosis were selected to study (**Figure 5.20A**). These mutations were chosen as they clustered around the phosphotyrosine binding site in the LNK SH2 domain, and so I hypothesized they may impede phosphotyrosine binding. The position of these residues is highlighted in **Figure 5.20F**. The E400K and R387C (E372K and R387C in mouse) mutants were reported in patients with idiopathic erythrocytosis, a disease characterized by the over proliferation of red blood cells where polycythemia vera has been ruled out (Camps et al. 2016). The R387H (R387H in mouse) mutation was identified in patients with polycythemia vera, who also had a JAK2 V617F mutation (Chen et al. 2016). Similarly, the V374M (V374M in mouse) mutation was identified in patients with essential thrombocythemia and primary myelofibrosis, along with a JAK2 V617F mutation (Lasho et al. 2011). While it is not clear what role these LNK mutations play in their respective disease states, there has been increased interest in LNK as a driver mutation that initiates the disease, or a secondary mutation that occurs during disease progression, particularly in progression to blast phase (Pardanani, Lasho, Finke, Oh, et al. 2010).

To determine whether any of these mutant LNK SH2 domains had reduced ability to bind the JAK2 pY813 or any other proteins (EPOR, FLT3 or JAK3), all four mutants were expressed in BL21 (DE3) *E. coli* and purified using affinity chromatography and size exclusion chromatography as previously described for the WT LNK SH2 domain (**Figure 5.20B-D**). All four mutant SH2 domains did not express as effectively as the WT LNK SH2 domains and were generally challenging to work with. Despite this, enough protein could be purified for biochemical and structural studies (**Figure 4.20G**).

A

Mutation in human	Mouse equivalent	Structural position	Disease	JAK2 status
E400K	E372K	β C strand	IE	WT
V402M	V374M	β C strand	ET and PMF	V617F
R415C	R387C	β D strand	IE	WT
R415H	R387H	β D strand	PV	V617F

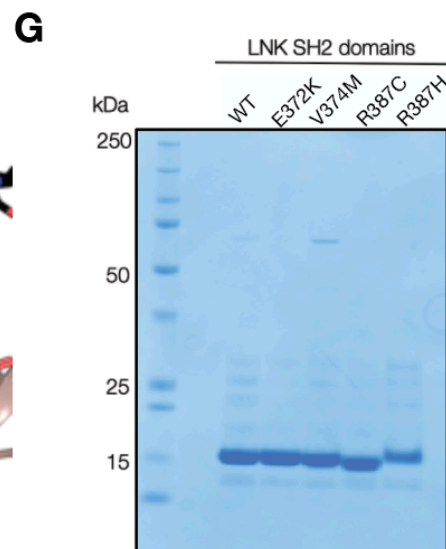
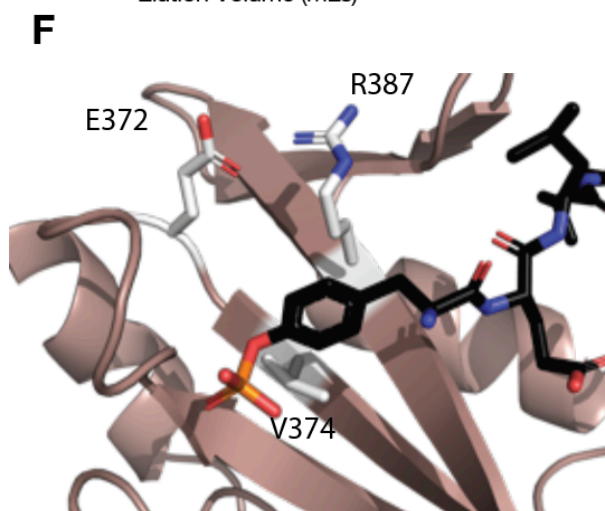
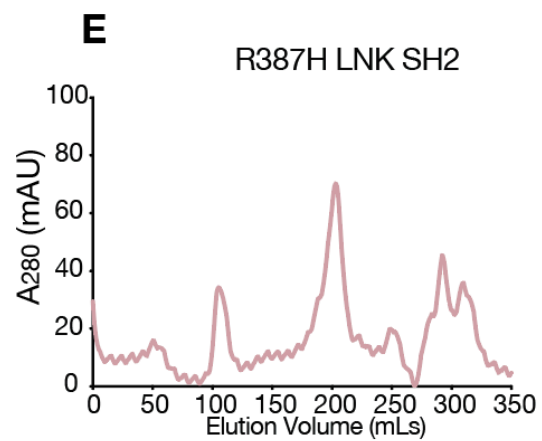
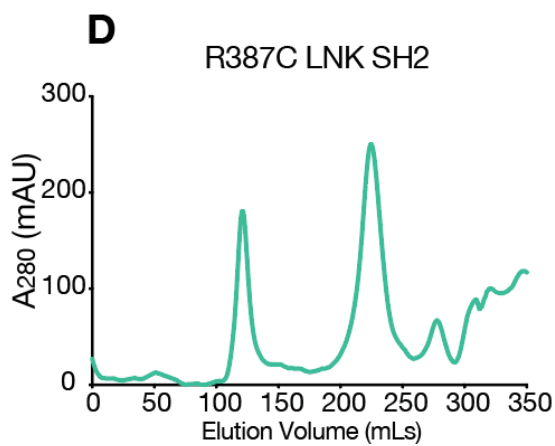
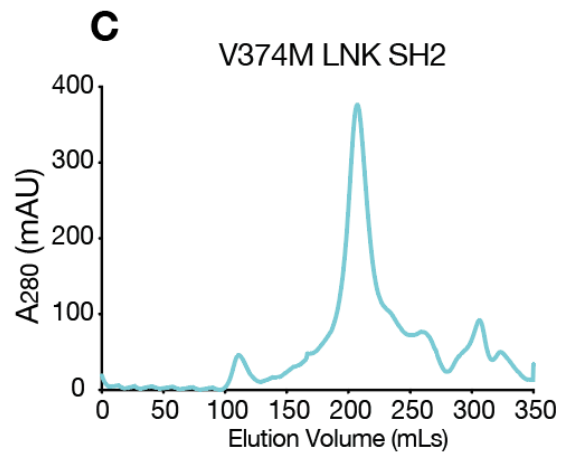
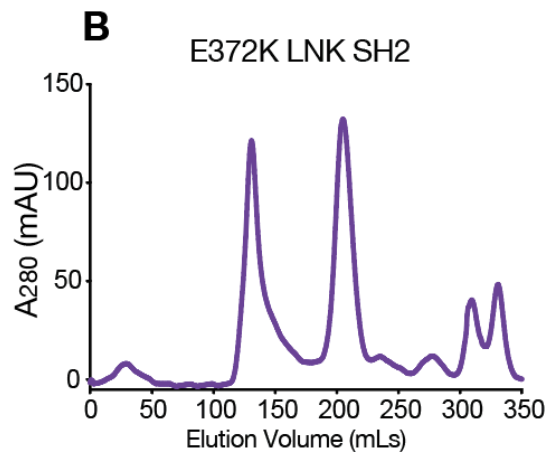


Figure 4.20 LNK SH2 domain mutants.

A) The four mutations investigated in this study, their location in the LNK SH2 domain, the disease they were identified in and the JAK2 status of the patient. **B-E)** Representative chromatographs of the mutant LNK SH2 domain purifications from an S200 Superdex HiLoad 26/60 size exclusion column. All four mutant LNK SH2 domain elute at approximately 240 mL. **F)** Location of the mutations mapped on to the WT LNK SH2 domain. All mutations cluster around the phosphotyrosine binding pocket. **G)** Coomassie stained SDS-PAGE gel of the four LNK SH2 domain mutants and the WT SH2 domain indicating their size and relative purity. All proteins are approximately 12 kDa.

4.7.1 Stability of the LNK SH2 domain mutants

Given that all mutants were more challenging to purify and handle than the WT, I hypothesized that the mutations may have an effect on the stability of the SH2 domain. To determine the thermal stability of these proteins, 100 μ M SH2 domain mutants in TBS + 2 TCEP were incubated in the presence and absence of a 5-fold molar excess of pY813 peptide. 10 μ L of each sample was transferred to a capillary and heated from 35°C to 95°C. For each sample the T_m was determined by measuring the ratio of fluorescence at 330 and 350 nm using a Tycho N6T as per section 1.5.1 (**Figure 5.21**).

The Apo WT LNK SH2 domain had a T_m of approximately 60°C and this increased to 70°C with the addition of pY813 peptide. All four mutant SH2 domains had a reduced stability in the apo state, each having a T_m of approximately 55°C. In the presence of the JAK2 pY813 peptide the T_m for both the E372K and R387C mutants increased to 60°C, suggesting they are still able to bind peptide to some extent. Similar to the WT, the R387H T_m increased by 10°C in the presence of peptide. The T_m for the V374M mutant did not change in the presence of the peptide, suggesting that this mutant may be significantly compromised in its ability to bind phosphopeptides.

Although these results indicated that the mutant SH2 domains are less stable than the WT in their apo form, they still have a melting point significantly higher than physiological temperatures and therefore it was judged unlikely that this is the cause of their deleterious function *in vivo*.

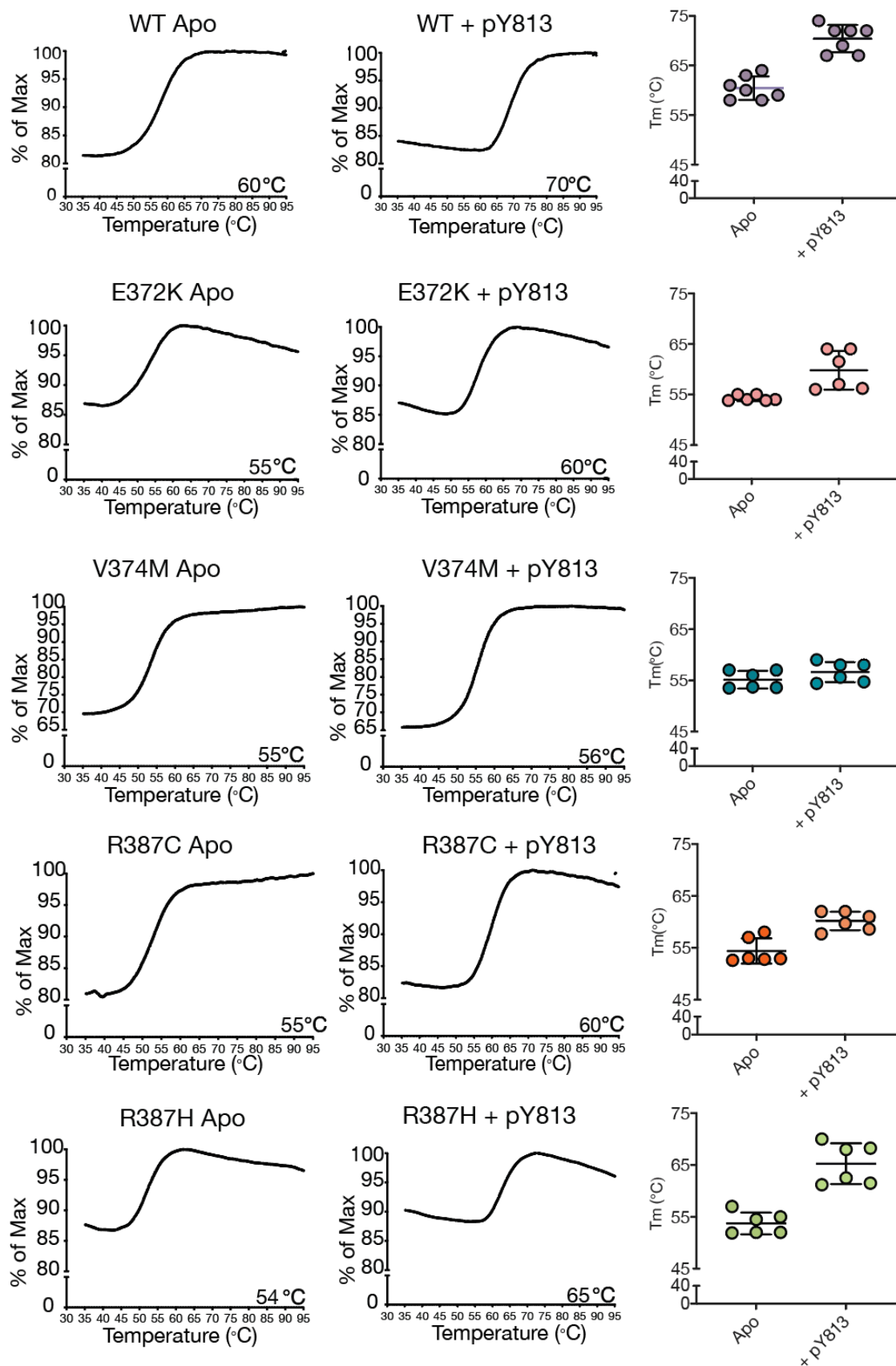


Figure 4.21 Thermostability of the LNK SH2 domain mutants.

*Representative melting curves for all the LNK SH2 domains and the average melting temperature for each LNK SH2 domain. **A)** Apo WT LNK SH2 domain is most stable with a melting temperature of 60°C. Melting temperature for WT LNK increases to 70°C in the presence of the pY813 peptide. **B)** Apo E372K has a melting temperature of 50°C, which increases to 60°C in the presence of the pY813 peptide. **C)** Apo V374M has a melting temperature of 55°C, which does not change in the presence of the pY813 peptide. **D)** Apo R387C has a melting temperature of 55°C, which increases to 60°C in the presence of the pY813 peptide. **E)** Apo R387H has a melting temperature of 55 v, which increases to 65°C in the presence of the pY813 peptide. N=6-7 error bars are SEM.*

4.7.1.1 Stability in the presence of other binding partners

The changes in T_m between the Apo and peptide bound SH2 domains gives a qualitative indication as to the ability of the SH2 domains to interact with different phosphopeptide sequences. Given the previous experiments with the JAK2 pY813 peptide revealed that the T_m of three of the four mutants did not increase to the same extent as the WT, the change in T_m was assessed for other peptide sequences by thermal shift assay. All SH2 domains were diluted to approximately 100 μM in TBS + 2 μM TCEP and incubated either alone or with a three-fold molar excess of phosphopeptides (**Table 4.3**). As the EPOR pY454 peptide contains two tyrosine residues, the ability of the SH2 domain to bind to the single (pY454) and double phosphorylated (pY454/pY456) form of the peptide was assessed.

These results indicated that for all the SH2 domains, other than the V374M mutant, the JAK2 pY813 peptide increases SH2 domain stability by the largest magnitude, followed by the JAK3 pY785 peptide. However, the EPOR pY454 and pY454/pY456 peptides do not increase the thermostability for most of the mutants, other than the R387H. This is consistent with the hypothesis that the binding capacity of the mutants are reduced in comparison to the WT SH2 domain.

Table 4.3 Comparison of LNK SH2 domain mutant melting temperatures in the presence and absence of phosphopeptides.

T_m for each LNK SH2 domain in the presence of four phosphopeptides as shown in $^{\circ}\text{C} \pm$ standard deviation.

LNK SH2 domain melting temperatures $\pm$ SD ($^{\circ}\text{C}$)					
	WT	E372K	V374M	R387C	R387H
Apo SH2	58.5 $\pm$ 1.5	54.6 $\pm$ 0.5	55.1 $\pm$ 1.8	54.0 $\pm$ 3.4	54.0 $\pm$ 1.4
JAK2 pY813 PD[pY]ELLTTEND	67.4 $\pm$ 2.0	59.5 $\pm$ 3.1	57.9 $\pm$ 1.8	58.9 $\pm$ 2.4	64.9 $\pm$ 3.5
JAK3 pY785 SSD[pY]ELLSDP	66.5 $\pm$ 2.4	57.3 $\pm$ 1.1	56.6 $\pm$ 1.5	59.7 $\pm$ 2.3	61.0 $\pm$ 2.6
EpoR pY454 PHLK[pY]LYLVVS	61.0 $\pm$ 1.7	55.2 $\pm$ 0.5	55.7 $\pm$ 1.2	53.7 $\pm$ 1.5	56.5 $\pm$ 2.1
EpoR pY454/pY456 PHLK[pY]L[pY]LVVS	62.8 $\pm$ 1.9	55.6 $\pm$ 1.9	56.0 $\pm$ 2.6	56.3 $\pm$ 1.1	58.7 $\pm$ 4.0

4.7.1 Mutant SH2 domain binding to pY813 of JAK2

The results of the thermal shift assays would suggest that the E372K, R387C and V374M mutants have reduced capacity to bind peptide, whereas the R387H mutant is the most similar to the WT. In order to investigate this further a direct-binding SPR experiment was developed.

Biotinylated pY813 peptide was immobilised on a chip and increasing concentrations of the LNK SH2 domains were passed over. Binding is measured by an increase in the response in the peptide-coupled flow cell minus the response in a control flow cell (**Figure 5.22A**). The three kinetic factors taken into consideration when interpreting these data are the on-rate, the off-rate and the overall response. The faster the on rate, slower the off rate and larger the response, the higher the binding affinity of the SH2 domain towards the peptide. Changes in any three of these factors may provide insights into the binding characteristics of these mutant SH2 domains (**Figure 5.22B**).

WT LNK SH2 domain binding to the pY813 peptide was characterised by a very rapid on-rate and a relatively slow off-rate (**Figure 5.21C**). The on-rate was calculated to be 7.12×10^5 1/Ms and was visibly faster than any of the other SH2 domain:peptide interactions studied in our laboratory (data not shown). Of the four mutant proteins, the V374M mutant showed the most dramatic reduction in binding. As shown in **Figure 5.21E**, the total response measured was only ca. 30% that of wild-type and the characteristics of the sensorgram were more consistent with a non-specific interaction (a slow, ever increasing association phase that does not plateau accompanied by a negligible off-rate). The data generated for this mutant could not be fitted to any simple 1:1 binding model. The three other mutants clearly retained an ability to bind to this peptide. However, the on-rate for those three mutants was clearly slower than for wild-type (E372K 9-fold slower, R387C 5-fold slower and R387H 12-fold slower) (**Figure 5.22B-G**). Despite the quality of the sensorgrams for the WT protein and the E372K, R387C and R387H mutants the data could not be acceptably fitted to a 1:1 kinetic model in order to determine affinity. Values for the best-fit data are shown in panel B for illustrative purposes however they cannot be used with any reliability. Instead, there

remains the general observation that on-rate, rather than off-rate, is perturbed in these mutants.

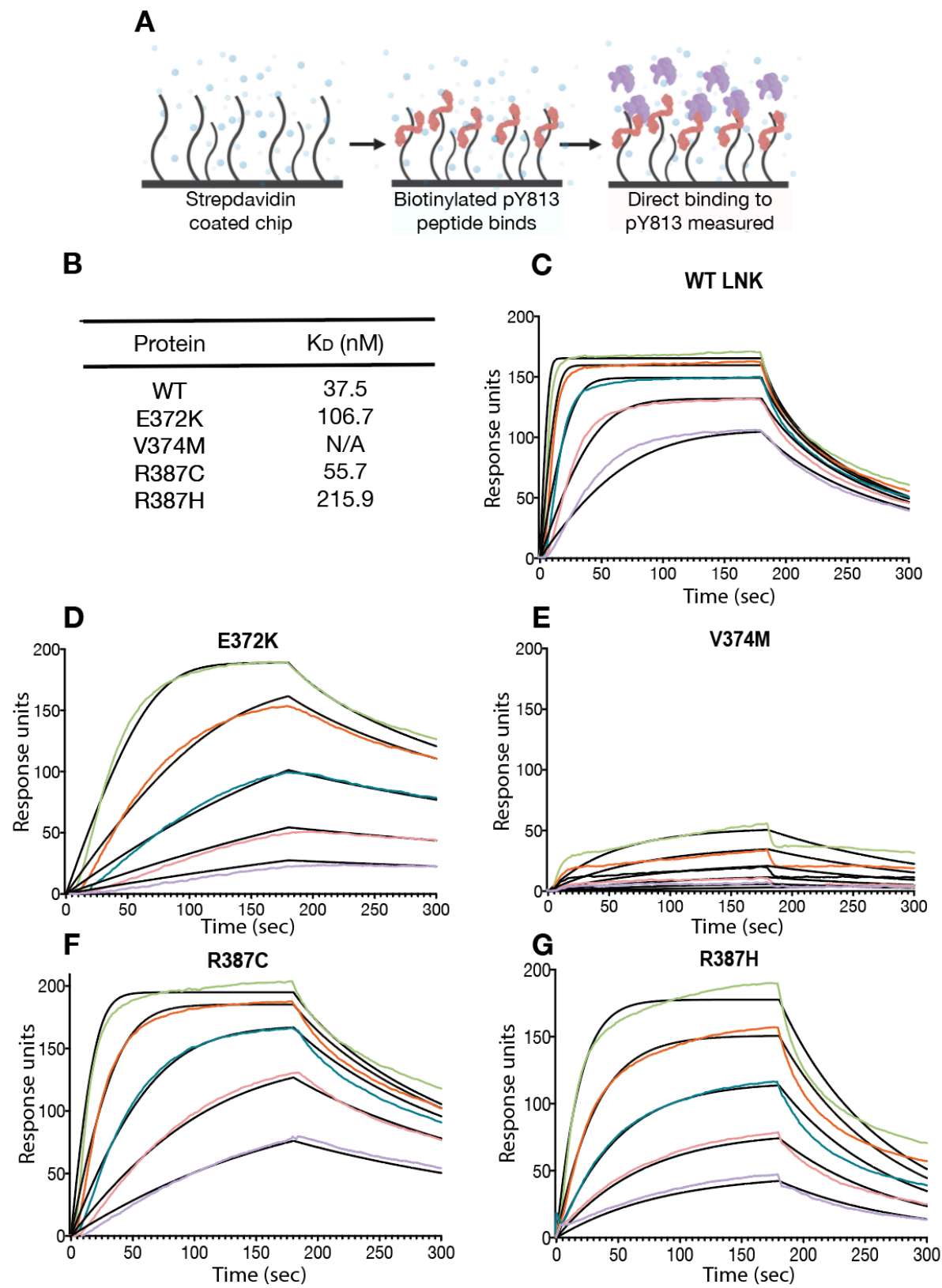


Figure 4.22 LNK SH2 domain mutants binding to pY813 by SPR.

A) Schematic diagram of the direct binding assay. Biotinylated JAK2 pY813 peptide is run over a chip and captured. Titrated amounts of the LNK SH2 domains are flown over the chip and their direct binding is measured. **B)** Although the experimental data did not fit a simple 1:1 kinetic model to a sufficient degree to allow unambiguous affinity determination, the best-fit values are shown here for illustrative purposes. **C-F)** Representative direct binding curves for the WT, E372K, V374M, R387C and R387H LNK SH2 domains to a JAK2 pY813 peptide. Raw data is shown in black, fitted data in colour. Curves represent; Light green 1 μ M peptide, orange 0.5 μ M peptide, green 0.25 μ M peptide, pink 0.125 μ M peptide and purple 0.0625 μ M peptide.

As the kinetic data above did not adequately fit to a simple 1:1 binding model (and therefore affinity could not be quantified), competition assays were used to determine the relative affinity of each mutant for the JAK2 pY813 peptide (**Figure 5.23**). Wild-type and mutant LNK SH2 domains were pre-incubated with increasing concentrations of the JAK2 pY813 phosphopeptide before being passed over the surface of an SPR chip containing bound IL6ST phosphopeptide and the maximum binding response used to calculate an IC₅₀ value. The V374M mutant could not be examined in this fashion as binding to the SPR chip was too poor (as seen in Figure 4.24) and likely represents non-specific association. The IC₅₀ for the WT LNK SH2 domain was approximately 130 nM, the R387H mutant was similar (IC₅₀ 180 nM) however the E372K mutant showed a significant decrease in affinity (IC₅₀ 680 nM). Unexpectedly, the IC₅₀ of R387C for pY813 was consistently lower than WT (IC₅₀ 50 nM). As there was no evidence of enhanced pY813 binding in previous direct-binding kinetic assays (**Figure 4.22**), the explanation for this is unclear.

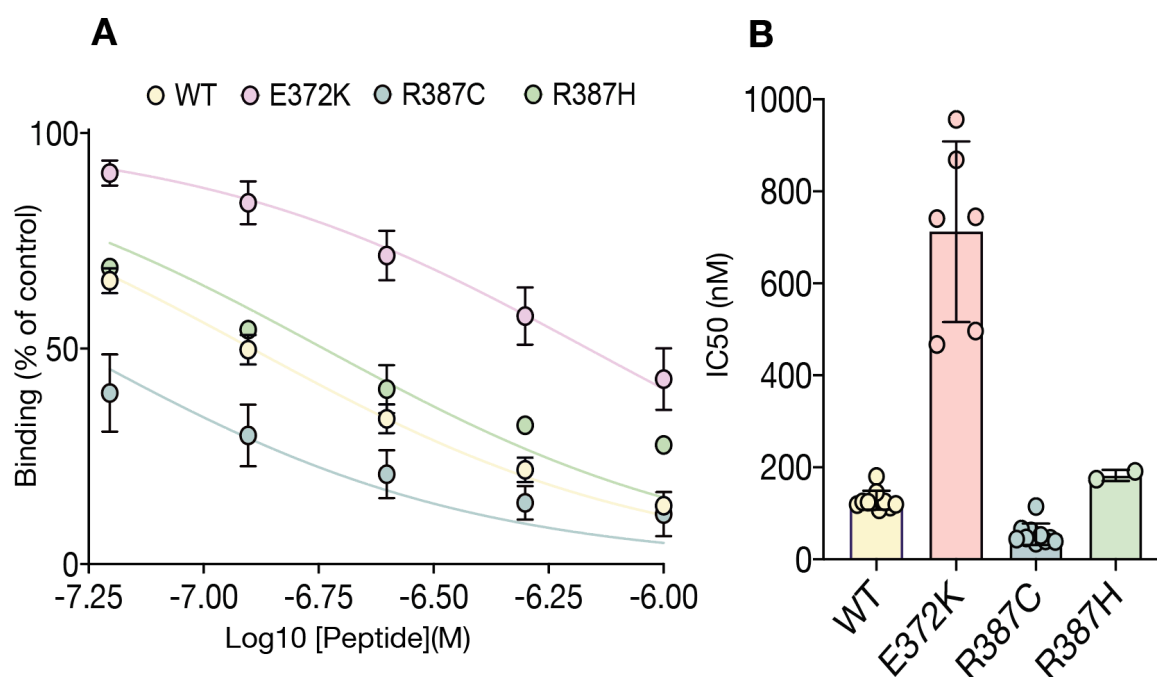


Figure 4.23 LNK SH2 domain mutants binding to pY813 by Competition SPR.

A) Inhibition curves for mutant LNK SH2 domains pre-incubated with the JAK2 pY813 peptide and flowed over an IL6ST pY757 chip. $N=2-10$, error bars are SD. **B)** IC₅₀ values for each competition assay, $N=2-10$, error bars represent SD.

4.7.1.1 Phenyl phosphate competition assays

The reduction in affinity for the LNK mutants for the pY813 peptide may be due to impaired phosphotyrosine binding or else impaired binding to other regions of the peptide. To distinguish between this, the ability of the mutant SH2 domains to bind phenyl phosphate (a phosphotyrosine mimetic) was investigated. Titrations of phenyl phosphate from 1 mM down were incubated with the LNK SH2 domains and flowed over an IL6ST chip as per section 1.4.3. As shown in **Figure 4.24**, there was a slight loss in affinity for phenyl phosphate (as measured by an increase in IC_{50}) for R387C and R387H but a significant loss in affinity for E372K. The V402M mutant was not examined as binding to the peptide immobilised to the chip was compromised.

These results mirror those shown in Figure 5.21 and suggest that impaired phosphotyrosine binding is a significant component of the inability of the E372K mutant to bind to the pY813 sequence, but less so for the R387C and R387H mutants.

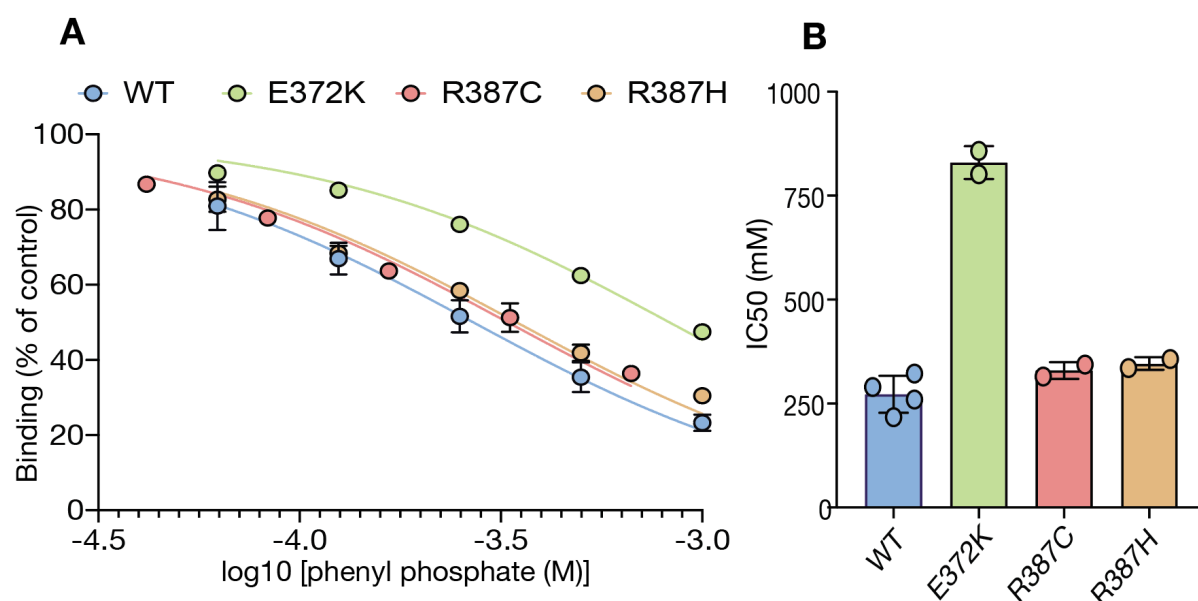


Figure 4.24 LNK SH2 domain mutant binding to phenyl phosphate by SPR competition.

A) Inhibition curves for mutant LNK SH2 domains pre-incubated with phenyl phosphate and flowed over an IL6ST pY757 chip. $N=2$, error bars are SD. **D)** IC_{50} values for the mutant LNK SH2 domains pre-incubated with phenyl phosphate. $N=2$, error bars are SD.

4.8 Mutant LNK SH2 domain structures

The E372K mutant of the LNK SH2 domain has been identified in patients with polycythemia vera and idiopathic erythrocytosis. Glu 372 is in the BC loop region that forms the phosphotyrosine binding pocket, and interacts with Arg 299 and Arg 387. The equivalent of Arg 387 is present in many SH2 domains and often assists in coordination of the pTyr. In the previous section it was shown that the E372K mutant has impaired binding in comparison to the WT SH2 domain including a reduced on-rate. To further understand this X-ray crystallography was performed.

Hanging drop vapour diffusion crystal trays were set up by hand at 5 mg/mL of the E400K LNK SH2 domain and a 2-fold molar excess of the JAK2 pY813 phosphopeptide with 1 μ L protein solution and 1 μ L of precipitant over a 450 μ L reservoir volume. Crystals formed in a condition containing 18% PEG 8000, 0.05 M magnesium acetate and 0.1 M Tris pH 8.5 (**Figure 4.25**). Crystals from this drop were cryoprotected in paratone, flash frozen in liquid nitrogen and taken to the MX2 beamline at the Australian Synchrotron. Data was processed to 1.65 Å in XDS (Kabsch 2010), and a solution was found using the SH2B apo structure as a model for molecular replacement, with a top TFZ score of 20.1 in the space group C 1 2 1, with one copy of the protein in the asymmetric unit. Refinement was performed using PHENIX (Adams et al. 2010) and the model was built in Coot (Emsley and Cowtan 2004). Further details of this structure can be found in **Table 4.1**.

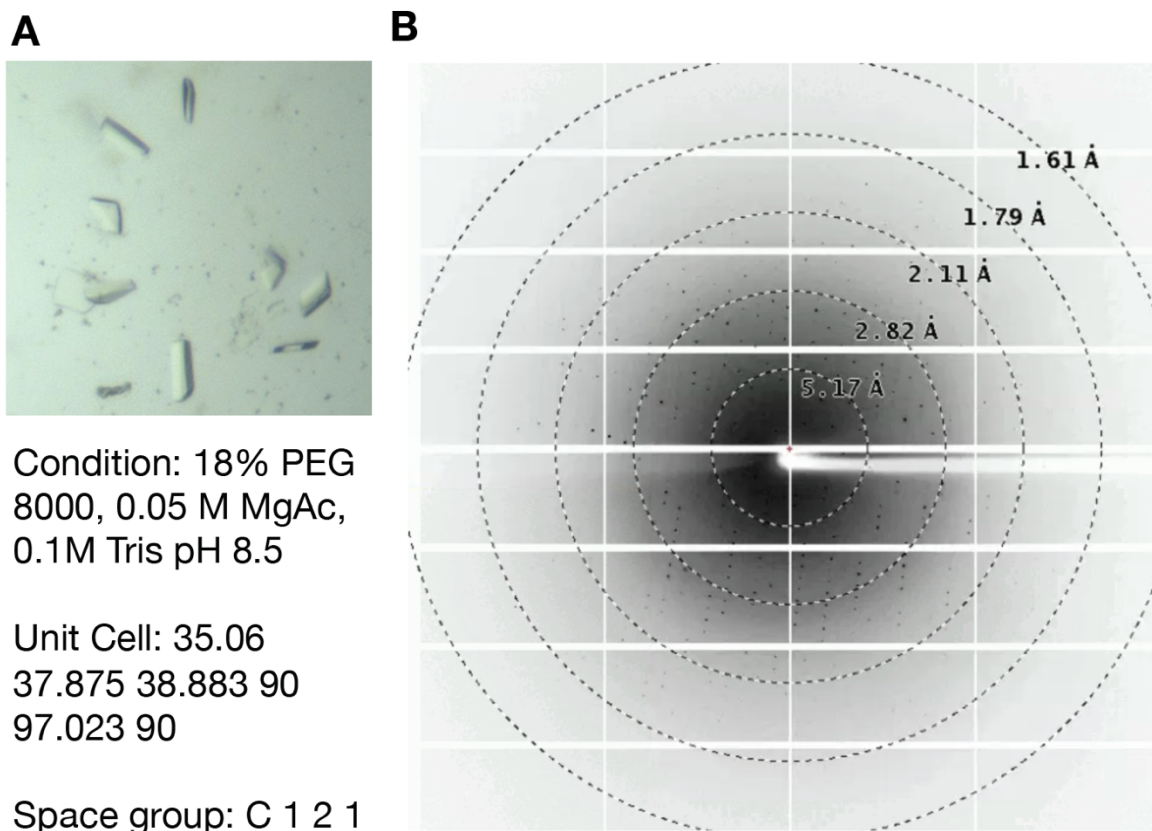


Figure 4.25 Crystals of the E372K LNK SH2 domain with a JAK2 pY813 peptide.

A) Crystals of the E372K LNK SH2 domain generated in house. Conditions, unit cell dimensions and space group indicated. **B)** Diffraction pattern from the MX2 beamline at the Australian Synchrotron. Diffraction to 1.61 Å.

4.8.1 Structure of the E372K LNK SH2 domain with a JAK2 pY813 phosphopeptide

Alignment of the WT and E372K LNK SH2 domains revealed very few structural changes between the two proteins, including at the BC loop where the E372K mutation occurs (**Figure 4.26A and B**). The two structures have an overall RMSD of 0.5 Å. Eight residues (-2 to +5) of the JAK2 pY813 peptide can be resolved including all side chains (**Figures 4.27 and 4.28**). Along with these interactions seen for the WT structure, two more interactions between LNK and the peptide side chains are apparent; The -1 Asp of the peptide forms a hydrogen bond with Arg 343, and the +5 Glu interacts with Gln 399 of LNK. This interaction between the +5 Glu and Gln 399 was not apparent from the WT structure, however, it is in agreement with the mutagenesis SPR competition assays (**Figure 1.7**). As seen in the EPOR pY454 bound structure, where the +5 Val stacked over His 400, the side chain of the +5 Glu also sits over His 400 at Van der Waals distance (~4 Å) and the orientation of the Gln 399 shifts to allow an additional H-bond to the +5 residue. Similar to the WT structure, Lys 384 salt bridges with the +1 Glu and several of the backbone interactions seen in the WT structure are also recapitulated in the E372K structure including those between Pro 419, Glu 421, and His 385 and the pY813 peptide. Additionally, the backbone carbonyl oxygens of Leu 420 and Pro 419 also hydrogen bond with the side chain of the +4 Thr, and the side chain of His 400 with the backbone carbonyl oxygen of the same +4 Thr. Each of these interactions are represented in **Figure 4.28**.

In the E372K structure, the BC loop occupies the same position as the WT SH2 domain. However, the Lys at position 372 of the mutant points in the opposite direction to the Glu in the WT domain as it cannot form the hydrogen bonding network with the Arg 369 and Arg 387 residues (**Figure 4.26C, D and E**). There is poor density for both Arg 369 and Arg 387 side chains, with the side chain for Arg 387 visible only to the epsilon nitrogen, with no density for the guanidinium headgroup (**Figure 4.27D**). However, what is clear in both structures (WT and E372K) is that the carbon chain of Arg 387 forms Van der Waals interactions with and shields the phosphotyrosine phenyl ring. In the WT structure, the network formed by Lys 372, Arg 369 and Arg 387 provides a hydrophobic environment for the phenyl ring of the phosphotyrosine, which is partially recapitulated by the side chains of Arg 369 and Arg 387 in the E372K structure (**Figure 4.27E**).

Despite these minor differences, perhaps the most striking feature of the mutant structure is how similar it is to wild-type in the phosphotyrosine binding region, as all the interactions with the phosphate group are maintained (**Figure 4.27C and 4.28**). The wild-type and mutant protein adopt very similar end-points when bound to peptide, despite the loss of affinity in the mutant. I hypothesize that when in the apo-state, the phosphotyrosine binding loop may not be “primed” to bind peptide. This would be due to the loss of the hydrogen bonding network within the BC loop made up of Glu 372, Arg 369 and Arg 387 causing the loop to be more flexible and existing less often in its “bound” conformation. This could explain the reduced on-rate of the E372K mutant and corresponding loss of affinity for various peptides (alongside a general decrease in thermostability) whilst it maintains the ability to adopt a very similar final structure (in the presence of peptide) to the wild-type protein.

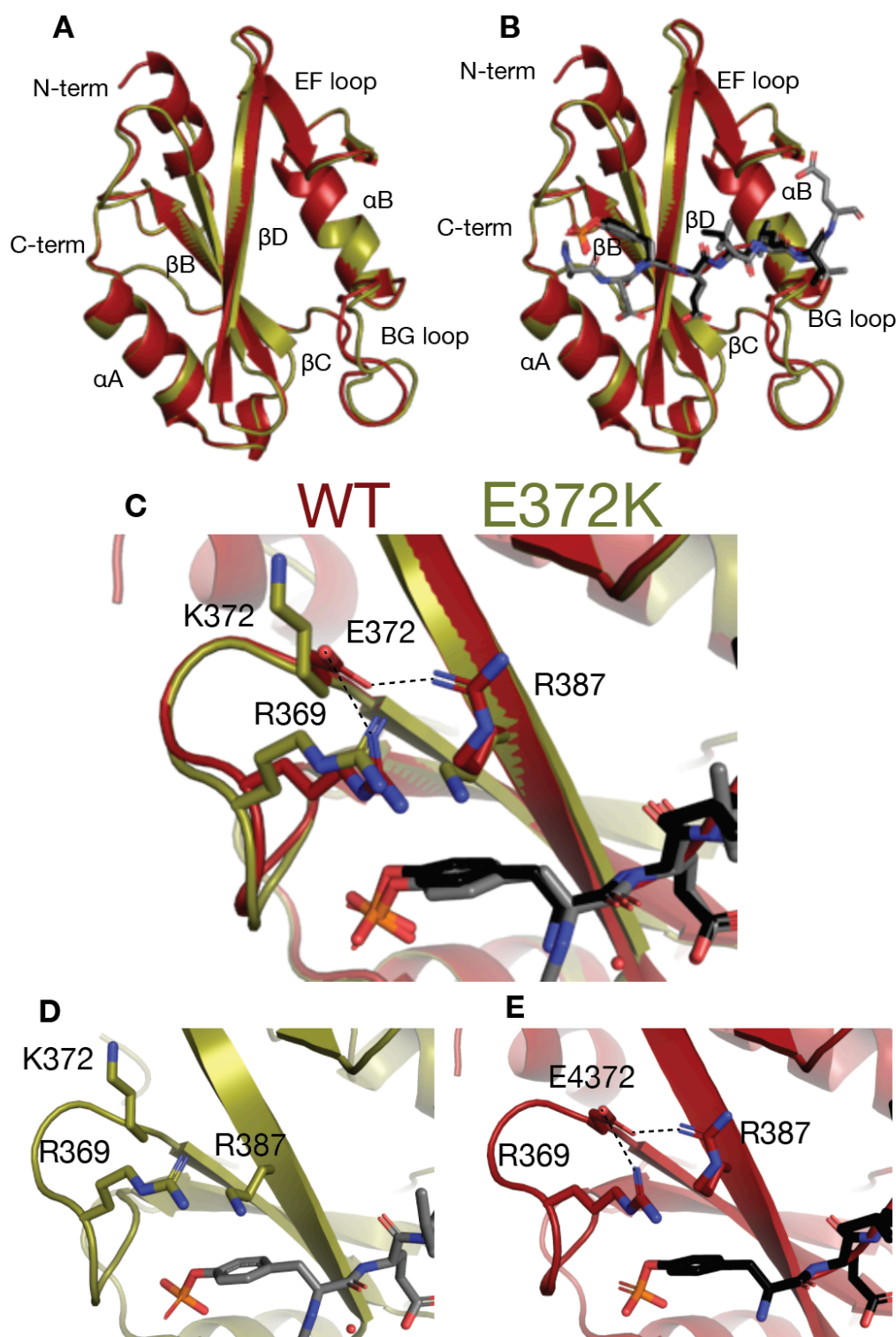


Figure 4.26 Comparison of the E372K and WT LNK SH2 domains bound to a JAK2 pY813 peptide.

A and B) The WT and E372K SH2 domain structures aligned to one another. Very few changes to the backbone are visible other than a small shift in the BG loop. The peptide is in an identical position in both structures, with all side chains in very similar positions. RMSD of 0.5 Å. **C)** The BC loop where the E372K mutation occurs is in the same position in both structures, however the Lys residue points into solvent whereas the WT Glu interacts with R369 and R387. **D and E)** Individual structures with the BC loop shown.

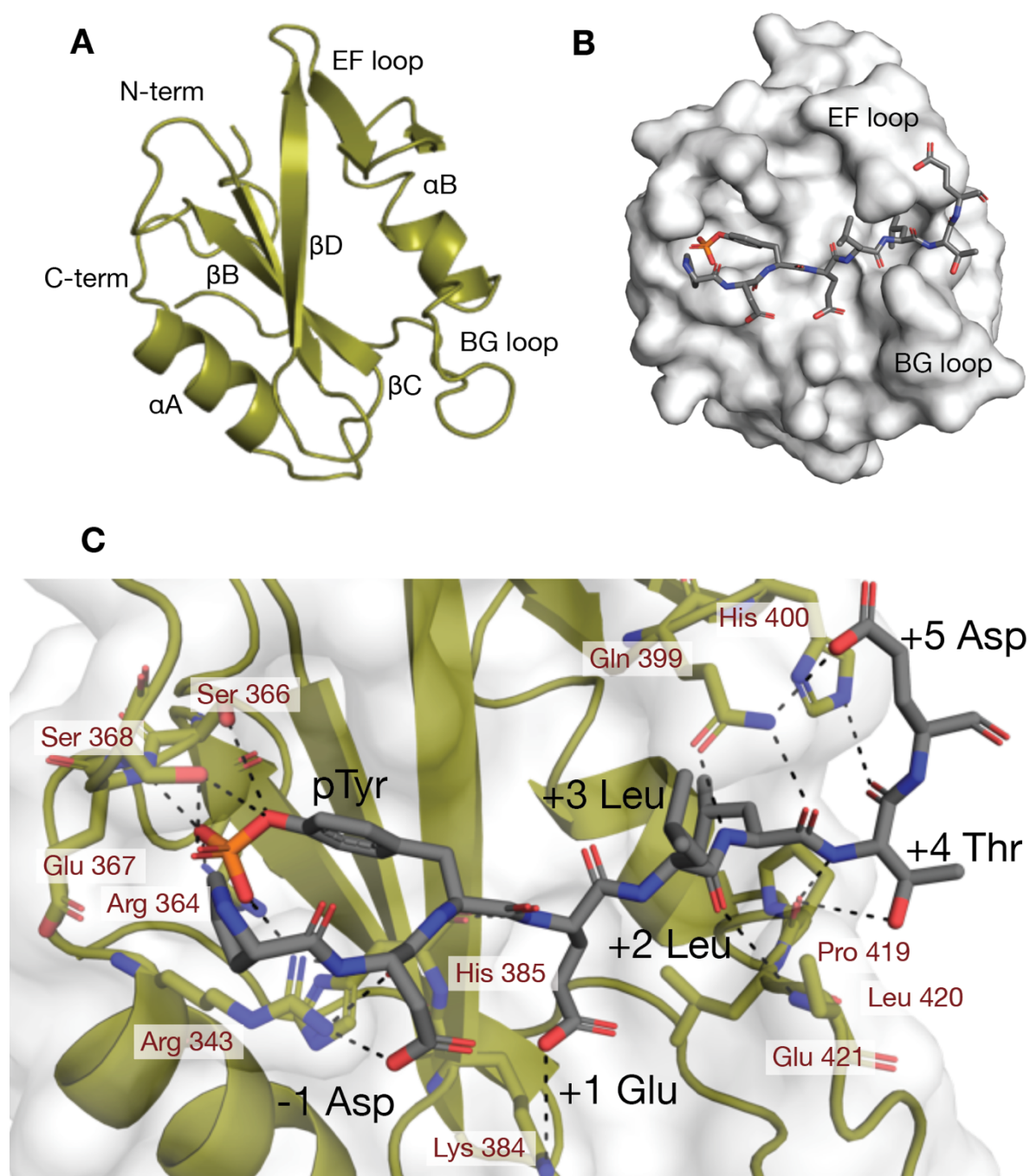


Figure 4.27 Crystal structure of the E372K LNK SH2 domain bound to a JAK2 pY813 peptide.

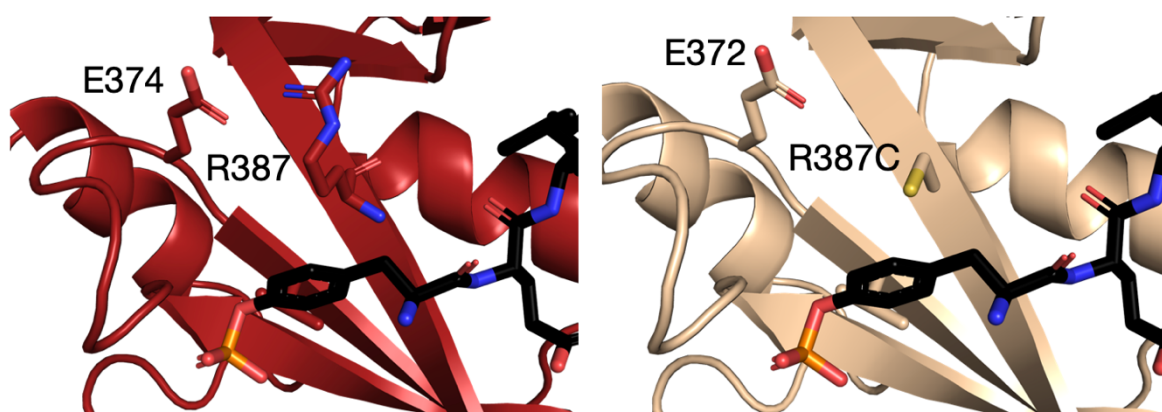
A) The backbone of the E372K LNK SH2 domain with the features indicated with peptide removed. **B)** Surface representation of the LNK SH2 domain with the JAK2 pY813 peptide bound. **C)** Peptide in black with LNK SH2 domain with surface representation shown. The pTyr sits in the phosphotyrosine binding pocket of the SH2 domain, the +1 Glu forms a salt bridge with Lysine residue 384 in LNK and the +3 Leu sits in a hydrophobic pocket formed in part by the EF and BG loops of LNK.

4.8.2 Other LNK SH2 domain mutants

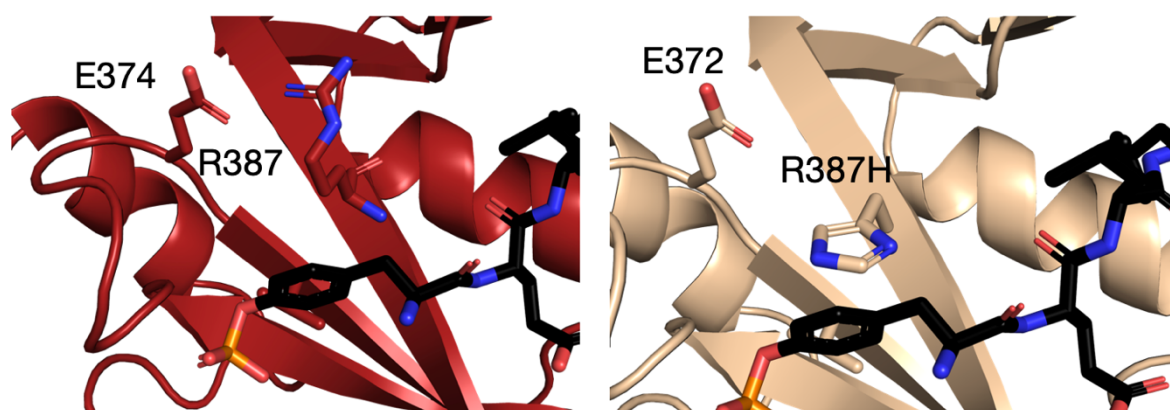
Despite attempts to crystallise the other mutants, no crystals were generated for the V374M, R387C and R387H LNK mutants, therefore it was only possible to infer what interactions may have been lost based on crystal structures of the WT LNK SH2 domain and the E372K mutant SH2 domain. The mutated residues were swapped in the WT structure bound to JAK2 pY813 peptide using PyMol (DeLano 2002), and compared to the WT structure (**Figure 5.29**).

As previously shown in the WT and E372K structures, R387 and E372 form a hydrogen bonding network which may stabilise the BC loop region of the SH2 domain. As shown for the E372K mutant, changing this residue to a lysine disrupts this interaction and the guanidinium group of Arg 387 cannot be resolved. Whatever the effect this has on the structure or stability of the domains, it is reasonable to assume that changing R387 may have a similar effect. The V374 residue sits directly beneath the phosphotyrosine binding pocket of LNK. While valine is a small hydrophobic residue, a mutation to a methionine is much bulkier, and would entirely preclude binding of a peptide in this position due to steric hindrance.

R387C



R387H



V374M

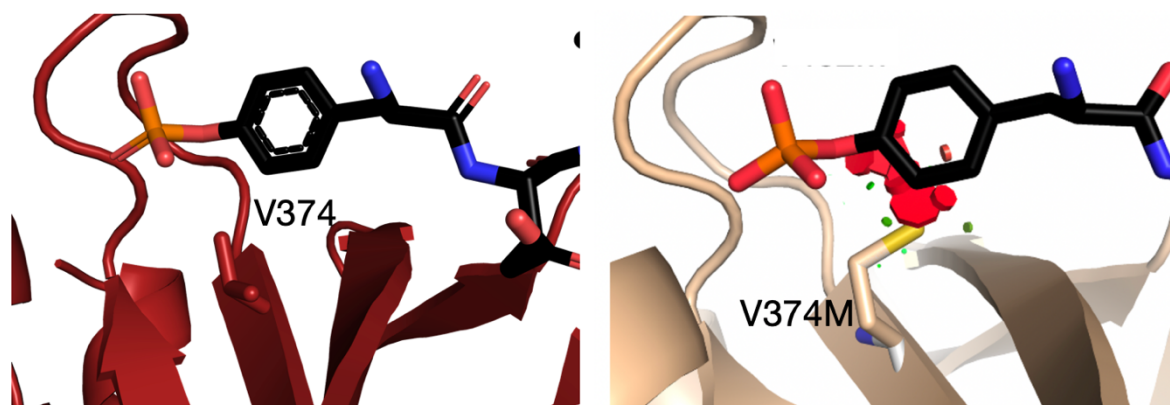


Figure 4.29 Models of V374M, R387C and R387H LNK mutants indicating placement of mutation on SH2 domain

A) R387H mutation model. WT structure shows interaction between Glu 372 and Arg 387. B) R387C mutation model. S. C) V374M mutation model. WT structure shows position of V374 under the phosphotyrosine binding site. The clash between a Met at position 4 and the pTyr is indicated.

4.9 Discussion

LNK is an important negative regulator of JAK2-mediated signalling downstream of TPO (Tong and Lodish 2004) and EPO (Tong, Zhang, and Lodish 2005), and has been suggested to regulate a number of other signalling pathways and proteins involved in haematopoiesis and immune function (Lin et al. 2012; Gueller et al. 2010; Gueller et al. 2008). Despite this, little is known about how LNK interacts with these proteins and how LNK is able to negatively regulate their activity. More recently, mutations in LNK have been identified in patients with a variety of hematopoietic diseases, including the myeloproliferative neoplasms (Pardanani, Lasho, Finke, Oh, et al. 2010; Oh et al. 2010) and some blood cancers (Tan et al. 2017). Without comprehensive knowledge of the full repertoire of LNK substrates, and thus an understanding of which biological processes are affected by LNK mutations, it is difficult to understand the role these mutations play in disease progression.

In this chapter, the interaction between the LNK SH2 domain and the JAK2 pY813 residue was studied using structural and biochemical methods. The results in this chapter illustrate how LNK interacts with specific phosphotyrosine motifs and highlights several phosphotyrosine motifs in other proteins that may serve as LNK docking sites. Moreover, studies of point mutants in the LNK SH2 domain show how single amino acid substitutions can lead to decreased SH2 domain stability and/or decreased affinity for phosphorylated motifs and therefore suggest these mutants have a reduced ability to regulate their targets.

4.9.1 LNK is a negative regulator of TPO signalling in M1 cells

As previously shown (Tong and Lodish 2004), LNK is able to negatively regulate TPO signalling (**Figure 4.1**). M1 cells expressing MPL, the TPO receptor, undergo terminal differentiation from monocytic-like cells into macrophages when cultured in the presence of TPO (**Figure 4.1B**). When LNK was overexpressed in these cells, TPO-induced differentiation and eventual cell death was slightly reduced (**Figure 4.1D**), consistent with previous literature using other cell-types (Tong and Lodish 2004; Seita et al. 2007). Despite the fact that SH2B and APS are also thought to be

regulators of JAK2 activity, M1 cells over-expressing these proteins responded to TPO in a similar manner to control cells (**Figure 4.1D**).

4.9.2 The structure of the LNK SH2 domain

The structure of the LNK SH2 domain adopts the typical SH2 domain fold comprising a central antiparallel beta-sheet flanked by two alpha-helices. In the case of both the JAK2 pY813 and EPOR pY454 peptides, the phosphotyrosine residue inserts into the canonical phosphotyrosine binding pocket, and the C-terminal residues cross the core beta-strands (**Figures 4.4, 4.19 and 4.27**). As predicted by Hu and Hubbard, the LNK SH2 domain does not dimerise like APS, and instead appears to remain in its monomeric state as seen with SH2B (Hu and Hubbard 2006). Hu and Hubbard also predicted that the loss of a Ser at position 421 in LNK would result in a loss of selectivity for a Glutamate residue (Glu 814 in JAK2 pY813) at the +1 position. However, our structures reveal that instead, Glu 814 interacts with Lys 384, allowing this selectivity to be retained (**Figures 4.4, 4.19 and 4.27**). At the same +1 position, the structure of the WT SH2 domain with an EPOR pY454 peptide bound revealed how a slight shift in the BG loop can accommodate a hydrophobic residue such as a Leu (**Figure 4.18**). In the case of both the JAK2 pY813 and EPOR pY454 peptides, the +3 peptide residue (Leucine) inserts into a hydrophobic pocket between the EF and BG loops (**Figures 4.5 and 4.17**). In addition to the pTyr, +1, and +3 interactions, the +5 Glu (818) of the JAK2 pY813 peptide hydrogen bonds with Gln 399 of LNK (**Figures 4.27 and 4.28**), and as established by SPR competition assay, a glutamate at this position may contribute to higher affinity interactions (**Figure 4.8**). In concordance with their similar binding modes, the total buried surface area between the LNK SH2 domain and the JAK2 pY813 peptide and the EPOR pY454 peptide is similar at 584.3 Å and 573.5 Å respectively. This is slightly lower than the total buried surface area between SH2B and JAK2 pY813 which is 672.4 Å. Unexpectedly in all three crystal structures of the LNK SH2 domain, Cys 421 and Cys 425 of the BG loop formed a disulphide bond, a feature not present in SH2B or APS as they both comprise a shorter BG loop that also lacks two Cys residues. It is likely this is a feature of crystallisation due to the oxidising environment.

4.9.3 The basis for specificity for the LNK SH2 domain

While many studies have demonstrated that LNK may bind to a suite of phosphorylation sites on proteins (Lin et al. 2012; Gueller et al. 2011; Gueller et al. 2008; Gueller et al. 2010), the molecular basis for the specificity of the SH2 domain of LNK for these proteins domain is still largely unknown. Without structural information to use as a guide, peptide libraries were previously used to determine which residues may be important for binding. One such study showed that the consensus sequence for the LNK SH2 domain is xxpY(V/I/E)xL (Cheng et al. 2016). The structural and biochemical data presented in this chapter are consistent with this finding and, in addition, indicate the +5 residue also contributes to affinity. All three structures of the LNK SH2 domain determined in this chapter contain peptides with a leucine at the +3 position. This was seen to insert into the hydrophobic pocket between the EF and BG loops of LNK, and is preferred over more bulky hydrophobic residues such as phenylalanine (**Figure 4.8**). Despite this, the two phosphorylated sequences in FLT3 that contain a Phe and a Met (**Figure 4.13**), so while there is a preference for a small residue such as a Leu, larger hydrophobic sidechains can be accommodated.

Other than the +1 and +3 residues, the results from the SPR competition assay revealed that a negatively charged residue at the +5 position also increases affinity (**Figure 4.8**). The E372K structure confirmed the +5 Glu of the JAK2 pY813 peptide could hydrogen bond with Gln 399 of LNK (**Figures 4.27 and 4.28**). Interestingly, an aspartate residue was shown to be preferred at the -1 position in the study screening the peptide library, and in the E372K structure, the Asp of the JAK2 pY813 peptide forms a salt bridge with Arg 343 of LNK (**Figure 4.28**). However, mutation of this residue in the SPR competition assay had little effect on binding (**Figure 4.8**) and so it is unclear how much this contributes to the binding affinity.

Collectively, the results from the previously published proteomics screen and the data presented in this chapter provide more detail to the previously proposed sequence recognised by the LNK SH2 domain (**Figure 4.30**). As for all SH2 domains, the pTyr is essential for binding. There is a preference for a Glu at the +1 position, however small hydrophobic residues are also able to interact with LNK at this

position in a very similar pocket, and a small uncharged sidechain such as Thr can also be tolerated. Additionally, a positively charged residue such as an Arg is not accommodated at the +1 position, reflected in the inability of the LNK SH2 domain to associate with multiple MPL peptides (**Figure 4.11**). At the +3 position, a leucine is preferred, however other hydrophobic residues such as methionine and phenylalanine can be accommodated. At the +5 position, a negatively charged residue increases binding affinity. It also would be interesting to investigate whether phosphorylation of other tyrosine residues, and perhaps serine and threonine residues surrounding the phosphotyrosine occupying the phosphotyrosine binding pocket can alter affinity. Initial analysis of the EPOR pY454/pY456 peptide (**Table 4.3**) indicated this was not the case, however given the position of this phosphotyrosine, this is perhaps unsurprising as the +2 residue doesn't interact with the SH2 domain. Moving forwards the MPL pY626 and FLT3 pY591 good candidates to test this hypothesis as the +5 residue is a tyrosine that may be able to be phosphorylated.

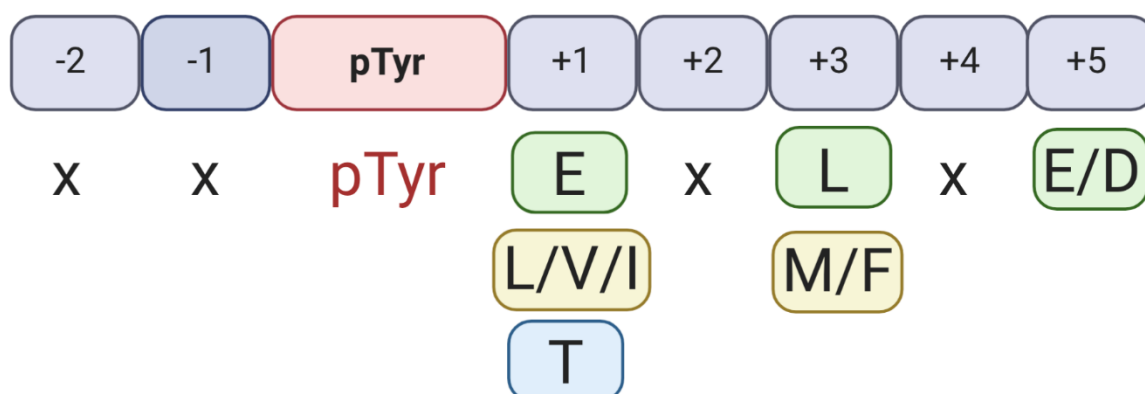


Figure 4.30 Residues important for association with the LNK SH2 domain. (Top) Position of residues relative to the pTyr (red). (Bottom) preference for residues at each position relative to the phosphotyrosine.

4.9.4 LNK SH2 domain binding partners

4.9.4.1 LNK and JAK STAT signalling

Having a more detailed understanding of how the LNK SH2 domain interacts with phosphotyrosine residues and which residues are important for binding may allow us to predict other phosphotyrosine motifs in other proteins LNK it may interact with. Using the sequence mentioned above as a guide (**Figure 4.30**), it is clear that

LNK has a strong preference for specific residues at the +1 and +3 positions. It was therefore unsurprising that LNK was unable to bind the JAK2 and IRK activation loops as they lack these preferred residues at the +1 and +3 positions (**Figure 4.9**).

The pY785 residue is a major site of phosphorylation in JAK3 (Cheng et al. 2008), though physiological the outcome of this phosphorylation is yet to be elucidated. JAK3 is activated downstream of IL-2 family cytokines (Miyazaki et al. 1994), and the ability of LNK to regulate B cell development is in accordance with this data. Why LNK is able to specifically regulate some pathways that activate JAK2, but not others is perhaps the most important question in this field. If LNK can interact with the EPOR directly, as suggested by work in this chapter, this may explain its involvement in EPO signalling, however given that the LNK SH2 domain is unable to bind any peptide derived from MPL, this does not hold true for its ability to regulate TPO signalling. LNK is upregulated upon TPO (Baran-Marszak et al. 2010) and EPO (Gery, Cao, et al. 2009) signalling, which may suggest that SH2B3 is a STAT5 responsive gene, and perhaps LNK is only expressed at high enough levels to inhibit signalling in those pathways that activate STAT5. Our structural studies have focussed solely on the SH2 domain of LNK and it is possible that the other domains of LNK may be involved in interactions with the receptors, or JAK2. Moreover, LNK could be recruited by an intermediate protein that mediates the interaction between LNK and other proteins which has yet to be identified.

LNK has also been implicated in modulating the activity of some haematopoietic receptors which are not involved in classic JAK-STAT signalling, such as FLT3 (Lin et al. 2012), c-KIT (Gueller et al. 2008), c-FMS (Gueller et al. 2010) and PDGFR (Gueller et al. 2011). FLT3 is a receptor tyrosine kinase essential for haematopoietic stem cell differentiation and survival. LNK has been shown previously to inhibit both normal and leukemic FLT3 signalling, with the proposed binding sites on FLT3 being pY572, pY591 and pY919 (Lin et al. 2012). Two of these were confirmed to be potential LNK SH2 domain binding sites (pY591 and pY919) in this chapter however the third could not bind the protein (**Figure 4.13**). The FLT3 pY591 peptide contains a hydrophobic Phe at the +3 position, and a Glu at the +5 position, which are likely able to facilitate binding. The pY919 peptide contains a hydrophobic Met at the +3 position, and an Ile at the +1 position, consistent with residues able to

interact with the LNK SH2 domain. Given that the FLT3 pY572 residue contained none of the residues preferred for binding, these results are not surprising.

Given these results and the binding motif discussed previously, purified LNK SH2 domain is likely able to associate with c-FMS, PDGFR and c-KIT. All three of these receptors contain phosphotyrosine residues that have hydrophobic residues at the +3 position, and either a hydrophobic residue or Thr at the +1 position (**Figure 1.15 of introduction**). If LNK is indeed able to bind these receptors *in vivo*, this may suggest that the activity of LNK extends to the regulation of both the MAPK and PI3K pathways, which are also important for haematopoiesis and further support the role of LNK as a tumor suppressor (Bersenev et al. 2010; Gery, Gueller, et al. 2009).

4.9.1 Mutants of the LNK SH2 domain

While most mutations of LNK are found in the PH domain, a number of single amino acid substitutions in the SH2 domain have been identified in patients with a variety of diseases (Oh et al. 2010; Hurtado et al. 2011; McMullin et al. 2011). While it is not obvious how LNK may play a role in these diseases, gaining an insight into the changes that occur to the SH2 domain caused by these single point mutations sheds light on the effect on mutations on LNK function and structure.

Four mutations identified in patients with myeloproliferative neoplasms were studied in this thesis; E372K (McMullin et al. 2011), V374M (Chen et al. 2016), R387C (Camps et al. 2016), and R387H (Chen et al. 2016). Each of these cluster around the phosphotyrosine binding pocket and so I hypothesised they may preclude or reduce binding to phosphotyrosine (**Figure 4.20**). Of these mutations, V374M had the most severe effect on peptide binding (**Figure 4.22**). Although the structure of this mutant was not determined it is clear from an examination of the wild-type structure that a bulky methionine residue cannot accommodate the aromatic ring of a phosphotyrosine (**Figure 4.29**). The E372K mutant also impaired phosphopeptide binding (**Figure 4.22**). Changing this negatively charged residue to a positively charged lysine abolished the interactions with Arg 387, which may destabilise the phosphotyrosine binding pocket (**Figure 4.26**). Both the R387C and R387H mutations were hypothesised to have a similar effect as the E372K mutation, abolishing their interaction and destabilising the loop region of the pTyr binding

pocket (**Figure 4.29**). Additionally, all mutants were less thermostable than the WT in their apo and peptide bound forms (**Figure 4.21 and Table 4.3**).

One of the most notable observations in this chapter was the fast on-rate when the pY813 peptide bound to LNK (**Figure 4.22**). This on-rate was significantly faster than that seen for other SH2 domains studied in our laboratory (Linossi et al. 2013). Given this data, I hypothesise that the K372-R369-R387 hydrogen-bonding network stabilises the phosphotyrosine binding pocket and shields the phosphotyrosine. Thus, in the E372K mutant, this pocket samples a range of conformations rather than being primed for phosphotyrosine binding. Of the four mutants studied, the R387H mutant may be the most similar to the WT, and may be able to form a similar interaction between the His 387 and Glu 372, however it is unclear to what extent this would be the case *in vivo*, as this effect may depend on protonation state of His 387.

An additional mutation highlighted in the literature is the LNK R392E mutation (Oh et al. 2010), which is completely unable to regulate signalling in any systems tested. The R392 residue corresponds to R364 in our structure (**Figure 4.5A**), which is essential for coordinating the pTyr residue in all SH2 domains (Liu, Engelmann, and Nash 2012) It is therefore unsurprising that mutation of this residue will abolish binding to any phosphotyrosine residues. Together these data lend insight into how single amino acid mutations can alter the ability of the LNK SH2 domain to associate with phosphotyrosine residues.

4.9.2 Implications in disease

Given the non-redundant role LNK appears to play in regulating the proliferation and self-renewal of several haematopoietic cells, it is unsurprising that inactivating mutations in LNK are implicated in disease onset or progression. From the data presented in this chapter, it is clear that mutations such as the V374M mutant may cause LNK to be entirely non-functional. As previously mentioned, the incidence of LNK mutations appears to increase in blast-phase transformation for patients with MPNs (Pardanani, Lasho, Finke, Oh, et al. 2010). Given the V374M mutant was identified in patients who also harboured a JAK2 V617F mutation, the synergy of these mutations could be a potential mechanism for any transformation that might

be seen. For the other three mutations whose activity was not completely abolished, it is possible that these mutations, over extended periods of time, or in conjunction with other mutations in cells, may at least exacerbate disease. Particularly the E400K mutant, where binding ability is reduced, but not abolished. An in-depth analysis of the effects of these mutants on cellular proliferation is required in order to gain a better insight into the roles of these mutations in disease.

While the identification of mutations in LNK provides information about how the function of the protein may be altered, determining other proteins that LNK may interact with will provide a broader picture of the biological process LNK is able to regulate and therefore which pathways are altered by these mutations. Given the potential breadth of LNK action on proteins linked with haematopoiesis (JAK2, JAK3, FLT3, EPOR), it is not surprising that LNK mutations are being identified in inflammatory disorders, immune dysfunction and blood cancers.

4.9.3 Outstanding questions

One of the main questions that remains is how LNK exerts its negative regulatory functions. One potential mechanism is that LNK is able to change the conformation of kinases such as the JAKs, and keep them in an inactive state. Binding of LNK to phosphotyrosine residues could also prevent other proteins from binding to activate downstream pathways. Finally, LNK may be able to recruit another protein that modulates JAK activity. The latter has been proposed for APS, which may interact with C-CBL an E3 ubiquitin ligase (Lv et al. 2017). These possibilities need to be investigated in order to fully understand the action of this protein. As previously discussed, LNK appears to display specificity for EPO and TPO signalling over other pathways, however the basis for this specificity is not fully understood. Additionally, while the role of the SH2 domain of LNK is clear, the functions of the other motifs and domains of LNK is less defined. Understanding the structure and function of other LNK domains may also provide some insight into the former two questions. Our future efforts lie in this direction.

4.10 Conclusions

The data presented in this chapter showed how the LNK SH2 domain interacts with the pY813 peptide of JAK2. Structural and biochemical studies revealed that the pTyr, +1 Glu, +3 Leu and +5 Glu of the JAK2 pY813 peptide are important for the interaction between these two molecules. It was further shown that the SH2 domain can also bind JAK3 pY785, three residues on the EPOR, and two on FLT3, although this requires confirmation *in vivo*.

This chapter also showed how mutations in the LNK SH2 domain can impair its ability to bind to phosphotyrosine residues, and therefore regulate their activity. Given the identification of these mutants in patients with MPNs, we now have a better idea of how LNK may be contributing to disease burden.

Chapter 5 Conclusions and Future Directions

The work presented in this thesis aimed to detail the interactions between PTP1B and LNK with proteins of the JAK-STAT signalling pathway in order to better understand how haematopoiesis is regulated at the molecular level.

Chapter 3 focused on describing how PTP1B interacts with and dephosphorylates the tyrosine kinase JAK. It was found that PTP1B could dephosphorylate the kinase domain of all members of the JAK family, specifically at the activation loop, which contains two consecutive phosphotyrosine residues. Using structural biology and biochemical techniques, PTP1B was shown to preferentially dephosphorylate the second phosphotyrosine of the JAK2 activation loop. This finding was unexpected given previously published data on the interaction between PTP1B and another tyrosine kinase (IRK) demonstrated that the first phosphotyrosine bound in the catalytic pocket, and the second in region termed the second aryl binding site (Salmeen et al. 2000). This finding raises interesting questions about the regulation of JAK activity by PTP1B, given that the second phosphotyrosine is dispensable for JAK activity (Feng et al. 1997). A potential justification for this preference may be that as JAK appears to have increased catalytic activity when both tyrosine's are phosphorylated (Hall et al. 2010), PTP1B may only be recruited to dephosphorylate JAK upon phosphorylation of both tyrosine residues. This would allow JAK to reach a certain threshold of activity before being negatively regulated *in vivo*. This hypothesis would be interesting to explore further.

Additional comparison of the dephosphorylation of the JAK2 and IRK activation loops revealed that while this preference exists for the second phosphotyrosine in the case of JAK2, PTP1B does not display a similar level of preference for either phosphotyrosine in the case of IRK. Because of the presence of the second aryl binding site in PTP1B, and the structure of the IRK activation loop peptide with the second phosphotyrosine bound in this site, it was assumed this was the only physiologically relevant interaction with a tandemly phosphorylated substrate. The results presented in chapter three establish that there are two orientations in which PTP1B is able to bind tandemly phosphorylated substrates such as the JAK2 and IRK activation loops. When the first phosphotyrosine occupies the catalytic pocket, the second can be coordinated by Arg 24 of PTP1B. When the second phosphotyrosine occupies the catalytic pocket, the first can be coordinated by Arg 47. These results are in agreement with previous studies that demonstrate the -1 and +1 residues of

PTP1B substrates are critical for selectivity. Additionally, these results may indicate that a new way to target PTP1B therapeutically may involve designing a molecule that spans the interactions at the catalytic pocket and the second aryl binding site (Arg 24) and Arg 47. Furthermore, given many other phosphatases may have different binding preferences, this may allow for better selectivity in cells.

Further data presented in chapter 3 demonstrated that the ability of PTP1B to dephosphorylate JAK depends on the conformation of the activation loop. While it is widely understood that the activation loop is a dynamic region of the JAKs, the conditions under which the position of the activation loop changes conformation once activated are not fully understood. As demonstrated for the insulin-like growth factor receptor kinase, there are at least three conformations the activation loop may adopt (Favelyukis et al. 2001; Munshi et al. 2003). Whether these conformations represent a physiologically relevant switch mechanism in the kinase that occurs during signalling is unknown. It would be of great interest to determine whether the conformation of the JAK activation loop changes over time *in vivo*, and whether this is a mechanism for controlling the availability of the activation loop to negative regulators including the phosphatases, and SOCS proteins.

The data presented in Chapter 4 detailed how the LNK SH2 domain associates with the pY813 residue of JAK2 and the structural basis for the specificity for this residue. While it was previously known that LNK interacted with JAK2, it was not fully understood which phosphotyrosine on JAK was responsible for the association with the LNK SH2 domain (Bersenev et al. 2008). Using structural and biochemical analysis, the phosphotyrosine, +1, +3 and +5 amino acid positions were shown to be important for binding to the SH2 domain. This same interaction was also demonstrated with a similar motif in JAK3. These results highlight the role of LNK in the negative regulation of several JAK-STAT signalling pathways, including EPO, TPO, and IL-7 signalling (Tong and Lodish 2004; Tong, Zhang, and Lodish 2005; Cheng et al. 2016).

Further investigation into the association of the SH2 domain with other phosphotyrosine residues in other proteins revealed that LNK may also interact with the receptors EPOR and FLT3. Although an exhaustive sequence analysis was not performed, through further studies of these interactions, I highlighted several residues which appear to allow association with LNK with the consensus sequence

pY(E/L/I/T)X(L/M/F)X(E/D). This would implicate LNK in the regulation of other signalling molecules including c-KIT, the PDGFR and c-FMS, all of which play important roles in regulating haematopoiesis (Gueller et al. 2008; Gueller et al. 2010; Gueller et al. 2011). Further studies into the ability of LNK to regulate the activity of these proteins *in vivo* are required to determine the full extent of LNKs negative regulatory capacity in the hematopoietic and immune systems.

The identification of those proteins starts to provide an insight into the full range of processes regulated by LNK and may lend insight into why mutations in LNK have been identified in patients with a wide variety of diseases. In chapter 4, mutations occurring in the SH2 domain of LNK which were initially identified in patients with myeloproliferative neoplasms were studied for their ability to bind phosphotyrosine residues. Given that mutations in LNK have been identified at a higher rate in patients who progress to blast phase, a better understanding of how mutations in LNK may play a role in disease is required. All four mutations studied in this thesis showed reduced capacity to bind phosphotyrosine to varying degrees. In depth studies of the effects of these mutations *in vivo* will be necessary to elucidate the full effects of each mutation in the haematopoietic and immune systems.

Finally, it is important to highlight the strengths and weaknesses of the studies presented in this thesis. Biochemical and structural experiments using purified components such as those presented here are fundamental in determining which interactions *can* occur in cells however, they do not prove that they *do* occur. For this reason, work remains to be done in validating these results.

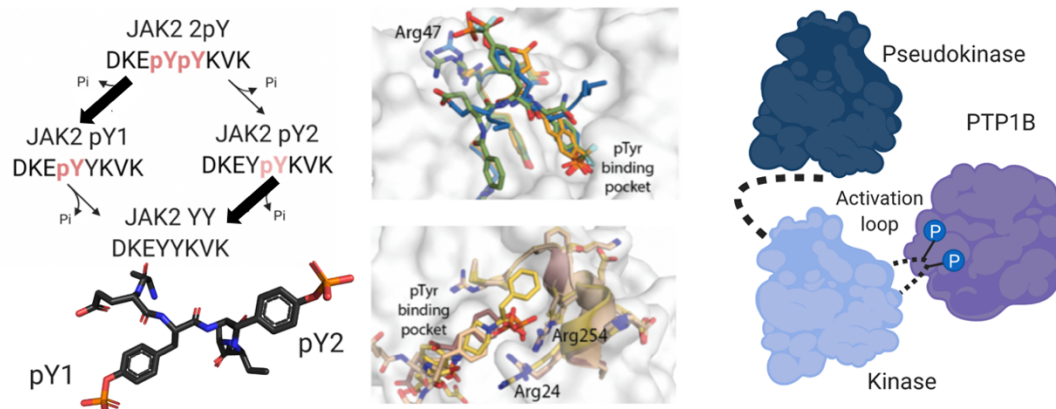
In Chapter 4, several phosphotyrosine residues in four proteins including JAK2, were shown to associate with the purified LNK SH2 domain. To validate the ability of the LNK SH2 domain to bind JAK3, EPOR and FLT3 in cells I propose the use of co-immunoprecipitation to determine whether similar association is seen *in vitro*. Additionally, to support the data highlighting the effects of the single amino acid mutations on the ability of the SH2 domain to bind phosphotyrosine residues, we aim to use mutagenesis studies in cells in parallel with *in vivo* studies to further understand the role these mutations may play in disease such as the myeloproliferative neoplasms.

Likewise, many of the experiments performed in chapter 3 used a complex of PTP1B with a peptide representing the activation loop of JAK2. These studies generated a detailed view of how PTP1B dephosphorylates a peptide, however, are all of these findings extrapolatable to the full-length protein? I suggest not. In fact, it was clear from several of our studies that in the intact protein the largest factor determining catalytic rate was the accessibility of the substrate. A small peptide has very different accessibility characteristics than the same motif within an intact protein. To address these caveats, I intend to further investigate the interaction of PTP1B with full length JAK proteins, using both structural and biochemical studies.

Despite the limitations of the experiments performed in this thesis we now have a much more thorough understanding of the molecular details of the interactions between JAK and two of its important regulatory proteins. What is more, the results presented here will help us move forward and study the interactions between these proteins as suggested above. Collectively, the work presented in this thesis provides a comprehensive analysis of how both PTP1B and LNK interact with the JAK proteins to negatively regulate cytokine signalling, a process critical for the control of haematopoiesis. A summary of the main findings presented in this thesis are illustrated in **Figure 5.1**.

PTP1B Aim 1: To determine how PTP1B interacts with and dephosphorylates the JAK kinase domains.

1. PTP1B preferentially dephosphorylates the second phosphotyrosine of the JAK2 activation loop over the first.
2. Arg 24 and Arg 47 of PTP1B control substrate specificity through interactions with + and - residues of substrates.
3. PTP1B dephosphorylates the JAK activation loop more rapidly in some conformations.



LNK Aim 2: To understand the substrate specificity of the LNK SH2 domain and how mutations in this domain contribute to disease.

1. The pTyr, +1, +3 and +5 residues of pTyr motifs are important for interaction with LNK
2. LNK may interact with pYs on JAK2, JAK3, EpoR and FLT3 in cells through different interactions at the +1 and +3 sites.
3. Mutations identified in MPN patients located in the SH2 domain of LNK can reduce ability to bind pTyr residues.

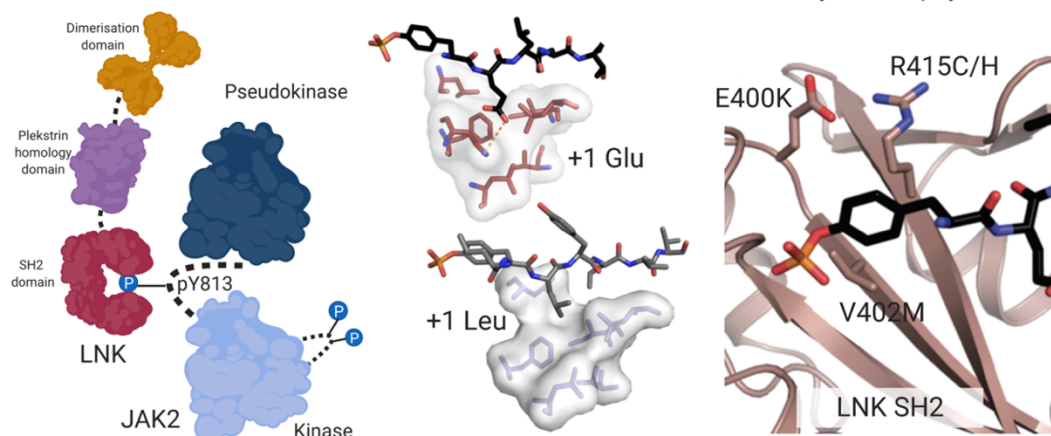


Figure 5.1 Summary of overall thesis conclusions.

The work presented in chapter 3 demonstrated how PTP1B interacts with the JAK activation loop for dephosphorylation and determined that PTP1B preferentially dephosphorylates the second pTyr of the JAK2 activation loop. In chapter 4 it was shown that the LNK SH2 domain interacts with the pTyr, +1 Glu, +3 Leu and +5 Glu of the JAK2 pY813 residue, and may be able to interact with JAK3, the EPOR, and FLT3. It was further shown that mutants of the SH2 domain identified in patients with myeloproliferative neoplasms and idiopathic erythrocytosis have reduced binding ability.

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Chapter 7 Appendices

7.1 Appendix I: Publication

Morris, R., Kershaw, N.J. and Babon, J.J., 2018. The molecular details of cytokine signaling via the JAK/STAT pathway. *Protein Science*, 27(12), pp.1984-2009.

REVIEW

The molecular details of cytokine signaling via the JAK/STAT pathway

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Abstract: More than 50 cytokines signal via the JAK/STAT pathway to orchestrate hematopoiesis, induce inflammation and control the immune response. Cytokines are secreted glycoproteins that act as intercellular messengers, inducing proliferation, differentiation, growth, or apoptosis of their target cells. They act by binding to specific receptors on the surface of target cells and switching on a phosphotyrosine-based intracellular signaling cascade initiated by kinases then propagated and effected by SH2 domain-containing transcription factors. As cytokine signaling is proliferative and often inflammatory, it is tightly regulated in terms of both amplitude and duration. Here we review molecular details of the cytokine-induced signaling cascade and describe the architectures of the proteins involved, including the receptors, kinases, and transcription factors that initiate and propagate signaling and the regulatory proteins that control it.

Keywords: cytokine; Cytokine Signaling; JAK/STAT; SOCS; cytokine receptor; hematopoiesis

Abbreviations: Note that a full list of the abbreviations for all cytokines is given in Table 1. Receptors for each cytokine are denoted by the cytokine abbreviation followed by "R". For example, TpoR, Tpo Receptor; AKT, protein kinase B; AML, acute myeloid leukemia; APS, SH2B adaptor protein 2; ATP, adenosine triphosphate; B-ALL, B cell lymphocytic leukemia; CBP, CREB-binding protein; CD45, cluster of differentiation 45; CHR, cytokine receptor homology region; Elk, ETS domain containing protein; ER, endoplasmic reticulum; FERM, band 4.1, ezrin, radixin, moesin; FnIII, fibronectin type III; FRET, fluorescence resonance energy transfer; Gp130, glycoprotein 130; Grb2, growth factor receptor-bound protein 2; HP1, heterochromatin protein 1; Ig, immunoglobulin; IRF9, interferon response factor 9; ISGF3, IFN-stimulated gene factor 3; JAK, Janus Kinase; JH, JAK homology domain; LNK, lymphocyte adaptor protein or SH2B adaptor protein 3; MAM, meprin, A-5 protein, and receptor protein phosphatase mu; MAPK, mitogen-activated protein kinases; mTOR, mammalian target of rapamycin; NK, natural killer; P300, E1A binding protein p300; PH, pleckstrin homology; PI(3)K, phosphatidylinositol-4,5-bisphosphate 3 kinase; PTP, protein tyrosine phosphatase; PTP-RT, protein tyrosine phosphatase, receptor type; PTP1b, protein tyrosine phosphatase 1B; pTyr, phosphotyrosine; SH2, Src homology 2; SH2B, SH2B adaptor protein 1; SHP1, Src homology region 2 domain-containing phosphatase 1; SHP2, Src homology region 2 domain-containing phosphatase 1; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TC-PTP, T cell protein tyrosine phosphatase; TM, transmembrane; TYK, tyrosine kinase; β c, beta common; γ c, gamma common.

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Introduction

Cytokines are secreted glycoproteins that act as inter-cellular messengers to control the hematopoietic and immune systems and the inflammatory response. The term “cytokine” arises from the Greek κύτος and κίνησις (cell and movement) consistent with their ability to mobilize cells to sites of infection and inflammation. Historically, the plethora of different cytokines have been divided into five groups: (A) TNF- α and related molecules, (B) IL-1 family members, (C) TGF- β s, (D) factors that signal through receptor tyrosine kinases such as M-CSF, (D) chemokines, and (E) cytokines that signal through the JAK/STAT pathway (Fig. 1). The latter group is perhaps the largest and comprises both hematopoietic growth factors such as EPO as well as immunomodulatory cytokines such as IL-2 and inflammatory cytokines such as interferon gamma (Table I). This review will focus entirely on this class and henceforth we use the term cytokine to describe only those that signal via the JAK/STAT cascade.

JAK/STAT signaling: from the cell-surface to the nucleus

The molecular details of the JAK/STAT pathway were largely uncovered in a series of ground breaking studies from the laboratories of James Darnell, George Stark and Ian Kerr more than two decades ago.¹ It is an elegantly simple signaling cascade in which the cytokine requires only three components (receptor, kinase, and transcription factor) to elicit a response (Fig. 2). Each cytokine binds to a specific receptor on

the surface of its target cell. These receptors contain intracellular domains which are constitutively associated with members of the JAK (Janus Kinase) family of tyrosine kinases.^{2–6} JAKs are inactive prior to cytokine exposure however binding of cytokine to its receptor induces their auto-activation by *transphosphorylation*.⁷ Once activated, JAKs phosphorylate the intracellular tails of the receptors on specific tyrosines which in turn act as docking sites for members of the Signal Transducers and Activators of Transcription (STAT) family of transcription factors (Fig. 2).⁸ Receptor-localized STATs are then phosphorylated by JAK^{9,10} which leads to their disassociation from the receptor and translocation to the nucleus, where they drive the expression of cytokine-responsive genes,¹¹ often leading to proliferation and/or differentiation. To ensure that signaling is switched off appropriately, a number of proteins act to attenuate cytokine signaling at multiple levels of the pathway. Notably, the suppressors of cytokine signaling (SOCS) family are negative feedback inhibitors of the signaling cascade.^{12,13} Although there are exceptions, a general rule of cytokine signaling is that each cytokine binds to a specific receptor, this induces activation of specific JAK(s) and STAT(s) and signaling is switched off by a particular SOCS protein (Fig. 3).

Evolutionarily, the JAK/STAT pathway first arose in Bilateria; *Drosophila* for example contains the complete set of pathway components (cytokine, receptor, JAK, STAT). Although the simplicity of the system's architecture has been maintained, there has

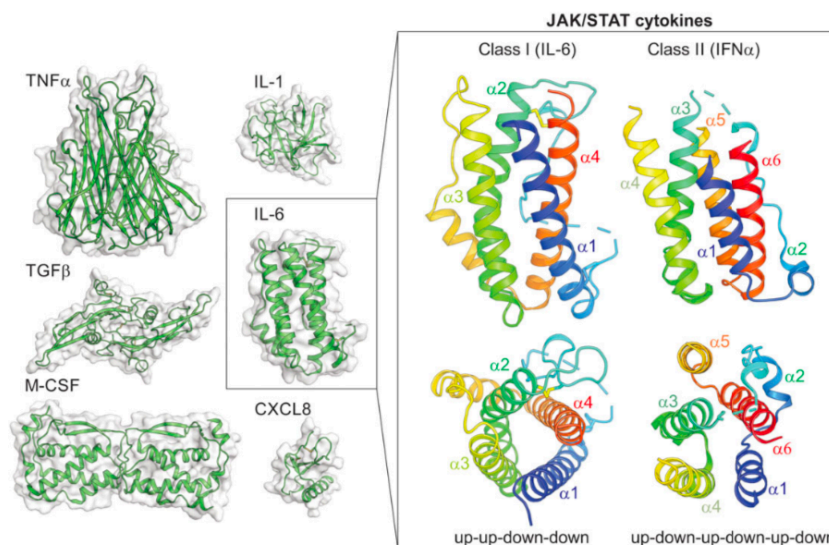


Figure 1. Cytokines. Structures of members of the TNF α -family, TGF β -family, IL-1-like cytokines, chemokines (CXCL8), cytokines that signal through receptor tyrosine-kinases (M-CSF) or the JAK/STAT pathway (IL-6) are shown on the left. JAK/STAT cytokines are helical bundle cytokines and can be divided into two classes. Examples of these two classes are shown on the right.

Table 1. List of Cytokines that Signal through the JAK/STAT Pathway

Abbreviation	Name	Major Functions
Class I cytokines		
<i>IL-2 family</i>		
IL-2	Interleukin-2	Immune response, T-cell differentiation
IL-4	Interleukin-4	T _H 2 differentiation
IL-7	Interleukin-7	T-, B-cell growth factor
IL-9	Interleukin-9	Pleiotropic, Stimulates, T-, B- and NK cells
IL-15	Interleukin-15	Stimulates T- and NK-cells
IL-21	Interleukin-21	Stimulates, T-, B- and NK cells
<i>IL-3 family</i>		
IL-3	Interleukin-3	Multi-lineage haematopoietic growth factor
IL-5	Interleukin-5	B-cell development, eosinophils
GM-CSF	Granulocyte/Macrophage Colony Stimulating Factor	Multi-lineage haematopoietic growth factor, especially monocytes, neutrophils, eosinophils and basophils
<i>IL-6 family</i>		
IL-6	Interleukin-6	Pleiotropic, haematopoiesis, acute phase response, lymphoid differentiation
LIF	Leukemia Inhibitory Factor	Pleiotropic, blastocyst implantation, bone remodeling, CNS
CNTF	Ciliary NeuroTrophic growth Factor	Neuronal growth factor
CT1	Cardiotrophin 1	Cardiac myocytes growth factor
CLC	Cardiotrophin-like cytokine	Neurological growth factor
OSM	Oncostatin M	Pleiotropic, bone formation
IL-31	Interleukin-31	Inflammatory, cell-mediated immunity
NP	Neuropoietin	Neural growth factor
<i>Homodimeric</i>		
G-CSF	Granulocyte Colony Stimulating Factor	Stimulates granulocyte production, mobilises stem cells
EPO	Erythropoietin	Stimulates formation of erythrocytes
TPO	Thrombopoietin	Stimulates formation of megakaryocytes/platelets
GH	Growth Hormone	Growth
PRL	Prolactin	Milk production
LEP	Leptin	Regulates appetite
<i>Others</i>		
IL-12	Interleukin-12	Stimulates T- and NK-cells
IL-13	Interleukin-13	Pleiotropic, airway epithelia, allergic response
IL-23	Interleukin-23	Inflammation
TSLP	Thymic Stromal LymphoPoietin	Inflammatory, stimulates T- and B-cells
Class II cytokines		
<i>Type I interferon</i>		
IFN α	Interferon alpha (23 subtypes)	Anti-viral, secreted by lymphocytes, fibroblasts and monocytes
IFN β	Interferon beta	Anti-viral, ubiquitously expressed
IFN ϵ	Interferon epsilon	Anti-viral, expressed in female reproductive tract
IFN κ	Interferon kappa	Anti-viral, expressed by keratinocytes
IFN ω	Interferon omega	Anti-viral, secreted by leukocytes
<i>Type II interferon</i>		
IFN γ	Interferon gamma	Pro-inflammatory, secreted by T- and NK-cells, activates macrophages/monocytes
<i>Type III interferon</i>		
IFN λ 1	Interferon lambda1	Anti-viral, similar to type I but acts on fewer cell-types
IFN λ 2	Interferon lambda2	Anti-viral, similar to type I but acts on fewer cell-types
IFN λ 3	Interferon lambda3	Anti-viral, similar to type I but acts on fewer cell-types
<i>IL-10 family</i>		
IL-10	Interleukin-10	Anti-inflammatory, inhibits macrophage activation
IL-19	Interleukin-19	Inflammatory, acts on dermal cells
IL-20	Interleukin-20	Inflammatory, acts on dermal cells
IL-22	Interleukin-22	Inflammatory, secreted by Th1 cells, acts on dermal cells
IL-24	Interleukin-24	Inflammatory, acts on dermal cells
IL-26	Interleukin-26	Antimicrobial, T _H 17 cytokine

been a large expansion in the numbers of each of the components throughout the course of evolution. For example, *Drosophila* has three cytokine-like molecules (*unpaired*, *unpaired-2*, *unpaired-3*), a single

receptor (*domeless*), one JAK (*hopscotch*), one STAT (*marelle/STAT92E*), and three SOCS. In contrast, the human genome encodes >50 cytokines, a roughly equivalent number of receptor chains, four JAKs,

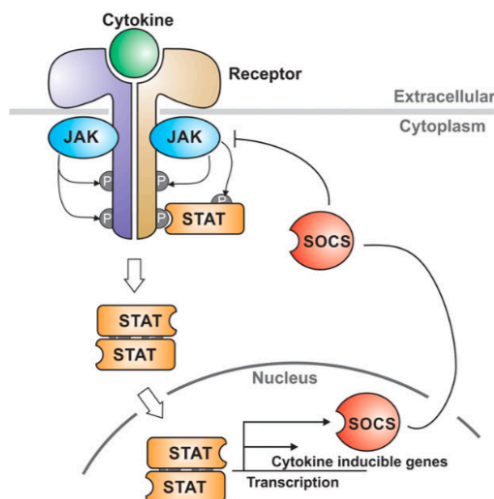


Figure 2. The JAK/STAT pathway. Schematic of the signaling cascade induced by cytokines that signal via the JAK/STAT pathway. Cytokine binds to a specific receptor and allows transactivation of the associated Janus Kinases (JAKs). Activated JAKs then phosphorylate tyrosines on the intracellular domains of the receptor which recruit the Signal Transducers and Activators of Transcription (STAT) transcription factors. STATs are translocated into the nucleus and upregulate the transcription of cytokine-responsive genes. SOCS proteins are direct targets of STAT and act as negative-feedback inhibitors to switch off the signaling cascade.

seven STATs and eight SOCS (Table II) and these numbers are not dramatically different in any vertebrates so far examined.⁴⁵

Cytokines and their receptors

Most cytokines are small helical-bundle proteins usually ca. 150–200 amino acids in length. They are divided into two classes based on motifs found in their receptors (see below). Class I cytokines consist of four α -helices in a characteristic up-up-down-down configuration. Some of these, such as IL-5 exist as dimers but the topology is conserved. The unusual up-up-down-down configuration necessitates two long loops to connect the up-up and down-down pairs. In class II cytokines, one or both of these loops is replaced by an extra α -helix resulting in 5–6 helices in total arranged in an anti-parallel fashion. Again, some (such as IFN γ and IL-10) function as dimers, where the 2 C-terminal helices of one molecule are domain-swapped into a second.

Cytokine receptors consist of multiple (usually two) protein chains. These receptor chains are type I single-pass transmembrane proteins with conserved intracellular and extracellular features. The extracellular domains contain a region termed the hemopoietin domain or cytokine receptor homology region (CHR),⁴⁶ formed by a pair of Fibronectin type III (FnIII) domains

oriented at nearly right angles to one-another (Fig. 4). FnIII domains are small ca. 100 residue domains that form a β sandwich comprised of a three- and a four-stranded β -sheet. The principal binding site for cytokines is at the junction between the two FnIII domains within the CHR, and flexible, variable loops from each domain determine specificity at this binding site.^{47,48}

The CHRs of the receptors for Class I cytokines contain four conserved cysteines arranged in a CX_(9–10)-CXWX_(26–32)-CX_(10–15)-C sequence within the first FnIII domain (forming two intra-domain disulfide bonds) and a “WSXWS motif” in the second FnIII domain.⁴⁹ The CHRs of Class II receptors do not have the WSXWS motif and although they too share conserved cysteines, they are arranged differently. Intracellularly, both Class I and Class II receptors share sequences that allow for recruitment of JAKs and STATs.

Class I cytokine receptors

Class I receptors represent the largest group, with 34 Class I receptor chains encoded in the human genome.⁵⁰ All Class I receptors contain a CHR but many also contain additional extracellular domains such as Ig domains, extra FnIII domains or even a second CHR. Class I receptors bind to a broad array of interleukins, hematopoietins, and growth factors whilst Class II receptors are more restricted, recognizing only interferons and IL-10 family cytokines (Fig. 3).

As shown in Figure 3, there are three major shared chains utilized by Class I cytokines. These are gp130, beta common and gamma common, utilized by IL-6, IL-3, and IL-2 family cytokines, respectively. In addition to these, there are two other shared chains used by cytokines in the IL4/13 and IL-12/23 subgroups. Finally, there are the homodimeric receptors, consisting of two identical chains such as those used by EPO, TPO, GH, PRL, Leptin, and G-CSF. Within each of these classes, the cytokine IL-12R β 2 receptor stoichiometry and organization can differ. A common theme within the non-homodimeric receptors is that there will be a cytokine-specific chain (nominally the “alpha” chain) that recognizes cytokine with high affinity, and the resulting dimer will then recruit a “shared” chain in order to initiate signaling. The alpha-chain may or may not contain the intracellular motifs required to recruit a JAK kinase. Although there are differences in the number of individual chains that comprise a Class I receptor, receptors usually contain precisely two signaling chains (those whose cytoplasmic domain binds to a JAK family member to initiate signaling).

Homodimeric receptors. The homodimeric cytokine receptors are a family of structurally diverse receptors that are categorized by their use of two identical receptor chains. Some, like EPOR,⁵¹ GHR⁴⁷ and PRLR⁵² are the most simple of all receptors in terms of architecture, the ectodomain of each receptor chain consisting

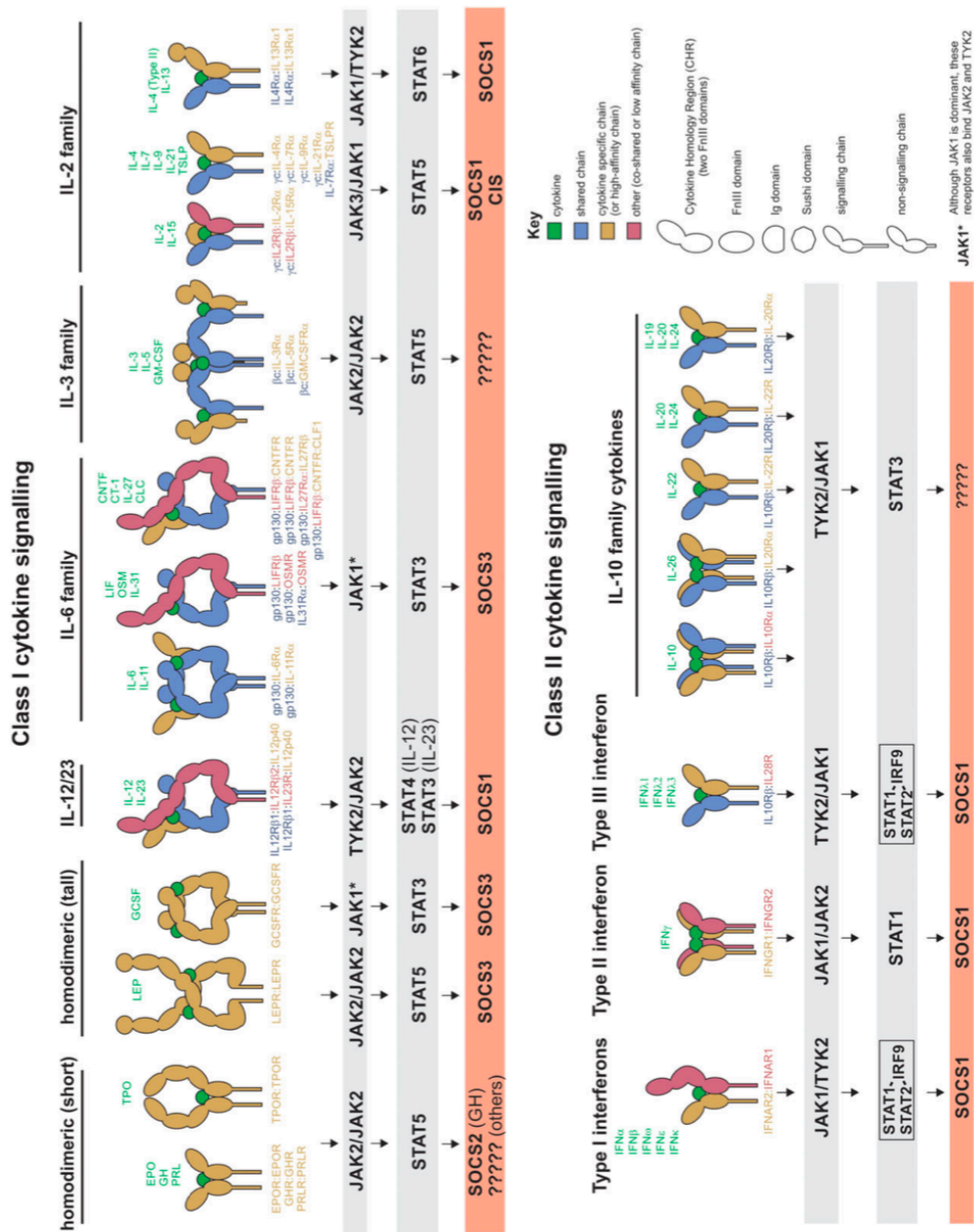


Figure 3. Class I and Class II cytokines. Families of cytokines and the receptors they bind to are shown above the JAK-, STAT-, and SOCS-family members they signal through.

Table II. JAK/STAT/SOCS Family Members

Gene	Knockout mouse phenotype	Cytokines dysregulated	Reference
<i>Jak1</i>	Early post-natal lethality, neurological defects; SCID	IL-2, IL-6 family cytokines, IFNs	14
<i>Jak2</i>	Embryonic lethal, failure of definitive erythropoiesis.	EPO, TPO, GH, PRL, IL-3, IL-5, GM-CSF and IFN γ	15–18
<i>Jak3</i>	SCID	IL-2 family cytokines	19–21
<i>Tyk2</i>	Resistance to LPS, reduced IL-12 response	IL-12	22–24
<i>Stat1</i>	Impaired antiviral response	Type I/II/III interferons	25
<i>Stat2</i>	Impaired antiviral response	Type I/III interferon	26
<i>Stat3</i>	Embryonic lethal	IL-6, IL-10 family cytokines, G-CSF	27
<i>Stat4</i>	Impaired Th1 development	IL-12	28
<i>Stat5a/b</i>	Impaired mammary gland development, infertility, no NK cells	GH, PRL, IL-3 family cytokines	29,30
<i>Stat6</i>	Impaired Th2 development	IL-4/13	31
<i>Cish</i>	Increased susceptibility to allergic asthma. Increased tumor immunity	IL-2, IL-4, IL-15	32,33
<i>Socs1</i>	neonatal lethality	IFN γ / α / β IL-12/23, IL4/13 IL-2 family cytokines	34–41
<i>Socs2</i>	gigantism,	GH	42
<i>Socs3</i>	embryonic lethality; placental defects.	IL-6 family cytokines, G-CSF, Leptin	43,44

only of a single CHR unit. Studies on EPOR and GHR in particular have formed the basis of the general paradigm for cytokine signaling. These three receptors, alongside the larger TPOR (which contains two CHRs per ectodomain chain), bind a single molecule of cytokine to form a signaling-competent ternary complex.

Although these complexes are by nature asymmetric, it is the hinge regions between the two FnIII domains of the CHRs on both chains that participate in cytokine binding. One chain binds to the cytokine with high-affinity, and the other chain binds to a lower-affinity site on the cytokine. This phenomenon is observed for all classes of cytokine receptor.

The Leptin and G-CSF receptors are also homodimeric but structurally have more in common with the IL-6 receptor family (see below and Fig. 3). They are

referred to as “tall receptors” as they contain “legs,” composed of two or three FnIII domains that link their cytokine-binding region to the membrane. This places the cytokine binding region of these receptors as far as 120 Å from the cell-surface. The leptin receptor is the largest of all cytokine receptors, consisting of (from N- to C-terminus): Ig-CHR-Ig-CHR-FnIII-FnIII. Both the leptin and G-CSF receptors bind two molecules of cytokine, forming signaling competent *tetramers* with a similar architecture to that seen in many IL-6 family receptors⁵³ (Figs. 3 and 4).

IL-6 family cytokine receptors. The IL-6 family of cytokines includes IL-6, IL-11, IL-27, LIF, OSM, CNTF, CT-1, and CLC. The majority of these cytokines act via receptors that include the common

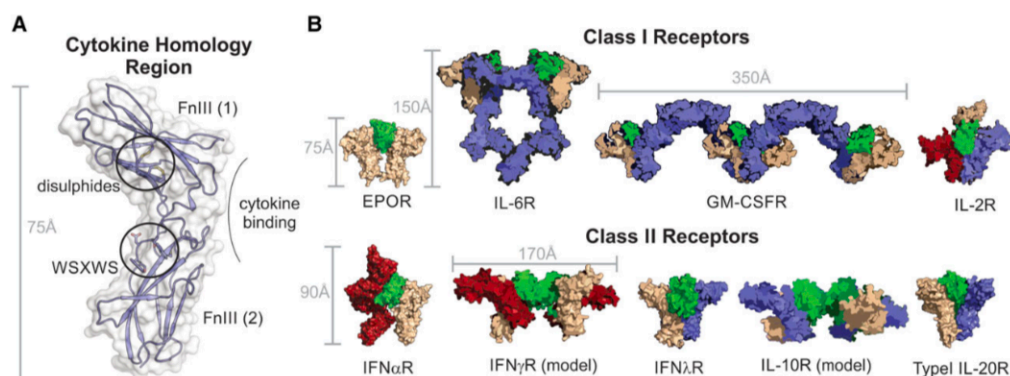


Figure 4. The cytokine homology region (CHR) forms the basis of all cytokine receptors. (A) The CHR from a Class I receptor (Growth Hormone Receptor) is shown with the two FnIII domains, disulfide bonds and WSXWS motif highlighted. All receptors contain a CHR however many receptors, especially those that recognize Class I cytokines, have additional FnIII and Ig domains and this results in a large variety of receptor architectures and stoichiometries. (B) Structures and models of a diverse range of cytokine:receptor complexes.

gp130 chain. Like the G-CSF and Leptin receptors, gp130 is also a tall receptor chain. Its extracellular domain consists of an Ig domain, a CHR, followed by three FnIII domains that form the legs. Within the IL-6 family are two other tall receptor chains which are shared between cytokines on a more limited basis, these are LIFR β and OSMR.

The signaling competent complex between IL-6 or IL-11 and their receptors are hexamers. These consist of two molecules each of cytokine, gp130, and a cytokine-specific alpha chain (IL-6R α or IL-11R α).^{54–56} Two copies of gp130 are required because the intracellular domain of the cytokine-specific alpha chains of these receptors do not bind JAK. Signal transduction is, therefore, achieved by transactivation of two JAK molecules bound to the two gp130 receptor chains.

Whilst the IL-6 and IL-11 signaling complexes are similar in structure and composition, other gp130 cytokines signal via different topological assemblies. For example, the complexes between CNTF, CLC, or CT-1 and their receptors are tetramers. These cytokine/receptor complexes are not built around two gp130 chains but instead are built around a heterodimer between gp130 and another JAK-binding tall receptor called LIFR β .^{57,58} As is the case for IL-6, these cytokines first bind to cytokine-specific non-signaling alpha receptor chains and the resulting complexes then recruit gp130 or LIFR β . Finally, LIF, OSM, and IL-31 signal via trimeric complexes with the receptor being a heterodimer of two tall chains without the requirement for an alpha chain.

IL-12/23 receptors. IL-12 and IL-23 also signal via tall receptors. Although there is no structure of the full receptors for these cytokines they are likely to be of similar architecture to the LIF receptor^{59,60} (Fig. 4). IL-12 and IL-23 are unusual cytokines as they are both composed of two disulfide-linked subunits that arise from different genes.⁶¹ The first of these subunits (IL-12p35 and IL-23p19) are classic four-helix bundle cytokines. The second subunit (IL-12p40, which is shared by both IL-12 and IL-23) is more like a receptor in structure, consisting of a CHR, replete with WSXWS motif, and an N-terminal Ig domain, but lacking a transmembrane domain. Indeed, the p40 receptor-like subunit associates with the 4-helix bundle subunit in a classic receptor-like manner, using the hinge region between the two FnIII domains of the CHR.^{62,63} The receptors for IL-12 and IL-23 are also heterodimers which share a common chain, the interleukin-12 receptor subunit β 1 (IL-12R β 1). The other monomer in each receptor is a cytokine-specific alpha chain (IL-12R β 2 or IL-23R). As one would expect, the common receptor subunit recognizes the p40 subunit whilst the specific alpha chains recognize the classic 4-helix bundle moieties within each cytokine heterodimer.⁶⁴

IL-3 family cytokines. The IL-3 family (IL-3,-5, GM-CSF) all signal via receptors that contain the common beta chain (beta-common, β c).⁶⁵ The extracellular region of β c is comprised of two CHRs (i.e. four FnIII domains). β c has no measurable affinity for any of the three cytokines alone, instead each of the three cytokines within this family has a specific alpha-chain that facilitates a high affinity interaction with β c.⁶⁶ The primary site of interaction with the alpha-chain is the usual hinge region between the two FnIII domains of the CHR however these alpha-chains also contain a N-terminal Ig domain which wedges over the top of the cytokine, providing extra affinity. It is this binary complex which associates with β c with high affinity.

β c forms a closely associated dimer with the individual FnIII domains of each monomer drawn out like beads on a string, allowing the dimer to associate in an almost domain-swapped fashion.⁶⁷ The two cytokine binding sites on the β c dimer are thus formed at the spatial junction between an FnIII domain from both monomers. The structure of the signaling competent GM-CSF/receptor complex shows the most surprising stoichiometry of all, forming dodecamers consisting of four cytokines, four β cs, and four alpha receptors.⁶⁷ This dodecameric structure is the minimum structure that can allow juxtaposition of the intracellular JAKs, leading to transactivation and downstream signaling.

IL-2 family cytokines. Finally, the IL-2 family cytokines (IL-2, -4, -7, -9, -15, -21) all signal via receptors that contain the common gamma chain (gamma common, γ c), a JAK3-associated receptor subunit. The extracellular region of γ c is much smaller than that of gp130 or β c, consisting of just a single CHR. Most members of this family of cytokines signal via heterodimeric receptors (γ c plus a specific alpha receptor), thus forming ternary signaling complexes upon the addition of cytokine. IL-4, IL-7, IL-9, and IL-21 all signal in this manner. The general paradigm for signaling by these cytokines is that γ c (like gp130 or β c) has little affinity for the cytokines alone but binds strongly to a complex between the cytokine and the alpha receptor chain.

Despite being the defining family member of this group of cytokines, IL-2 itself, along with IL-15, are unusual in that they also require a third receptor subunit called IL2R β . IL2R β is a JAK1-associated receptor subunit and is essential for signaling. The alpha receptors for IL-2 and IL-15 are non-signaling chains distinct from other cytokine receptors as they consist of sushi domains (another beta-sandwich fold) rather than a CHR. The full IL-2 receptor is found on activated T- and NK-cells, and binds IL-2 with extremely high affinity (10pM). IL-15 signals similarly to IL-2 but IL-15R α usually presents IL-15 from a different cell (i.e. *in trans*) thus IL-15 requires cell-cell contact in order for signaling to occur.⁶⁸

Just as is seen for the gp130 family, the common gamma receptor family also contains chains that are shared on a more limited basis. For example, IL-7R α can either heterodimerize with γ_c to form the IL-7 receptor or with TSLPR to form the receptor for TSLP. Likewise, IL-4R α can heterodimerize with γ_c to form the Type I IL4R (found on hematopoietic cells); however, it can also heterodimerize with IL-13R α 1 to form the Type II IL4R (found on non-hematopoietic cells). Both IL-4 and IL-13 can signal via this complex and this is one of only a few examples of different Class I cytokines signaling via the same receptor, a phenomenon that is common for Class II cytokines as we shall now discuss.

Class II cytokine receptors

The Type II family cytokines encompass the interferons (IFN α , β , γ , λ , ϵ , κ , ω) and IL-10 family cytokines.⁶⁹ Signaling via Class II cytokine receptors (unlike Class I) adheres to a more common set of rules regarding stoichiometry and receptor assembly. Each Class II receptor is a heterodimer and each of these receptors associate with one molecule of cytokine to initiate signaling. The only exceptions to this rule are IL-10 (and possibly IL-26) and IFN γ which are dimeric cytokines and the stoichiometry of the entire signaling complex is, therefore, doubled (Figs. 3 and 4). All Type II cytokine receptor chains bind to JAK, unlike many Type I receptor alpha chains. Finally, the ectodomain architecture of all Class II receptors consist of just a single CHR (with the sole exception of IFN α R1 which has two) and are not decorated by extra Ig or FnIII domains.

Similar to Class I, the Class II receptor family consists of both shared chains and cytokine-specific chains. However, a characteristic of class II cytokine signaling is the plasticity seen within the system, in many cases a single receptor can bind multiple cytokines and a single cytokine can in some cases bind multiple receptors.

The Type I interferon receptor (IFN α / β receptor). The Type I interferon receptor is a heterodimer consisting of IFN α R1 and IFN α R2. IFN α R1 has a large extracellular domain that consists of two CHRs while IFN α R2, similar to all other Class II cytokine receptors has only a single CHR. IFN α R2 is the high affinity chain, interacting with ligand with sub-nanomolar affinity whilst IFN α R1 binds with an affinity approximately two orders of magnitude lower.⁷⁰

There is a vast array of type I IFNs, including IFN- α (which itself has at least 13 different subtypes), IFN- β , IFN- ϵ , IFN- κ , and IFN- ω . Other interferons are found in different mammalian species. Remarkably, these all bind to the same receptor, even though the downstream consequences elicited by each Type I interferon differs. Structural studies have shown that each ligand binds the receptor at the

same position; however, the stability of the resultant complexes differ.⁷¹ Thus, the half-life of the cytokine/receptor complex appears to determine the downstream signaling outcomes.

The Type II interferon receptor (IFN γ receptor). The interferon gamma receptor does not consist of any shared receptor chains and interacts only with a single ligand, interferon gamma. Likewise, IFN γ does not bind any other receptors. IFN γ acts as a homodimer and binds two copies of IFN γ R1 with sub-nanomolar affinity. The active signaling complex is then formed by recruiting two copies of IFN γ R2,⁷² making the signaling complex hexameric.

The Type III interferon receptor (IFN λ receptor). The Type III cytokines IFN- λ 1, - λ 2, and - λ 3 (also known as IL-29, IL-28A, and IL-28B, respectively) signal via a single receptor. This Type III receptor consists of two chains, IL-28R and IL-10R β , the latter of which is shared with receptors for the IL-10 family. The structure of IFN λ bound to its receptor is reminiscent of the GH:GHR complex structure with the ligand occupying a specially similar position albeit with a very different angle of occupation.⁷³ The IL-28R chain binds cytokine with high affinity and this binary complex then recruits IL10R β .

IL-10 family receptors. IL-10 family cytokines (IL-10, IL-19, IL-20, IL-22, IL-24, and IL-26) can be subdivided into two classes. Those that use the shared IL10R β chain (IL-10, 22, 26) and those that use the shared IL20R β chain (IL-19, 20, 24).

Like IFN γ , IL-10 (and probably IL-26) are homodimeric cytokines and their receptors likewise contain two copies of each of the two individual chains. The shared chain for these two receptors is IL10R β and the cytokine-specific chains are IL-10R α and IL-20R α , respectively. In each case, the cytokine-specific chains bind with high affinity to ligand (sub-nanomolar), while IL-10R β is the lower affinity (high micromolar – mM) receptor.^{74,75} IL10R β also forms half of the receptor for IL-22, a monomeric cytokine.⁷⁵ The cytokine specific chain can be IL22R α 1, or alternatively it can recruit a soluble receptor (IL22BP) that can mediate its biological effects.

The remaining IL-10 family cytokines (IL-19, 20, 24) bind to two distinct receptors. The so-called Type II IL-20 receptor consists of a shared IL20R β and a cytokine-specific subunit IL22R. The type I receptor contains the same shared IL20R β chain along with the IL20R α subunit. IL-20 and IL-24 can signal via both receptors whereas IL-19 binds to the type I receptor only. In the Type I receptor, IL20R β is the high affinity subunit.⁷⁶ Overall the structure of the cytokine:receptor complex is similar to that of IFN λ . Modeling of the type II receptor suggests a similar overall architecture.⁷⁷

Cytokine receptor cytoplasmic domains

Both Class I and II cytokine receptors are complexes of single pass transmembrane-domain containing proteins. Many cytokine-specific alpha receptor chains contain a short cytoplasmic region with no known function, but each functional receptor complex always consists of at least two (most often precisely two) individual receptor chains with long intracellular regions (several hundred amino acids in length) that are the scaffolds upon which signaling is initiated. These unstructured^{59,60} cytoplasmic domains exist to provide sequence-specific docking sites for JAKs and STATs. The JAK-binding regions are known historically as the Box 1 and Box 2 motifs and are membrane proximal whilst the STAT binding motifs are located towards the C-terminus, distal to the membrane. In some cases, in between these two motifs are additional binding sites for negative-regulators such as the SOCS proteins.

The JAK binding motif: Box 1/2. Mutagenesis studies first identified two regions on the cytoplasmic tail of receptors, termed Box 1 and Box 2, critical for the association of JAKs with receptor.⁷⁸ Box 1 is proline rich and is located approximately 10 residues from the C-terminus of the transmembrane region of the receptor, whilst Box 2 is about 10–50 residues further downstream and is rich in hydrophobic residues. Apart from these features, these regions share low sequence homology between different receptors. Moving the Box 1/2 motif further from the membrane abolishes the ability of JAK to associate⁷⁹ suggesting that membrane proximity is important for cytokine inducible activation. Specific receptors bind to specific JAKs, although some receptors (most notably the G-CSF and IL-6 family of receptors) can bind multiple JAKs.⁸⁰ It is sequence differences within the Box 1 and Box 2 motifs of different receptors that determine which JAK (JAK1, JAK2, TYK2, or JAK3) is bound. For example, structural studies identified a PxxLxF sequence in JAK1-binding Class II receptors as the key motif required for JAK1 interaction.⁸¹ No obvious motifs have yet been defined for other receptors. Very recently, it has been shown for two homodimeric receptors (EPOR and LeptinR) that the small region between the membrane and the Box 1 motif coordinates JAK homodimerization and is necessary for efficient signaling.⁸² It may be assumed (but has not yet been shown) that the same region of other receptors may mediate JAK dimerization as well.

STAT-binding motifs. Once JAKs are activated (see below) they phosphorylate distal tyrosines on the receptor intracellular domains and these sites act as docking sites for the signal transducer and activator of transcription (STAT) transcription factors. Thus, all receptors contain conserved tyrosines that fulfill this

function. Just as different receptors bind different JAKs, so they also bind different STATs.⁸³ The ability of a certain cytokine to induce activation of a particular STAT is driven purely by the STAT-binding sites contained within its receptor. STAT-binding sites from one receptor can be replaced with binding sites for different STATs from other receptors and thereby activate non-physiological STATs.^{84,85} In many cases, a single pTyr can recruit multiple members of the STAT family, albeit with differing affinity.⁸⁶ It is likely that the affinity for one STAT over another is purely a function of the sequence immediately surrounding the phosphotyrosine, for example pYxxP,⁸⁷ pYxxQ,⁸⁵ pYxxL,⁸⁸ and pYxxF⁸⁹ sequences are associated with recruitment of STAT1, STAT3, STAT5, and STAT6, respectively.

Some receptors contain multiple STAT binding sites, for example the IL-6 receptor signaling chain (gp130) contains four STAT3 binding motifs⁹⁰ and EPOR contains four STAT5 binding motifs on each chain.⁸⁸ In other cases, such as in IFN γ R, only a single STAT binding site is found.⁸⁷

Other stimulatory sites. In addition to stimulating signaling by STATs, many cytokines also induce additional signaling pathways via the same receptors,^{91,92} such as the MAPK and PI(3)K pathways. For example, IL-6 family cytokines stimulate both these pathways. Although the mechanism of PI(3)K stimulation is unclear, the MAPK pathway is activated via the phosphatase SHP2.⁹³ SHP2 binds to phosphotyrosine 759 on the gp130 subunit of the IL-6 receptor; cytokine exposure activates SHP2; and this leads to Ras/Raf signaling which stimulates the MAPK cascade and ultimately transcriptional activators such as Elk.

Negative-regulatory sites. In addition to stimulatory sites on the intracellular domains of cytokine receptors, there are often sites for regulatory proteins that inhibit signaling. In general, these inhibitory proteins interact with phosphotyrosine motifs on the receptors via SH2 (Src-homology 2) domains (as do the STATs) and hence they are only recruited once the receptors are phosphorylated. The SOCS proteins are a family of negative-regulatory proteins that all contain SH2 domains and many bind to specific receptor sites to inhibit signaling.⁹⁴ Particularly well-characterized sites are found on the IL-6 and G-CSF receptors (for SOCS3) and the GHR (for SOCS2). In all cases these SOCS binding sites are located C-terminal to the JAK-binding region of the receptor.

Janus Kinases (JAKs)

There are four members of the JAK family found in all vertebrates: JAK1, JAK2, JAK3, and TYK2^{3–5,95} (see Table II). Each JAK is ca. 1000 residues in length and consists of four distinct domains: An N-terminal FERM (band 4.1, Ezrin, Radixin, Moesin) domain followed by an SH2 domain and two kinase domains (Fig. 5). The

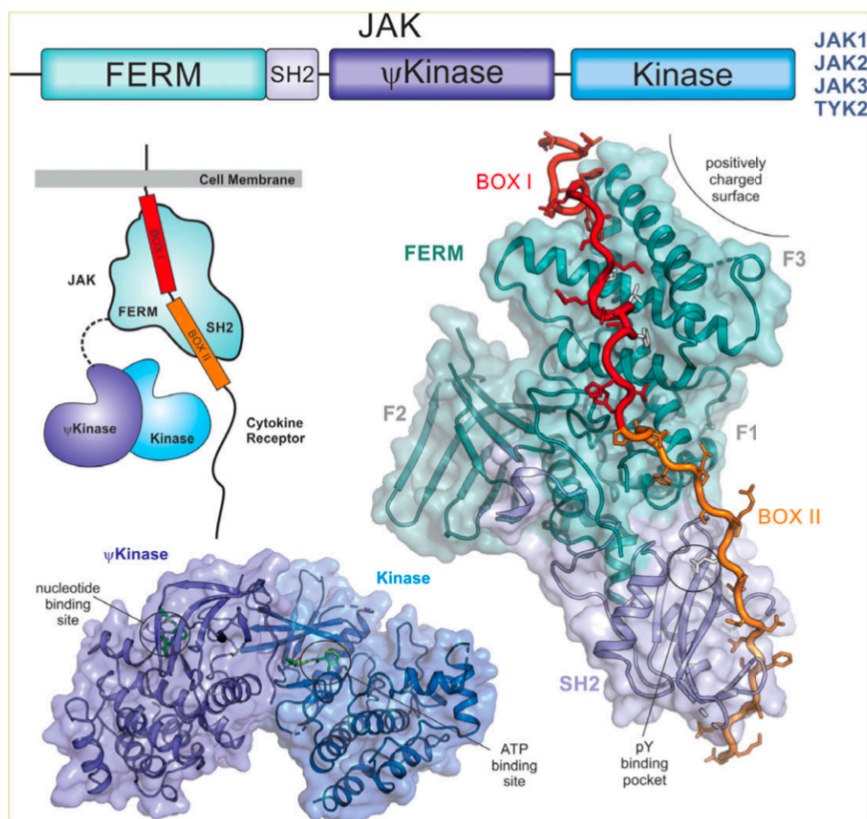


Figure 5. Janus kinases (JAKs). There are four members of the JAK family (JAK1, JAK2, JAK3, and TYK2) and all share similar domain architecture (top). The FERM and SH2 domains tether JAK to the receptor, binding Box I and Box II respectively (structure shown on the right, PDB ID: 5L04). The pseudokinase (ψ kinase) regulates the activity of the catalytically active kinase domain (bottom, PDB ID: 4OLJ) via a mechanism that is unclear. There is no structure of a full-length JAK protein and hence the relative orientation of the N- and C-terminal halves of the protein is unknown (indicated schematically on the left).

first of these kinase domains is catalytically-inactive and is therefore a pseudokinase domain (also termed the JAK Homology 2 or JH2 domain). The C-terminal kinase domain is the catalytic domain in each JAK, historically termed the JH1 domain.

FERM/SH2 domains. The FERM and SH2 domains together are responsible for binding to the receptor. This provides the specificity required to target a particular JAK to a particular receptor chain. With a few exceptions, a receptor chain only binds to a single, specific member of the JAK family. The FERM and SH2 domains are closely associated and form a single structural unit.^{81,96,97} Together they provide the binding site for the Box 1 and Box 2 motifs found in all cytokine receptors. The FERM domain is a three-lobed structure consisting of an F1 lobe (ubiquitin-like), an F2 lobe (acyl-CoA-binding protein-like), and an F3 lobe (pleckstrin homology domain). The F2 lobe is primarily responsible for binding the membrane-

proximal Box 1 motif on the receptor and in addition contains a large surface with significant positive charge that probably interacts with the cell membrane.^{81,96,97} The SH2 domain interacts with the Box 2 motif. Interestingly, it coordinates a glutamate in the same way that other SH2 domains coordinate phosphotyrosine. Together, the Box 1 and Box 2 motifs form a long (85 Å), largely extended epitope that buries over 3000 Å² of JAK.⁹⁷ The relevant contributions of Boxes I and II toward overall affinity differ between receptors. For example, in the IFN λ receptor, Box 1 provides most of the affinity whilst the addition of Box 2 adds a further 10-fold increase.⁸¹ In contrast, TYK2 binding to the IFN α receptor appears to be dominated by Box 2.⁹⁶

Pseudokinase domain. The pseudokinase domain, critical for modulating the activity of the C-terminal tyrosine kinase domain,^{98–100} is the most enigmatic of the JAK domains. Although it adopts a typical kinase

fold,^{100–106} it is catalytically defective. The pseudokinase domain of JAK2 shows some residual activity that may be responsible for autophosphorylation *in cis* on two auto-inhibitory phosphorylation sites, Ser523 and Tyr570¹⁰⁰; however, it is likely to be completely inactive in other members of the JAK family,¹⁰³ despite maintaining the ability to bind ATP.¹⁰⁷ An emerging idea is that ATP binding by pseudokinase domains functions as a “molecular switch”¹⁰⁷; however, this remains to be established for the JAK family.

The ability of the pseudokinase domain to regulate the activity of the kinase domain has been recognized for some time, since the observation that a mutation in the pseudokinase domain of the *Drosophila* JAK homolog, hop, resulted in hyperactive kinase activity and a leukemia-like disease.¹⁰⁸ Importantly, deletion of the pseudokinase domain increases the basal level of kinase activity but prevents a further increase in activity in response to cytokine.^{98,99} The importance of the pseudokinase domain was further highlighted in 2006 when it was discovered that a V617F point mutation in human JAK2 was causative of a range of myeloproliferative neoplasms.^{109–111} This group of diseases, which includes Polycythemia Vera, Essential Thrombocythemia and the more severe Primary Myelofibrosis display aberrantly high levels of myeloid cells such as erythrocytes and platelets. The V617F mutation leads to an increased basal activity of JAK2 and cytokine-independent signaling through the EPO and TPO receptors. The analogous mutation in other JAK family members has also been shown to cause aberrant signaling.¹⁰³ Further mutations in the linker between the SH2 and pseudokinase domains, the so-called exon 12 mutations, have since been discovered to lead to the same diseases.^{112,113} Given that the pseudokinase domain plays a role in both switching on the kinase domain and controlling its maximal activity and that mutations within this domain cause disease there have been many efforts to understand this at a molecular level. To date, there is only a single experimentally-determined structure of the tandem pseudokinase-kinase domains (from Tyk2).¹⁰² This structure showed that the two domains adopt a back-to-back orientation, and activating mutations cluster together at the interface between these domains indicating that a physical interaction between the pseudokinase and kinase domains is required for correct regulation of JAK activation. Although equivalent structures of other JAKs have not been forthcoming, sophisticated modeling of the analogous construct from JAK2 gave a near identical prediction of the overall structure¹¹⁴ even prior to the TYK2 structure being solved.

Kinase domain. The kinase domain of JAK is the domain required for phosphorylation of receptor and subsequently the STAT transcription factors. All JAKs are tyrosine kinases and their kinase domains

display a typical fold.^{115–119} Tyrosine kinases catalyze the transfer of a phosphate from ATP to a tyrosine-containing protein. The general organization of a kinase includes two lobes: an N-terminal lobe consisting mostly of β -strands and a larger, mostly α -helical C-terminal lobe. The ATP-binding site (and thus the active site) exists between these lobes. The conserved residues for ATP-binding lie mainly on the N-terminal lobe whilst the motifs for tyrosine binding, phosphotransfer and Mg^{2+} -binding are found mostly on the C-terminal lobe. Magnesium is an essential factor for kinases, aiding the co-ordination of ATP. A number of important motifs are characteristic of all kinases and these include (i) the VAIK motif of the β 3 strand in the N-lobe, in which the lysine anchors and orients the α - and β -phosphates of ATP; (ii) an aspartic acid in a HRD motif of the catalytic loop (β 6– β 7) which provides the catalytic base; (iii) an aspartic acid in the DFG motif within the activation loop (β 8– β 9) that binds the Mg^{2+} coordinating the β - and γ -phosphates of ATP¹²⁰; and (iv) a glycine-rich loop (G-loop; GXGXXG) between the β 1 and β 2 strands of the N-lobe. The latter is a key factor in binding ATP through hydrogen-bond formation between the backbone of the loop and the ATP γ -phosphate.¹²¹ In addition to these, two hydrophobic regulatory “spines” determine whether a kinase is capable of enzymatic activity, the Regulatory-spine and the Catalytic-spine.^{122,123}

Another important motif is the activation loop. This loop contains two consecutive tyrosines and the phosphorylation-state of the first of these determines whether the enzyme is active or inactive. JAKs phosphorylate one-another at this position *in trans* upon cytokine stimulation.⁷ Although many structures have been solved of JAK kinases domains with their activation loop in the phosphorylated (active) conformation,^{115–119} the inactive conformation has not been observed structurally but can be inferred from studies on other tyrosine kinases, in particular the insulin and IGF receptor kinases, in work led by Stevan Hubbard.^{124–127} In the inactive conformation, the activation loop forces the N- and C-lobes apart and blocks the ATP binding site. In addition, the substrate-binding site (which is partly formed by residues within the loop) is not present and the orientation of the DFG motif does not allow magnesium binding.¹²⁴ Phosphorylation results in reorientation of the activation loop such that it swings out of the ATP-binding site and lies flat against the solvent exposed surface of the C-lobe. This allows ATP and substrate to bind and catalysis to occur.¹²⁷ Structurally trapping a tyrosine kinase in the process of auto-activation (in trans) has only been successfully performed for the IGF1 receptor and the activation loop in this conformation is highly extended, allowing the first tyrosine to access the active site of a second kinase molecule and become phosphorylated.¹²⁶ The

second tyrosine within the activation loop has been found fully or partially phosphorylated in a number of JAK structures^{116–119}; however, its importance in terms of catalytic activity is unclear. In our studies on JAK2, we observe no difference in the activity of the kinase domain when this residue is mutated to phenylalanine (unpublished data).

The final motif of interest in the JAK kinase domains is the JAK insertion loop that is peculiar to this family.¹¹⁷ This loop links the α H and α I helices in the C-lobe of the kinases and in JAK1, JAK2, and TYK2 is capped by a “GQM” motif that allows them to bind to SOCS1 and SOCS3, two regulatory proteins that can inhibit the catalytic activity of these kinases. JAK3 does not contain a GQM motif in its JAK insertion loop and is, therefore, immune to SOCS-mediated inhibition.¹²⁸

Signal Transducers and Activators of Transcription (STAT) proteins

The STATs are a family of proteins named for their dual roles of (1) transducing signals from cytokines and (2) promoting transcription of specific genes. The

STATs predominantly reside in the cytoplasm as inactive dimers but are rapidly activated upon initiation of cytokine signaling and translocate into the nucleus.^{129–131} There are seven mammalian STATs (STAT1-4, STAT5a, STAT5b, and STAT6)^{132–134} and each contains several conserved features; an N-terminal region followed by a coiled-coil domain, a DNA binding domain, a linker region, an SH2 domain, and a C-terminal transactivation domain (Fig. 6). Located between the SH2 domain and the transactivation domain is a single conserved tyrosine residue which is the site at which the STAT proteins are phosphorylated by the JAKs and is essential for their activation.¹³⁷ STATs exist as dimers both in their active and inactive forms, but the structural arrangement of the two dimeric species is very different. Most STATs function primarily as homodimers; however, heterodimeric complexes do occur and are particularly important for STAT2, which only acts as a heterodimer. STAT2 acts downstream of Type I and III interferons but it does so as part of a complex called ISGF3 (IFN-stimulated gene Factor 3). ISGF3 is a three-protein complex that contains STAT2,

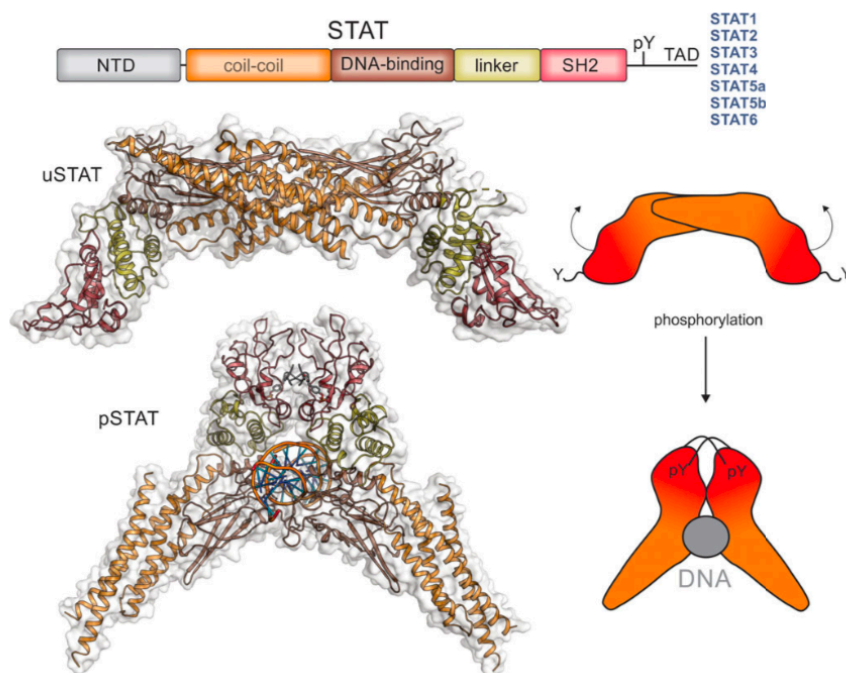


Figure 6. STATs. The Signal Transducers and Activators of Transcription (STATs) are a family of latent transcription factors that are activated by phosphorylation following cytokine exposure. The same domain architecture is shared by all STAT proteins and is shown schematically above. Unphosphorylated STAT (uSTAT) exists as an antiparallel dimer in the cytoplasm (upper). The SH2 domain (red) of uSTAT binds to phosphotyrosines in cytokine receptors which allows JAK to phosphorylate a specific tyrosine located between the SH2 and transactivation domain (TAD). This phosphotyrosine is then targeted by the SH2 domain of the other monomer inducing a large rotation between the two subunits of the dimer and allowing phosphorylated STAT (pSTAT) to occupy its DNA-binding competent dimeric structure (lower). The structures shown here are of STAT1 (PDB ID: 1YVL,¹³⁵ 1BF5¹³⁶) with the colors matching the schematic representation above. The N-terminal domain of STAT does not appear to form a stable interaction with the rest of the molecule and is not shown here. The transactivation domain (TAD) is unstructured but allows binding of accessory factors.

STAT1 and a third transcription factor called IRF9 which binds the STAT2 coiled-coil domain.¹³⁸ Figure 3 highlights the dominant STAT family members activated in response to individual cytokines; however, it should be noted that there is often low-level activation of additional STATs.

N-terminal region. The N-terminal domain of the STATs is largely conserved between all seven proteins and forms a bundle of alpha-helices oriented roughly at right angles to one-another.^{139,140} Generation of chimeric STAT molecules where the N-terminal domain of STAT1 was replaced with that of STAT2 or STAT5 revealed a role for the N-terminal region in nuclear translocation and deactivation.¹⁴¹ The N-terminal domain of the STAT proteins also plays a role in cooperative DNA binding¹⁴² between STAT dimers in regions of DNA where there are clusters of STAT binding sites, perhaps accounting for some of the specificity of cellular response to different cytokines.¹³⁹

Coiled-coil domain. The coiled-coil domain of the STAT proteins is a region of approximately 180 amino acids immediately following the N-terminal domain. It comprises four antiparallel alpha-helices which form a bundle in a down-up-down-up topology that is a major site of dimerization in the inactive form but then projects outwards from the core of the protein after activation. Allowing DNA binding and providing a surface for other proteins such as transcription factors to bind.¹³⁶

DNA binding domain. The DNA binding domain allows STATs to function as transcription factors and targets specific DNA sequences.¹⁴³ All STATs recognize palindromic DNA sequences with a TTCN₂₋₄GAA motif.¹⁴⁴ While all STATs bind this motif, their sequence preferences differ. STAT1 and STAT5 show preference for sites with a three-base pair spacer between the C and G (N3), with STAT1 also displaying preference for binding sites with a C at the -7 position, relative to the palindrome centre.¹⁴⁵ STAT6, unlike the other STAT proteins, binds to sites with a four-base pair spacer (N4)^{145,146}; however, STAT5^{147,148} has also been shown to bind weakly to N4 sequences.¹⁴⁵ STAT4 prefers the palindromic sequence (T/A)TTCC(C/G)GGAA(T/A) where the first and last T/A sites outside of the usual motif are also necessary for binding.¹⁴⁹

Linker and SH2 domain. Immediately downstream of the DNA-binding domain are the linker and SH2 domains. SH2 domains are modules that bind phosphotyrosine (pTyr) when it is embedded in a particular amino acid sequence motif. Each SH2 domain will have its own preferred sequence surrounding the pTyr, usually dictated by residues in the +1 and +3 positions (relative to the pTyr). Once JAK is activated

it immediately phosphorylates tyrosine residues in the receptor to which it is bound and the presence of an SH2 domain allows STATs to bind to those phosphorylated cytokine receptors. Thus which STATs will be activated by a particular cytokine depends on which receptor(s) their SH2 domain will bind.^{150,151} Different STATs can bind to the same sequence with different affinities which accounts for some of the pleiotropy of the STAT family and redundancy of biological outcomes.¹⁵²

STAT activation and transcription. Prior to activation, the STATs are found as inactive dimers in the cytoplasm. The DNA-binding and coiled-coil domains of two STAT monomers interact to form a reciprocal dimer (Fig. 6).^{135,153,154} STATs are activated by JAK-catalyzed phosphorylation of a specific tyrosine between the SH2 and transactivation domain. Instead of binding the phosphorylated receptor, the SH2 domain of each STAT monomer now binds the newly created pTyr in the other monomer resulting in dissociation from the receptor and re-orientation to form the active conformation with an exposed DNA-binding domain. These dimers then translocate into the nucleus and induce transcription of genes whose promoters contain the appropriate STAT binding sites. The STATs form a scissor-like structure around the DNA¹³⁶ (stabilized by the reciprocal interactions between the SH2 domain of one monomer and the pTyr of the other) and transcription is facilitated by the recruitment of transcriptional co-activators such as CBP/P300. Determining the complete set of genes upregulated by each of the STAT proteins has been difficult due to activation of other transcription factors by pathway crosstalk; however, several hundred to several thousand genes appear to be activated by each activated STAT¹⁵⁵⁻¹⁵⁸ as well as a number of genes being downregulated.¹⁵⁹

The dephosphorylation of STAT by phosphatases in the nucleus allows them to shuttle back into the cytoplasm for further rounds of activation.¹⁶⁰⁻¹⁶² While STATs are known to be active transcription factors in their phosphorylated state, there are some cases where unphosphorylated STATs also appear to play a role in gene transcription.^{163,164} It has also been suggested that unphosphorylated STATs are important for maintaining heterochromatin stability by associating with HP1, a heterochromatin protein necessary for heterochromatin formation.^{165,166} Unphosphorylated STAT5 has an important role as a transcriptional repressor in megakaryocytes where it blocks differentiation.¹⁶⁷

Negative regulation of JAK-STAT signaling

SOCS proteins

Signaling via the JAK-STAT signaling pathway is a dynamic process that involves the rapid transmission

of signal from the cell membrane to the nucleus followed by a highly organized response and subsequent controlled downregulation and attenuation of the initial signal.^{137,168} SOCS proteins are the primary drivers of signal attenuation, they are induced by cytokine exposure (via STAT) and then act as negative-feedback inhibitors to switch off the signaling cascade.^{13,94,169,170} There are eight SOCS proteins encoded in the human genome, SOCS1-7 and CIS.^{13,169–171} All eight contain N-terminal domains of various length and often unknown function but are defined by the presence of a central SH2 domain and a short, C-terminal domain called the SOCS box.¹⁷¹ CIS and SOCS1/2/3 are the members of the family associated with inhibiting signaling by JAK/STAT-inducing cytokines while other SOCS proteins appear to regulate signaling by factors such as EGF and insulin.

Although induced by many cytokines,¹⁷⁰ and potent inhibitors of many cytokine signaling pathways when artificially overexpressed, knockout studies have shown that each SOCS protein is specific for only a subset of cytokines.^{172,173} For example SOCS3 is induced by IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12, IL-13, IL-21, IL-22, G-CSF, GM-CSF, LIF, PRL, IFN α , IFN γ , GH, EPO, TPO, OSM, CT1, CNTF, and leptin^{170,174–185}; however knockout studies have shown that only IL-6 family cytokines,¹⁷² G-CSF,¹⁷³ and Leptin¹⁸⁶ are aberrantly regulated in its absence. This specificity is provided by the SH2 domains of SOCS proteins which in general bind to phosphotyrosine-containing motifs in specific cytokine receptors and therefore only inhibit signaling by cytokines that act via those receptors. For example the SH2 domains of CIS, SOCS2, and SOCS3 bind to motifs in the IL-2,³² GH,¹⁸⁷ and IL-6^{92,188,189} receptors, respectively.

Once bound to cytokine receptors, SOCS proteins induce their ubiquitination and degradation through the activity of their SOCS box domain. The SOCS box is structurally similar to a domain first described in VHL¹⁹⁰ and has two distinct binding properties. The first is a short motif that allows it to bind to elonginBC,^{191,192} an adapter complex that is also recruited by VHL. Once the ternary SOCS/elonginBC complex has formed the second motif can then recruit Cullin5, a 100 kDa E3 ligase scaffold.¹⁹³ This differs from VHL which uses its SOCS box-like domain to recruit Cullin2.¹⁹⁴ Cullin5 is constitutively associated with a RING domain protein, Rbx2,^{194,195} and this provides the E3 ubiquitin ligase activity by recruiting E2 ubiquitin conjugating enzymes to catalyze the ubiquitination of protein substrates. Therefore, SOCS proteins act as the substrate recruitment modules for Cullin5-based E3 ligases and proteins bound by the SOCS SH2 domains (usually receptors) are ubiquitinated and subsequently degraded by the proteasome.

SOCS1 and SOCS3. SOCS1 and SOCS3 are unique amongst the SOCS family in containing a short motif called the Kinase Inhibitory Region (KIR).^{196,197} This allows these two SOCS proteins to bind directly to the JAK kinase domain and inhibit its catalytic activity. The KIR is unstructured in the absence of JAK^{198,199} but upon binding adopts an extended conformation that sits in the substrate binding groove of the kinase.²⁰⁰ This occludes the substrate-binding site and prevents JAK from phosphorylating downstream substrates. SOCS1 and SOCS3 inhibit JAK1, JAK2, and TYK2 but not JAK3 due to the absence of a “GQM” sequence in the latter kinase.¹²⁸ SOCS1 is approximately 10-fold more potent than SOCS3 at inhibiting JAK, mainly due to sequence differences in the KIR.^{128,201}

In vivo, SOCS3 is a specific inhibitor of IL-6 family cytokines,^{43,202–204} G-CSF,^{172,173} and leptin¹⁸⁶ as these cytokines all signal via receptors that contain binding sites for the SOCS3 SH2 domain.²⁰⁵ SOCS3 engages the receptor with its SH2 and simultaneously inhibits the JAK1, JAK2 or TYK2 associated with that receptor.²⁰⁰ SOCS1, on the other hand, is a potent inhibitor of interferon, IL-12/23, IL-4/13 and IL-2 family cytokine signaling^{34–41,206–208} however, does not target the receptors for those cytokines with its SH2 domain. The target of the SOCS1 SH2 domain is unclear, it binds tightly to the activation loop sequence of all four JAKs when they are synthesized as synthetic peptides *in vitro*, however, appears to be sterically hindered from doing so when the activation loop is part of an intact kinase domain.²⁰¹ Further work is needed to clarify to this issue.

Phosphatases

Association of cytokine with the appropriate receptor triggers a cascade of intracellular tyrosine phosphorylation: JAKs are auto-phosphorylated *in trans*, receptors are then phosphorylated by these activated JAKs and finally the STATs are phosphorylated allowing them to adopt their active conformation. Therefore it is no surprise that phosphotyrosine phosphatases (PTPs) play a crucial role in regulating these signaling pathways.^{209,210} Six phosphotyrosine phosphatases in particular have been shown to regulate JAK/STAT signaling: the receptor tyrosine phosphatases CD45 and PTP-RT, two related cytoplasmic phosphatases PTP1b and TC-PTP, and the SH2 domain containing phosphatases SHP1 and SHP2.²¹¹ These phosphatases are constitutively expressed and are, therefore, not feedback-inhibitors. As such, they tend to restrain the amplitude of the signaling cascade rather than controlling its duration. It is the balance between the action of these phosphatases and the activity of the JAKs that determines the flux through the pathway. Determining the true targets of these phosphatases (JAKs, STATs, or receptors) has been difficult and at times contentious.

The receptor phosphatases CD45 and PTPRT. CD45 and PTPRT are both receptor phosphatases comprising an extracellular receptor-like region, a transmembrane domain and two intracellular tandem phosphatase domains. For both CD45 and PTPRT the first phosphatase domain is the catalytically active domain, whereas the second is a catalytically dead pseudophosphatase domain that is thought to have regulatory roles in both proteins. Full length CD45 is ~140 kDa; however, alternative splicing gives rise to several different sized isoforms. The extracellular region of CD45 is comprised of three FnIII domains (Fig. 7). CD45 is highly expressed in hematopoietic cells and thought to dephosphorylate all four JAK proteins.^{13,212} Cells deficient in CD45 display extended signaling in response to IL-7, EPO, and interferon stimulation. PTPRT is a large protein containing an N-terminal MAM domain followed by an Ig domain, four FnIII domains, a transmembrane domain and two tandem PTP domains. PTPRT directly interacts with and dephosphorylates the critical tyrosine residue in STAT3, pY705.²¹³

domain containing phosphatases. They are approximately 70 kDa, and composed of two SH2 domains and a single PTP domain that is negatively regulated by interactions with the SH2 domains.

The expression of SHP1 is limited to the hematopoietic lineage, where it regulates IL-3, EPO, IFN α , and potentially other cytokine-induced signaling by dephosphorylating JAK1, JAK2, and TYK2.^{214–218} SHP2 is ubiquitously expressed, but also plays an essential role in the regulation of hematopoiesis. Knockout of *shp2* leads to increased JAK1 autophosphorylation and upregulation of interferon signaling^{217,219} implying a role as a negative regulator. However, SHP2 is better characterized as a *positive* regulator of cytokine signaling. For example, it binds to pY759 on gp130 and activates the MAPK signaling cascade in response to IL-6 and LIF. In fact, SHP2 was the first tyrosine phosphatase to be identified as a proto-oncogene and somatic activating mutations of SHP2 have been identified in acute myeloid leukemia (AML) and B cell acute lymphoblastic leukemia (BALL).

The SH2 domain containing phosphatases SHP1 and SHP2. SHP1 and SHP2 are cytoplasmic SH2

The cytoplasmic phosphatases PTP1B and TC-PTP. PTP1B and TC-PTP are two highly related,

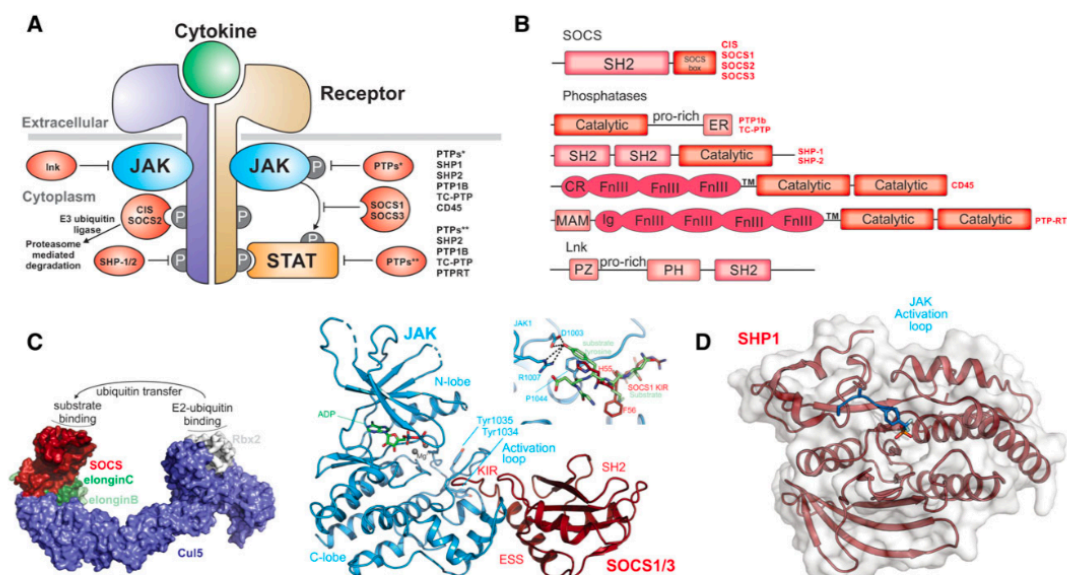


Figure 7. Regulation of cytokine signaling. (A) Schematic diagram showing regulators of cytokine signaling and where they act. (B) Domain architecture of the proteins indicated in A. (C) The primary negative feedback regulators of cytokine signaling are a subset of the SOCS (Suppressors of Cytokine Signaling) family, CIS, SOCS1, SOCS2, and SOCS3. These proteins function as the substrate recruitment modules of an E3 ubiquitin ligase (model structure shown in surface representation) and promote the ubiquitination and degradation of cytokine receptors and potentially other substrates. Substrates bind to the SH2 domain of SOCS proteins (red) and ubiquitin is transferred via an E2 ubiquitin-conjugating enzyme that docks onto the RING-domain protein Rbx2 (white). SOCS1 and SOCS3 (right) can also directly inhibit the JAK kinase domain by using their kinase inhibitory region (KIR) to block the substrate binding site of the kinase (PDB ID: 6C7Y) (model of a substrate overlay shown inset). (D) Six tyrosine phosphatases have been shown to be important regulators of cytokine-pathway activity, acting by dephosphorylating JAKs, STATs, or receptors. The structure of one of these, SHP1, has been solved in complex with the JAK activation loop of JAK2 (PDB ID: 4GSO).

~40 kDa phosphatases that are tethered to the cytoplasmic face of the endoplasmic reticulum (ER).^{220–222} Substrate-trapping mutants of PTP1B have been shown to interact directly with the activation loop of JAK2 and TYK2 suggesting these as the targets for its catalytic activity however it may also directly dephosphorylate STAT3.^{223–226} PTP1B is a powerful regulator of leptin signaling and knockout mice show increased JAK2 phosphorylation in response to that cytokine.²²⁵ TC-PTP is also tethered to the ER, however, a different isoform which lacks the ER-targeting motif is found in the nucleus and can dephosphorylate STAT3. Both JAK1 and JAK3 are dephosphorylated by TC-PTP and its knockout leads to increased IL-2, IFN α and IFN γ signaling.^{227,228}

The adaptor protein, LNK

The lymphocyte adaptor protein, LNK, also known as SH2B3, is a member of the SH2 domain containing adaptor protein family which also comprises APS (SH2B1) and SH2B (SH2B2). This family of proteins contains three distinct domains: a dimerization domain (phenylalanine zipper) which allows homodimerization, a Pleckstrin Homology (PH) domain and an SH2 domain.²²⁹ While APS and SH2B appear to activate cytokine signaling, LNK is a negative regulator of cytokines that signal via JAK2, particularly EPO and TPO.^{230,231} LNK knockout mice have enhanced numbers of hematopoietic stem cells and are hyperresponsive to EPO and TPO and over-expression of LNK inhibits megakaryocyte development. Consistent with its suppressive role, inactivation mutations in LNK are found in ca. 5% of MPNs and also in rare cases of idiopathic erythrocytosis. The SH2 domain of LNK binds directly to JAK2 (at pTyr813, located between the kinase and pseudokinase domains)²³², however, it is unclear how this regulates signaling.

Case study: IL-6 signaling

IL-6 represents perhaps the archetypal cytokine, it being the closest homologue to cytokines present in extant insect species. IL-6 (and related family members, Fig. 3) are all highly pleiotropic with roles in hematopoiesis, the acute phase response, development and both pro and anti-inflammatory processes.^{233–235} Here we provide a summary of the molecular events involved in IL-6 signaling.

IL-6 production can be induced by a variety of stimuli and by many different cell types. The receptor for IL-6, as discussed above, consists of the gp130 shared signaling chain and the non-signaling cytokine-specific IL-6R α chain. Although gp130 is found ubiquitously expressed, IL-6R α is found only on hepatocytes and some leukocytes.²³⁶ However, soluble IL-6R α is released from the liver and this allows IL-6 to signal to many different cell-types in a process called trans-signaling.²³⁷ Although IL-6 on its own has little affinity for gp130, it binds tightly to IL-6R α

(either membrane bound or soluble). This dimeric complex can then bind with high affinity to gp130, using regions on both the cytokine and the α chain. The resulting ternary complex then dimerizes to form the signaling hexamer.^{54,238} Thus, the formation of the signaling competent IL-6 receptor complex is an ordered process. The molecular details of IL-6 binding to its receptor were first described by Boulanger et al., who solved the structure of IL-6 bound to the CHR of IL6R α and the first three domains of gp130.⁵⁴ Combined with the full structure of gp130²³⁹ these studies allow a full model of the IL-6/IL-6 receptor complex to be constructed (Fig. 8).²³⁹

Once IL-6 has bound to its receptor, bringing together the two JAK-associated gp130 chains, this allows transactivation of the associated JAKs. JAK1, JAK2, and TYK2 can all be found associated with gp130 although knockout studies suggest JAK1 may be dominant.¹⁴ The ability of gp130 to associate with different members of the JAK family is unusual (Fig. 3). Upon activation, these JAKs then phosphorylate five tyrosine residues in the gp130 cytoplasmic domain.⁹⁰ The four distal sites (Y767, Y814, Y905, and Y915) are motifs targeted by the STAT3 (and to a lesser extent, STAT1) SH2 domain whilst the proximal phosphotyrosine (Y759) binds the negative regulatory protein SOCS3 and the phosphatase SHP2. STAT3 exists as a pre-formed dimer; however, once it is docked onto the receptor it is phosphorylated by JAK on Y705 and pY705 is then bound by the SH2 domain of the opposing monomers. This results in a re-orientation of STAT3 from an anti-parallel to a parallel dimer (Fig. 6) and subsequent translocation to the nucleus where it induces the transcription of target genes.¹¹ Activated STAT3 is observed within 15 min of IL-6 exposure and STAT3 target genes are observed almost immediately after²⁴⁰ highlighting a rapid transcriptional response. Importantly, STAT1 is also activated alongside STAT3.

A well characterized activity of IL-6 is its ability to induce the differentiation of monocytes into macrophages. An important component of this activity is the ability of IL-6 to induce the master transcription factor PU.1 alongside hundreds of other genes (RM, unpublished data). One of the most important of the early genes upregulated by IL-6 is SOCS3.¹⁷¹ SOCS3 mRNA and protein are observed within 30 min of IL-6 exposure and it is one of the most highly expressed early response genes (RM, unpublished data). SOCS3 protein binds to pY759 on gp130 via its SH2 domain and then uses its kinase inhibitory region to inhibit the associated JAK1 (or JAK2, TYK2). This switches JAK off and prevents any further STAT3 phosphorylation. STAT3 is dephosphorylated in the nucleus by TC45, the nuclear isoform of the T-cell protein tyrosine phosphatase (TC-PTP)¹⁶² and is then shuttled into the cytoplasm by exportin-1 to allow for subsequent activation cycles.²⁴¹ SOCS3 inhibits the

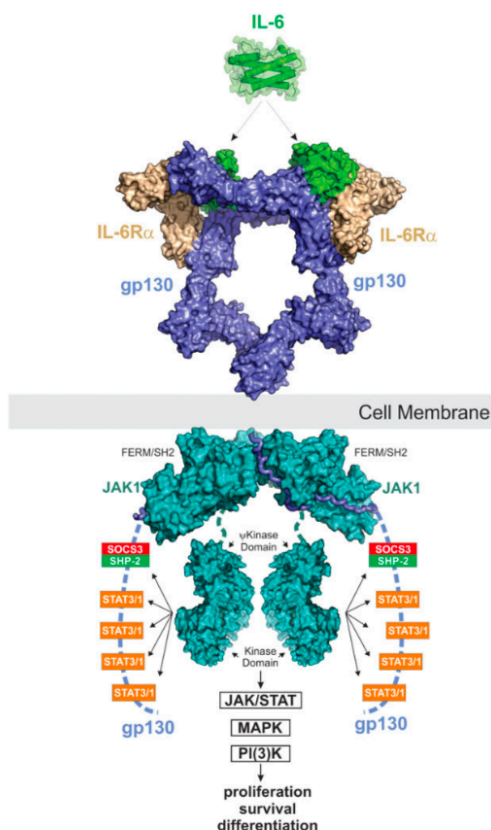


Figure 8. IL-6 signaling. IL-6 signals via a 2:2:2 complex between itself, gp130 and either membrane-bound IL-6R α (classic signaling) or soluble IL-6R α (trans-signaling). JAK1, JAK2 and TYK2 can all bind the intracellular domain of gp130; however, JAK1 appears to be the dominant kinase. The structure of JAK1 bound to the gp130 cytoplasmic domain is a model based on the structures of JAK1/IFN γ R (PDB ID: 5L04) and the JAK2/EPOR dimeric structure (coordinates kindly provided by R. Ferrao and P. Lupardus). JAK is activated by trans-phosphorylation and then phosphorylates five tyrosine residues on the receptor intracellular domain. The four distal tyrosines are docking sites for STAT3 and to a lesser degree STAT1. Activated STAT3 is then phosphorylated by JAK and translocates into the nucleus to drive the biological response. The MAPK pathway is also stimulated by IL-6 via SHP2 which binds to pY759 using its SH2 domain. The PI(3)K pathway is also activated in response to IL-6. SOCS3 is a direct STAT3 target gene and binds to the SHP2-binding site on the receptor via its SH2 domain. This inhibits MAPK signaling via displacement of SHP2 and also inhibits further STAT3 activation by direct inhibition of JAK catalytic activity.

signaling cascade as described above but may also play a role in shaping the cellular response. Genetic deletion of SOCS3 leads to a wider transcriptional response after IL-6 exposure, in particular there is increased expression of a number of genes associated

with IFN γ (STAT1) signaling. Under normal conditions, therefore, SOCS3 appears responsible for dampening STAT1 transcriptional programs and allowing STAT3 to dominate,²⁴² while eventually inhibiting both pathways.

Alongside activation of STAT3 (and STAT1), IL-6 stimulates two other signaling cascades: the MAPK and PI(3)K pathways. The phosphatase, SHP2 binds to pY759 on gp130 and promotes activation of the MAPK cascade via a mechanism that is not completely understood but may involve Grb2.²⁴³ SOCS3 also binds to this site and can thereby inhibit both STAT3 and MAPK induced transcriptional responses. How IL-6 induces the PI(3)-kinase pathway is less clear but the end result is activation of the serine/threonine kinase AKT (protein kinase B) at the cell membrane and stimulation of downstream signaling including mTOR.

Unanswered questions

The most important unanswered question in the field is how the activation of JAK (by trans-phosphorylation) is induced by cytokine binding and how this process goes awry in the presence of the activating mutations seen in the pseudokinase domain in human myeloproliferative diseases. The classical explanation given for the process of JAK activation was that simple dimerization of the receptor chains (by cytokine) brought the JAKs into close-enough proximity for their kinase domains to phosphorylate one-another. However it is now clear that many receptors exist as pre-formed dimers even in the absence of cytokine²⁴⁴ and that it is rather a re-orientation of these chains that allows JAK auto-phosphorylation. In fact, in 2014, Brooks et al. performed a series of FRET-based analyses to show that Growth Hormone induced a *separation* of the intracellular receptor domains and this led to a geometry where the kinase domains of the two JAK molecules were juxtaposed.²⁴⁵ Such a model supported their earlier analyses which showed that the GHR could be activated by tuning the relative orientation of the TM and juxtamembrane regions even in the absence of cytokine.²⁴⁶

This model suggests that prior to cytokine stimulation the pseudokinase domain from one JAK interacts with (and inhibits) the kinase domain from the other. After cytokine stimulation this inhibition is released. The importance of the pseudokinase domain in regulating the kinase domain is of course well-established as described above and by the existence of activating mutations within this domain. The important structure of the TYK2 pseudokinase-kinase domain pair highlighted that activating mutations tend to cluster near the interacting surface between the two domains however did not provide a molecular mechanism for what the pseudokinase domain was actually doing. The only structural information available for transphosphorylation of a tyrosine kinase was provided by crystallographic studies

from the Hubbard laboratory of the IGF1 receptor kinase¹²⁶; however, as far as JAK kinases are concerned, we have no picture of what allows their kinase domains to adopt this position and what prevents them from doing so in the first place. It seems clear that only structures of complete JAK proteins (with and without bound receptor) will provide a full molecular description of how JAK is activated and why this process goes awry upon mutation in human disease.

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Disclosure statement

The authors declare no conflicts of interest.

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7.2 Appendix II – Omit maps for five PTP1B structures with phosphopeptides bound.

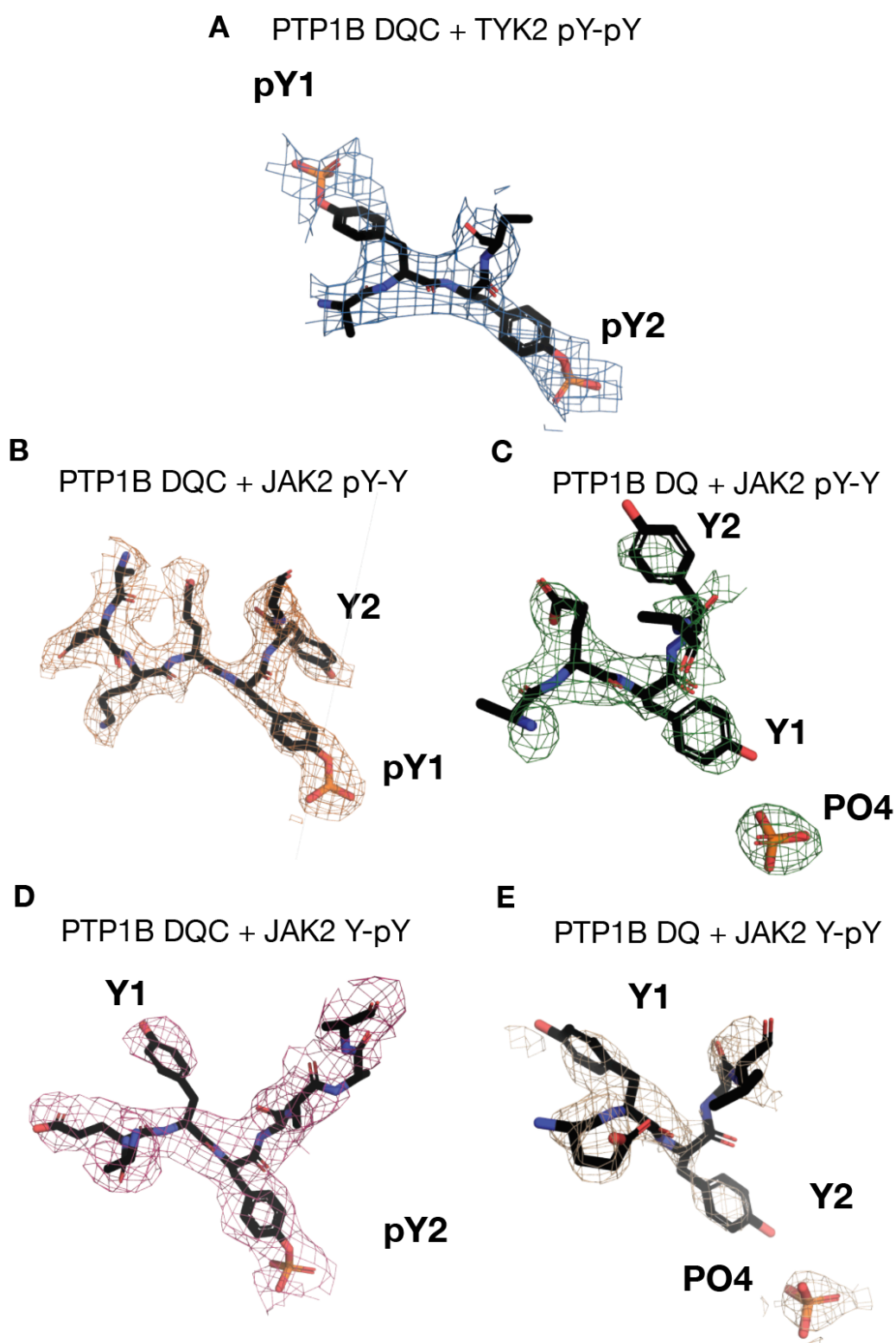


Figure 7.1 Omit maps for phosphopeptides bound to the PTP1B phosphatase domain.

*Simulated annealing 2Fo-Fc composite omit map, contoured to 0.8 σ for **A)** The TYK2 pY-pY phosphopeptide bound to PTP1B DQC. **B)** The JAK2 pY-Y phosphopeptide bound to PTP1B DQC. **C)** The dephosphorylated JAK2 pY-Y phosphopeptide bound to PTP1B DQ. **D)** The JAK2 Y-pY phosphopeptide bound to PTP1B DQC. **E)** The dephosphorylated JAK2 Y-pY phosphopeptide bound to PTP1B DQ.*

7.3 Appendix III - Raw data for NMR pYpY dephosphorylation assays of IRK and JAK2.

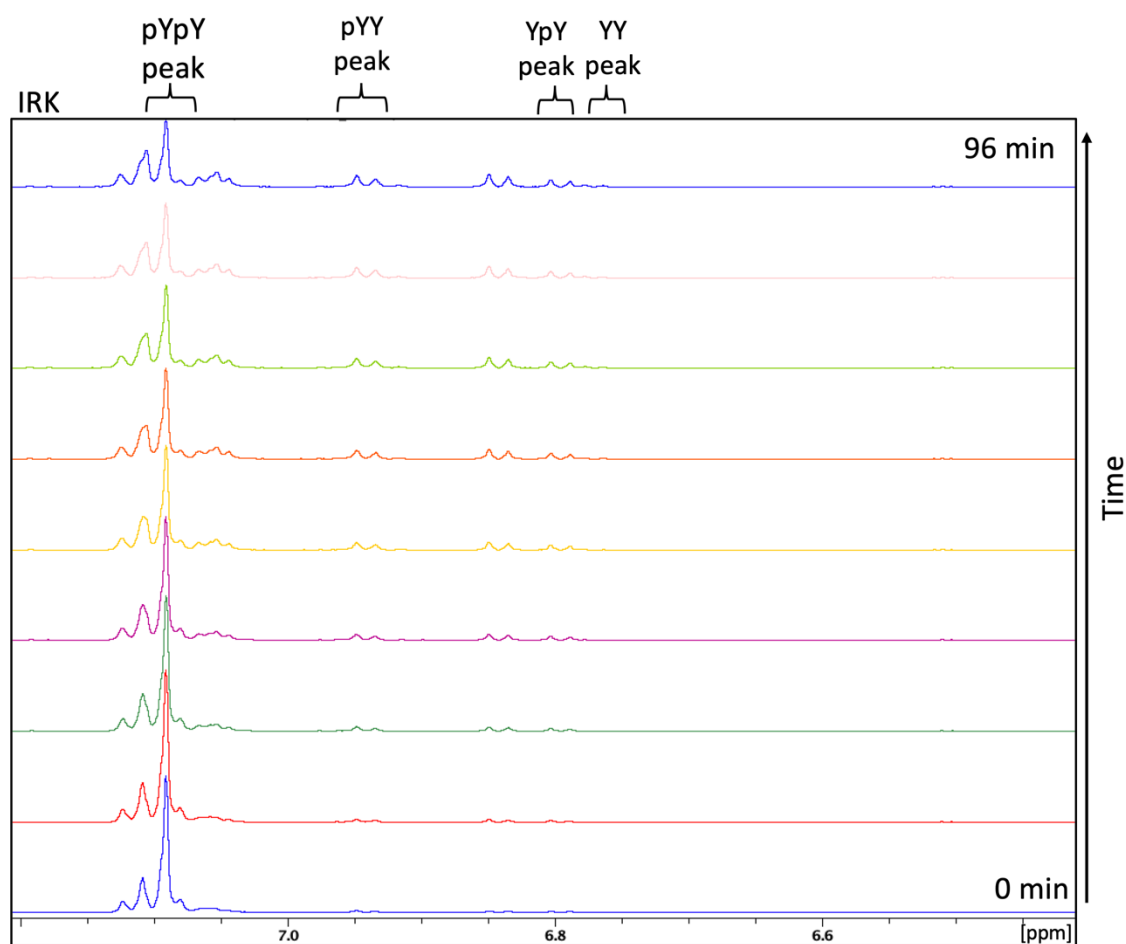


Figure 7.2 Peptide spectra for IRK a pYpY dephosphorylation time course.

Nine representative 1D ^1H solution NMR spectra of tyrosine residues for IRK pYpY dephosphorylation assays. Peaks representing the pYpY, pYY, YpY and YY peptides indicated at top.

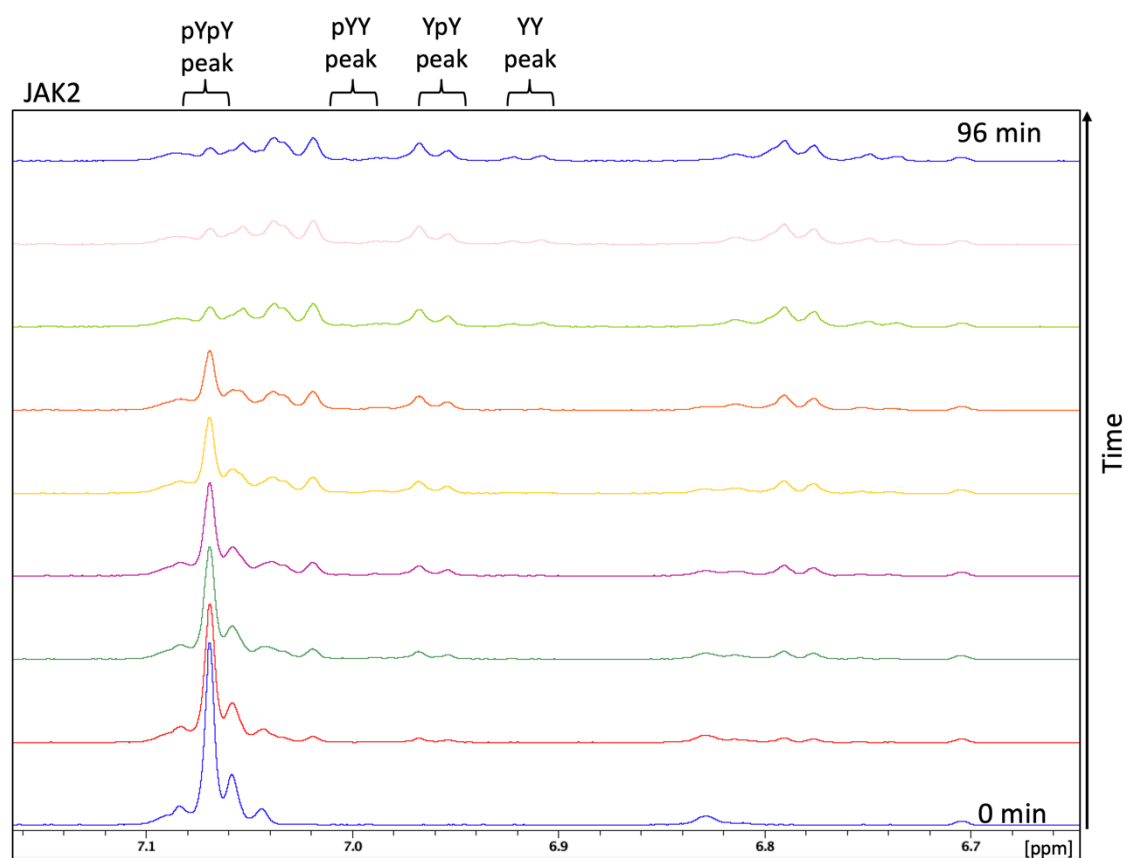


Figure 7.3 Peptide spectra for JAK2 pYpY dephosphorylation time course.

Nine representative 1D ^1H solution NMR spectra of tyrosine residues for JAK2 pYpY dephosphorylation assays. Peaks representing the pYpY, pYY, YpY and YY peptides indicated at top.

7.4 Appendix IV – Fo-Fc map for water modelled into catalytic pocket of PTP1B D/Q plus JAK2 pYpY

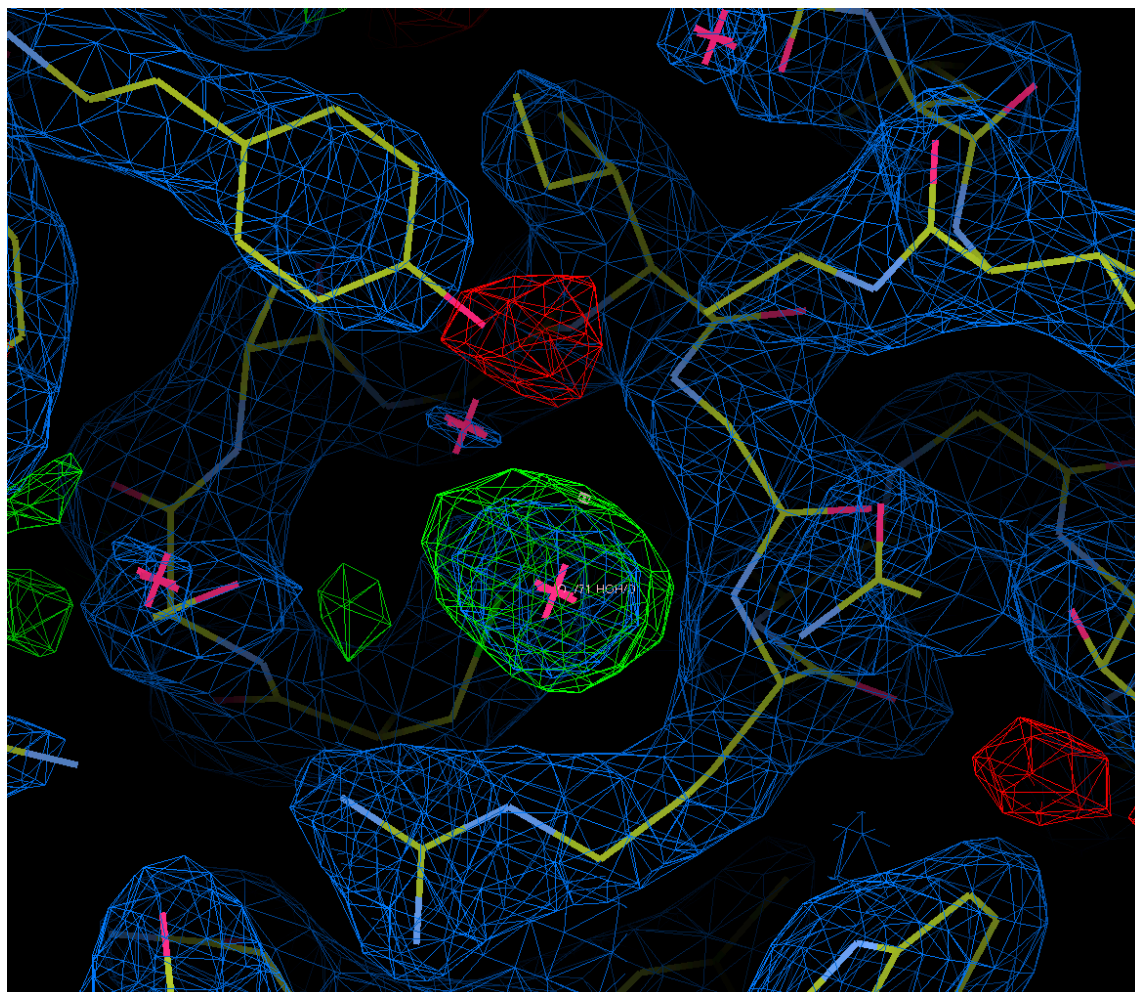


Figure 7.4 Fo-Fc map for water molecule modelled into PTP1B D/Q plus JAK2 pYpY as seen in PHENIX.

Replacement of the phosphate in the active site of PTP1B with water resulted in a residual peak of 8.84 sigma in the Fo-Fc map.