The Regulation of Cell Signalling by LAR Protein Tyrosine Phosphatase

BY

ADIL RASHID SARHAN

A thesis submitted to

The University of Birmingham

For the degree of

DOCTOR OF PHILOSOPHY

College of Life and Environmental Sciences

School of Biosciences

University of Birmingham

Abstract

Signal transduction pathways are mainly depending on phosphorylation events, which are controlled by the activity of phosphatases and kinases. Although kinases have been widely studied, however, much less is known about the contribution of phosphatases to the regulation of cell signalling pathways. Leukocyte common antigen-related protein (LAR) is a member of the LAR subfamily of receptor-like protein tyrosine phosphatases (RPTPs). LAR is involved the regulation of a number of receptor tyrosine kinases (RTKs) including platelet-derived growth factor receptor (PDGFβR). To gain insight into the signaling pathways regulated by LAR, including those that are PDGF-dependent, we have carried out the first systematic analysis of LAR-regulated signal transduction using SILAC-based quantitative proteomic and phosphoproteomic techniques. The differential phosphorylation between wild-type mouse embryo fibroblasts (MEFs) and MEFs in which the LAR cytoplasmic phosphatase domains had been deleted (LARΔP) was analysed. A significant change in abundance of phosphorylation on 270 phosphorylation sites from 205 proteins was associated with the lack of LAR phosphatase activity. Gene ontology analysis revealed an enrichment of LAR-mediated phosphorylation events on proteins involved in many signalling transduction pathways including those regulating the actin cytoskeleton, cell adhesion, endocytosis and cell metabolism. Analysis of putative kinases upstream of LAR-dependent phosphorylation events revealed a role for LAR in regulating signalling through mTOR and JNK. In summary, this thesis identifies an important role for LAR phosphatase in the regulation of signal transduction, cell adhesion and cell metabolism.

Acknowledgements

It has been such a great time to work at the School of Biosciences, 5th floor for the past few years. It has been a period of intense learning for me not only in the scientific field, but also on a personal level. I would like to reflect on the people who have supported and helped me so much throughout my PhD study.

I would first like to thank my funding agency "The Higher Committee for Education Development in Iraq" (HCED), I am grateful to them for this opportunity to study at the University of Birmingham.

I want to express my extreme gratitude to my supervisors, Neil Hotchin and Debbie Cunningham for their encouragement, patient guidance and advice throughout my PhD. It is just not possible to show my appreciation and grateful by words!

I would also like to thank my co-advisors John Heath and Mike Tomlinson for their wonderful collaboration and guidance. Special thanks to the late Carina Hellberg, my previous supervisor. Although you passed away, I really appreciate your help during the beginning of my PhD.

I am grateful to my colleagues Trushar Patel, Andy Creese and Tijs van Wieringen, for their invaluable advice and patience for scientific and non-scientific conversations and for all the opportunities I was given to conduct my research.

In addition, I would like to thank Andy Philp and Stephen Ashcroft (School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham), for their help with Seahorse assay, and Christopher Weston (Institute of Immunology and Immunotherapy, University of Birmingham) for his help with counting cells using IncuCyte.

I am thankful to Shabana, Justyna, Peter, Alex, Alessandro, Cleidiane, Jinglei and for all my colleagues from the 5th floor for their support and good times we had together.

I would also like to thank my parents for their wise counsel and sympathetic ear.

Finally, and most importantly, I would like to thank my wife "Noor" for all the late nights and early mornings. Her support, patience, motivation, and love were undeniably.

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List of Abbreviations

C-Abl	Abelson murine leukemia viral oncoprotein
CKIγ2	Casein kinase Iγ2
Cbl	Casitas B-lineage lymphoma
DMSO	Dimethyl sulfoxide
DSPs	Dual specificity phosphatases
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ESCRT I	Endosomal sorting complex required for transport I
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
GPCR	G protein coupled receptor
GPS	Group-based Prediction System
GRK	G protein coupled receptor kinase
HGF	Hepatocyte growth factor
Hrs	Hepatocyte growth factor regulated tyrosine kinase substrate
iGPS	GPS algorithm with the interaction filter
LAR	Leukocyte common antigen related
MEF	Mouse embryonic fibroblast
MKP	MAP kinase phosphatase
mRNA	Messenger RNA
MTOC	Microtubule organizing centre

mTOR	Mammalian Target of Rapamycin
NHERF	Na+/H+ exchanger regulatory factor
NPTP	Non-transmembrane PTPs
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDZ	PSD95/*Drosophila* discs large/ZO1
PH	Pleckstrin Homology
PI(4,5)P2	Phosphatidylinositol (4,5) bisphosphate
PI3K	Phosphoinositide-3-kinase
PIP3	Phosphatidylinositol (3,4,5) triphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PTEN	Phosphatase and Tensin homolog
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RasGAP	RasGTPase activating protein
RPTP	Receptor protein tyrosine phosphatase
RTK	Receptor tyrosine kinase
SH2	Src homology 2
SILAC	Stable Isotope Labelling of Amino Acids in Cell Culture
siRNA	Small Interfering RNA
STAT	Signal transducers and activators of transcription

CHAPTER 1

INTRODUCTION

1.1. Platelet-derived growth factors

1.1.1. Introduction

Platelet-derived growth factor (PDGF) was firstly isolated from human platelets and is expressed in all organs with the highest level in the heart and placenta (Fredriksson et al., 2004, Ross, 1986). Many activities that occur in cells, such as growth, survival, motility, proliferation and differentiation, are regulated by growth factors. PDGF is a dimeric molecule that is composed of disulfide bonded A, B, C and D isoforms. PDGF has been shown to make a homodimeric (PDGF-AA, PDGF-BB) and heterodimeric (PDGF-AB). These PDGF isoforms bind to Platelet-derived growth factor receptor α (PDGFαR) and Platelet-derived growth factor receptor β (PDGFβR). PDGFαR binds to isoforms A, B and C whereas PDGF β receptor binds to ligands B and D (Heldin et al., 1998). Since the ligands are dimeric molecules thus have two-receptor epitopes bind to the two receptors leading to receptor dimerization and oligomerization (Duan et al., 1991).

PDGF AA gives rise to the dimerization of PDGFRαα, while BB leads to αα and ββ dimer, CC ligand form αα and DD induces ββ dimerization (**Figure 1.1**). Both, PDGFR α and PDGF β receptor consist of five extracellular immunoglobulin domains and intracellular domains with tyrosine kinase activity (Kanakaraj et al., 1991).

An important function of dimerization is to activate PDGF receptor by allowing the autophosphorylation of certain tyrosine residues (see section 1.2) located in the intracellular domains (Jiang and Hunter, 1999).

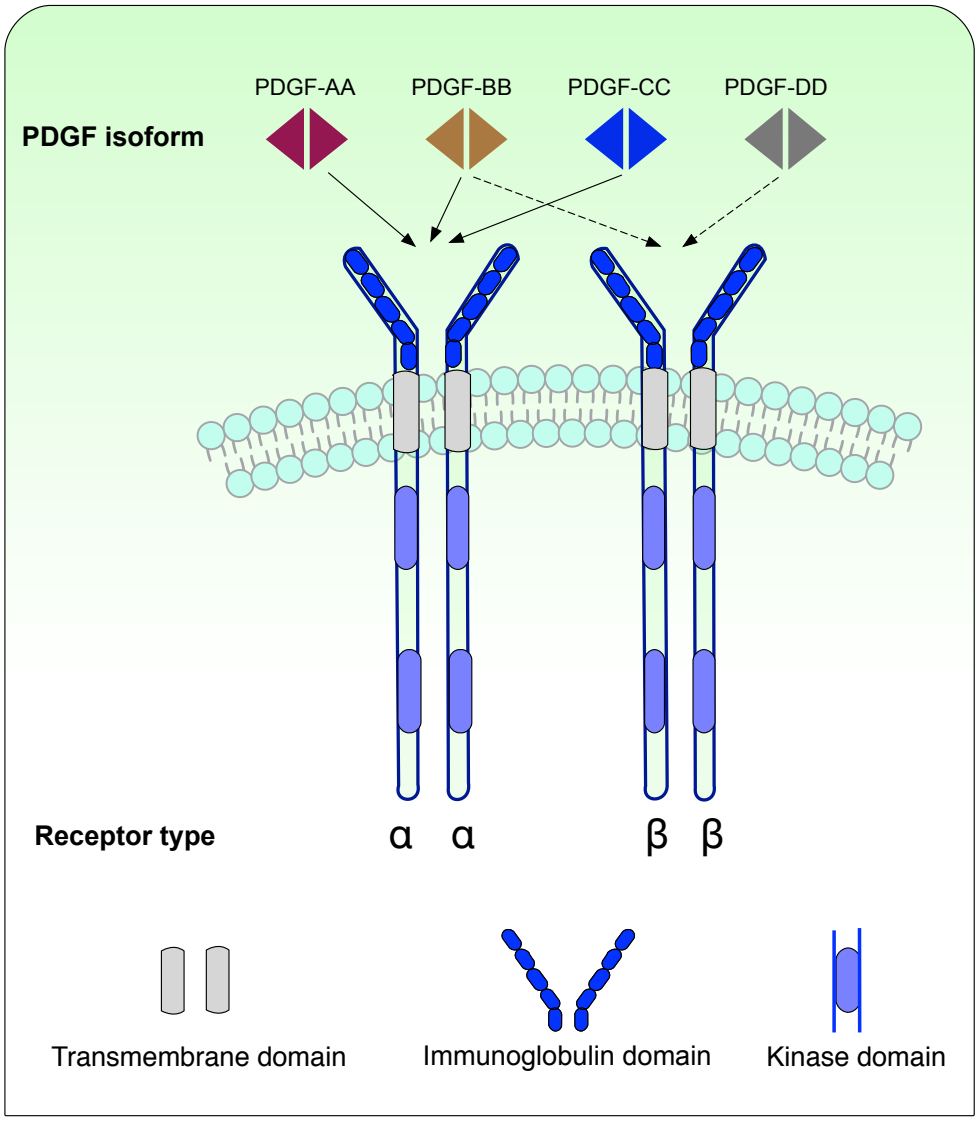

Figure 1.1. The specificity of PDGF and their receptors. PDGF AA gives rise to the dimerization of PDGFRαα, while BB leads to αα and ββ dimer, CC ligand form αα and DD induces ββ dimerization. Adapted from (X. Li and Eriksson, 2003).

PDGF has been shown as a stimulator of cellular processes in cells of mesenchymal origin. PDGF and their receptors are expressed in most tissues (Heldin and Westermark, 1999). PDGFs are highly mitogenic molecules during embryonic stages leading to cell proliferation and differentiation. PDGF and PDGFR play a crucial role in later maturation stages including wound healing, constriction of blood vessels, angiogenesis, cell migration and tissue remodelling (Fredriksson et al., 2004, Hellberg et al., 2010). Several studies have shown that PDGFβR stimulates chemotaxis (Hansen et al., 1996). In contrast, PDGFαR plays an inhibitory role in chemotaxis signal in many cell types (Koyama et al., 1996, Siegbahn et al., 1990, Yokote et al., 1996).

1.1.2. Platelet-derived growth factors in disease

PDGFs and corresponding receptors were expressed in most cells and tissues (Guha et al., 1995, Heldin, 1992, 2013, Heldin et al., 2016). In contrast, overexpression of the PDGF receptors has been reported to cause several diseases such as atherosclerosis and cancer. Previous research findings from animal studies indicated that knockout of PDGF or PDGFR can cause disorders such as defective of alveoli development in lung, abnormality in cerebral vascularization, and also defective in the development of blood vessels (Hoch and Soriano, 2003).

A considerable amount of literature has been published on diseases caused by PDGF receptor overactivation including breast cancer, glioblastoma, sarcoma, atherosclerosis and renal fibrosis (Aguilar, 2016, Alehagen et al., 2016, Appiah-Kubi et al., 2016, Lewandowski et al., 2016a, Lewandowski et al., 2016b, Pietras et al.,

2003). It has also been reported that overactivation of PDGF and PDGFR linked to breast cancer and the inhibition of PDGFR reduced the invasion of the cancer cells (Paulsson et al., 2009). PDGF and their receptors have been identified in glioblastoma multiforme (GBM) cell lines suggested the autocrine activity caused by PDGF stimulation leading to development of glioblastoma multiforme (Hermanson et al., 1992, Lokker et al., 2002).

According to animal studies, the hyperactivity of PDGF has also been reported to induce oligodendroglial tumor and many brain tumours. Chronic myeologenous leukaemia has linked to PDGFβR overexpression (Appolloni et al., 2009, Dai et al., 2001). Moreover, PDGF is upregulated in prostate carcinoma and liver cancer and the inhibition of PDGFR kinase activity by imatinib or sunitinib was shown to reduce cell migration and tumor progression (Gotzmann et al., 2006, Ko et al., 2001). These finding demonstrate that PDGF and PDGFβR overactivation enhance the development of cancer and invasiveness. Overexpression of PDGFβR has also reported in lung cancer, demonstrating a crucial role for PDGF and their receptor in the development of lung cancer (Tsao et al., 2011).

1.1.3. PDGFβR downregulation

1.1.3.1. Dephosphorylation by protein tyrosine phosphatases

PDGFβR signalling is highly controlled through several mechanisms; one of them is the dephosphorylation by protein tyrosine phosphatases (PTPs). It has been identified that many of PTPs bind with and dephosphorylate several selected tyrosine residues on PDGFβR to terminate the receptor downstream signalling molecules. For example, Low molecular weight protein tyrosine phosphatase (LMW-PTP) has been found to dephosphorylate Tyr^{857} on PDGFβR to inhibit the receptor kinase activity (Chiarugi et al., 2002). In addition, LMW-PTP can also dephosphorylate Tyr^{716} on the receptor leading to prevent the interaction of Grb2 (Chiarugi et al., 2002).

T-cell protein tyrosine phosphatase (TC-PTP) can dephosphorylate Tyr^{1021} on PDGFβR, which is a binding site of phospholipase C gamma1 (PLCγ1), leading to decreased cell migration in response to PDGF (Persson et al., 2004). Grb2 can bind to Tyr^{716} on PDGFβR and form a complex with Sos1 in order to activate Ras signalling (Schlessinger, 1993). Ras-GAP has been shown to binds with PDGFβR at Tyr^{771} (Fantl et al., 1992, Kashishian et al., 1992) which in turn deactivates the Ras signalling pathway by converting the active form of Ras-GTP to Ras-GDP (van der Geer et al., 1997). However, SHP-2 can dephosphorylate the binding site of Ras-GAP Tyr^{771} leading to enhance the activity of Ras signalling pathway by preventing Ras-GAP interacting with PDGFβR (Ekman et al., 2002).

Moreover, PTP1B dephosphorylates Tyr^{579} residue of PDGFβR where Src and Shc can bind, to terminate the signalling initiation by these proteins (Ekman et al., 2002, Persson et al., 2004). Histidine domain-containing protein tyrosine phosphatase (HD-PTP) is recruited to the PDGFβR in response to PDGF-BB and is required for

PDGFβR degradation (H. Ma et al., 2015a). In summary, protein tyrosine phosphatases play an important role in regulating PDGFβR signalling.

1.1.3.2. Serine phosphorylation

Serine phosphorylation is also a key regulator of the receptor tyrosine kinases to desensitize receptor activity such as insulin receptor (IR) and EGFR after stimulation (Countaway et al., 1992, Takayama et al., 1988). It has been reported that some serine/threonine kinases can phosphorylate the serine residues on PDGFβR leading to decreased activity of the receptor by reducing the autophosphorylation rate (Bioukar et al., 1999). For instance, Casein kinase Iγ2 (CKIγ2) is serine/threonine kinase that can regulate PDGFβR activity by phosphorylating serine residues on the receptor (Bioukar et al., 1999).

Several studies have published on the importance of G protein-coupled receptor kinase 2 (GRK2) for regulating receptor desensitization and internalization. GRK2 is one of seven members of the GRK family (GRK1-GRK7), which are widely expressed in mammalian tissue and regulate a variety of cellular effects (Black et al., 2016). GRK2 regulates PDGFβR by mediating serine phosphorylation leading to receptor desensitization and a reduction in receptor autophosphorylation (J. H. Wu et al., 2005). A GRK2 inhibitor restored tyrosine phosphorylation of the PDGFβR but not EGFR demonstrating a significant role of the GRK2 in regulating PDGFβR signalling (Freedman et al., 2002). GRK2 translocates to the plasma membrane by an interaction with Gβγ subunits and PIP_2 in order to activate GRK2 (Pitcher et al., 1998). On the

other hand, GRK2 and GRK5 have a specific sequence for binding to Ca^{2+}/calmodulin leading to inhibition of their activity suggesting an alternative mechanism to regulate GRK2 activity (Pronin and Benovic, 1997).

1.1.3.3. PDGFβR internalization and degradation

PDGFβR can also be downregulated by ubiquitination and internalization. Upon activation, PDGFβR is internalized and sorted for the lysosomal degradation. The internalization and intracellular sorting of the cell membrane components such as epidermal growth factor receptor (EGFR) and PDGFR have been characterized (Maxfield and McGraw, 2004). In general, RTKs are trafficked to the early endosomes and at this point some receptor rapidly recycle back to the membranes via Rab4a GTPase (Maxfield and McGraw, 2004). By using Rab11 dependent endocytosis, some receptors are sorted and recycling to reach a long recycling loop to the cell membrane such as EGFR (Dikic, 2003, Waterman and Yarden, 2001). Other receptors reach late endosomes to lysosomal degradation (van Ijzendoorn, 2006). The termination and sorting of the receptors signalling toward lysosomal degradation depends on the interaction of the receptors with hepatocyte growth factor regulated tyrosine kinase substrate (Hrs) and endosomal sorting complex required for transport I (ESCRT I) (Bache et al., 2003, Raiborg et al., 2003). Hrs can bind with signal transducing adapter molecule 2 (STAM2) and promote PDGF-induced PDGFβR degradation (Takata et al., 2000). The Cbl family of E3 ubiquitin ligases have been shown to induce PDGFβR

ubiquitination and degradation by sorting receptor towards lysosomal degradation (Miyake et al., 1999, Schmidt and Dikic, 2005).

The kinase activity of PDGFβR is required for PDGF-induced internalization (Mori et al., 1993). Later, researchers have found short amino acid sequences 952–965 essential for the receptor internalization (Pahara et al., 2010). It has been reported that activation of protein kinase C α (PKCα), the downstream of PDGFβR, is required for the PDGFβR recycling via Rab4a-dependent manner (Hellberg et al., 2009).

1.2. Signal transduction pathways

The interaction of PDGF and PDGF receptor leads dimerization and autophosphorylation of specific tyrosine residues on the receptor in trans. This initiate a cascade of signal transduction leading to modulation of cellular functions such as cytoskeletal rearrangement, gene transcription, proliferation, ion channel activation and cell movement.

Autophosphorylation is an important mechanism and plays a key role in receptor kinase activity by provide docking sites for the downstream transduction molecules. Several studies have revealed that there 11 out of 15 tyrosine residues situated in the intracellular domain (non-catalytic) of the PDGFβ receptor and are considered as autophosphorylation sites (**Figure 1.2**) (Claesson-Welsh, 1994). PI3K binds to the PDGFβR tyrosine sites Tyr^{740} and Tyr^{751} (Fantl et al., 1992, Kashishian et al., 1992). Phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) is a substrate for the PI3K which

can be converted to the $PI(3,4,5)P_3$ (Vassbotn et al., 1992). PI(3,4,5)P3 phosphorylates Tyr^{308} on Akt/PKB leading to initiates Akt/PKB signalling (Klippel et al., 1997). Phospholipase C (PLCγ) is another important example which produce two molecules by using $PI(4,5)P_2$ as a substrate. Firstly, inositol (1,4,5) trisphosphate which increased the concentration of cytoplasmic Ca^{2+}, and secondly, diacylglycerol that leads to protein kinase C activation (Berridge, 1993). Taken together, the activation of Akt/PKB and PLC-γ via PDGF receptor leading to certain cellular functions such as cytoskeletal rearrangement, gene transcription, proliferation, ion channel activation and cell movement. In addition, Src kinases, Ras-GAP, are also involved in signalling network as well as adaptor molecules including Grb2, Grb7, Shc, Nck, Crk, SAPK/JNK and STATS (Heldin and Lennartsson, 2013).

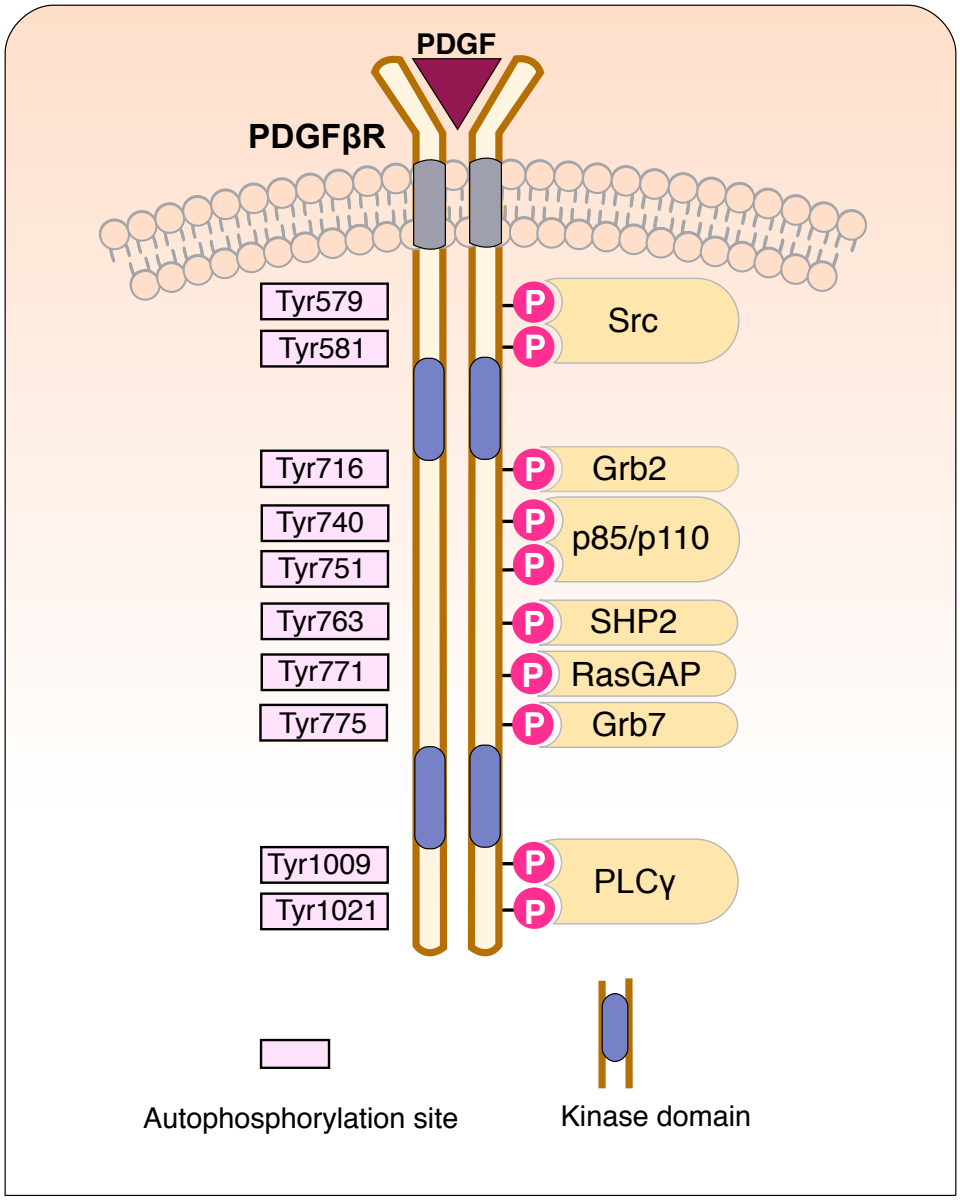

Figure 1.2. PDGFβR dimerization and interaction with signal transduction molecules. Shows autophosphorylation tyrosine residues and the known binding SH2 containing downstream proteins.

1.2.1. PI3K/Akt pathway

PI3Ks are a family of cytoplasmic kinases that enable to phosphorylate the 3′-position (hydroxyl group) of phosphoinositides. PI3Ks are classified into three distinct groups (Bayascas and Alessi, 2005, Engelman et al., 2006, McManus et al., 2004). Class I PI3K can be activated in response to multiple stimuli such as G-protein-coupled receptors, Ras or growth factors (e.g. PDGF) (Cantley, 2002). PI3K class I phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) leading to the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Cantley, 2002, Vanhaesebroeck and Alessi, 2000). Whereas class II and class III produce phosphatidylinositol-3-phosphate PI-3-P by using phosphatidylinositol (PI) as a substrate (Bago et al., 2014, Engelman et al., 2006).

PI3K class II and III are mainly involved in regulation of membrane trafficking, endocytosis, phagocytosis and can be activated in response to integrins, cytokines and growth factors (Backer, 2008, Bago et al., 2016, Engelman et al., 2006). PI3K class I have been shown to bind and become activated by PDGFβR (Auger et al., 1989, Coughlin et al., 1989, Kazlauskas and Cooper, 1989). PI3K class I consist of catalytic subunit p110 and regulatory subunit p85 with two SH2 domains (Songyang et al., 1993). Two autophosphorylated tyrosine residues in the PDGFβR and PDGFαR Tyr^{740}, Tyr^{751} are the binding sites for PI3K class I (Fantl et al., 1992, Kashishian et al., 1992, Kazlauskas et al., 1992, Yu et al., 1991).

After the activation of PI3K by PDGFβR, PI3K generate PI(3,4,5)P3 which in turn recruits PH (Pleckstrin homology) domain containing proteins, such as Akt (**Figure 1.3**) (Alessi et al., 1996, Burgering and Coffer, 1995). This causes a conformational change leading to the activation of Akt by phosphorylation of two residues, Thr^{308} via

phosphoinositide dependent kinase 1 (PDK1) (Franke et al., 1997, Stokoe et al., 1997) and Ser^{473} via mTORC2 (Feng et al., 2004, Persad et al., 2001, Sarbassov et al., 2005). Akt has been shown to regulate many downstream proteins. For example, Akt phosphorylates Bcl-2 family protein BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death) which leads to suppression of apoptosis by preventing the interaction of BAD with Bcl-2 or Bcl-X_L, the unphosphorylated form of BAD can bind to BLC_{XL} leading to apoptosis (Datta et al., 1997, Vanhaesebroeck and Alessi, 2000, Zha et al., 1996). Researchers examined whether PDGFβR activation prevents apoptosis by transfecting rat adrenal gland (PC-12) cells with Y740F mutant PDGFβR (lacks the binding site of PI3K) and they found these cells are unable to prevent apoptosis (R. Yao and Cooper, 1995). This notion supports the fact that PDGF β-receptor is important not only for proliferation and many other functions but also protects the cells from undergoing apoptosis.

In addition, Akt has been shown to regulates mammalian target of rapamycin (mTOR) by two ways; First, by phosphorylation and suppression of the activity of tuberous sclerosis complex 2 (TSC2), a negative regulator of mTOR; and second, by inhibition of the activity of AMP-activated protein kinase (AMPK), an activator of TSC2 (Hahn-Windgassen et al., 2005, Hay and Sonenberg, 2004). PI3K has been found to be involved in many biological processes in response to PDGF stimulation including actin reorganization, cell movement, cell growth and the suppression of cell survival (Kamohara et al., 1995).

Signalling through PI3K/Akt also contributes to angiogenesis via the activation of endothelial nitric oxide synthase (eNOS) in endothelial cells and also involved in Glucose Metabolism (Kamohara et al., 1995, Manning and Cantley, 2007). Recently,

researchers showed that hyperactivity of Akt is linked to increased breast cancer recurrence rate by the inhibition of FOXO3a, Forkhead transcription factor (also known as FKHRL1). FOXO3a can prevent mammospheres formation which is responsible for metastasis and retrieval of the tumor in breast cancer stem cells (BCSC) (Smit et al., 2016).

The PI3K/Akt pathway is highly controlled by dephosphorylation of Akt at Ser^{473} by PH domain and Leucine rich repeat Protein Phosphatases (PHLPP) and at Thr^{308} by protein phosphatase 2A (PP2A) to downregulate Akt activity (Carracedo and Pandolfi, 2008, Osaki et al., 2004). Akt signalling can also downregulated by phosphatase and tensin homolog (PTEN). PTEN terminates PI3K signalling by dephosphorylation of PIP3 (Carracedo and Pandolfi, 2008, Chalhoub and Baker, 2009).

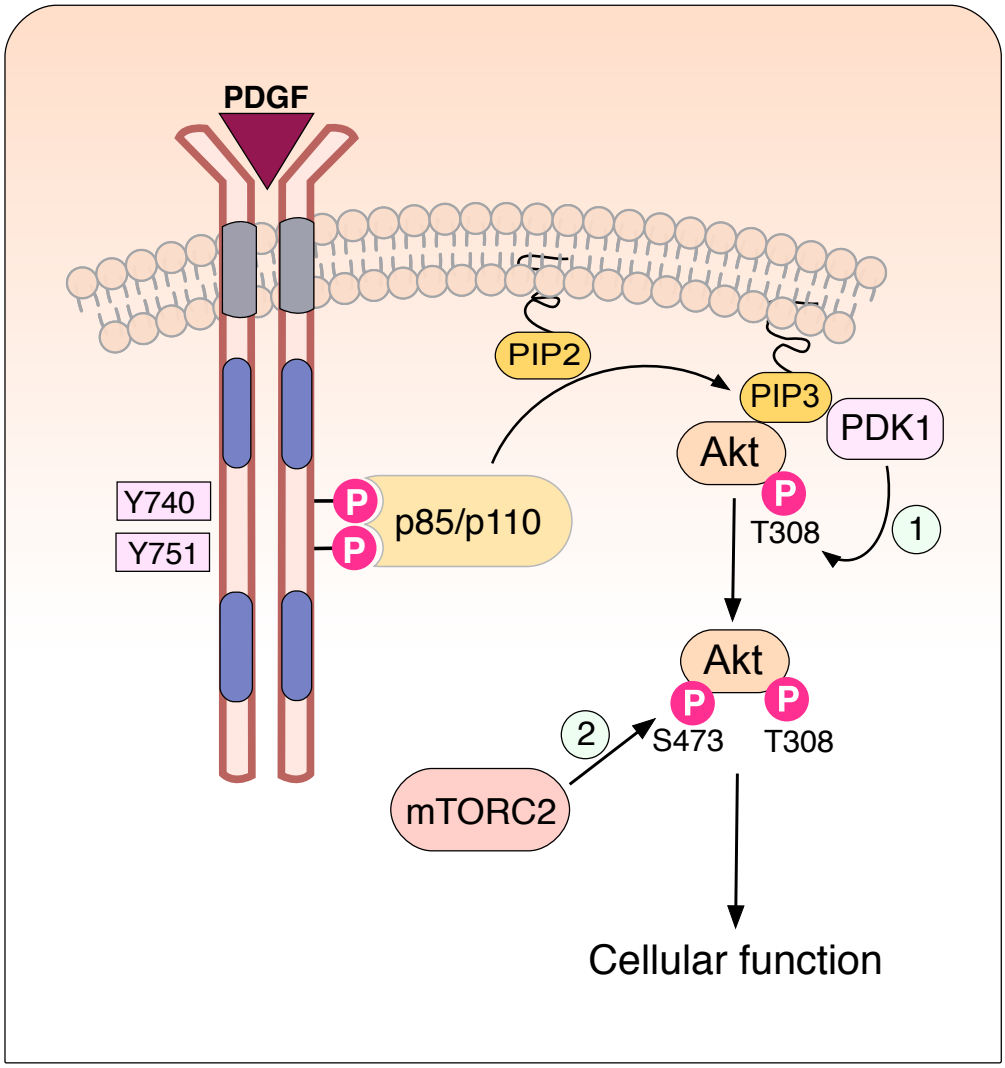

Figure 1.3. Schematic representation of the PI3K/Akt pathway. PI3K class I isoforms bind to phosphorylated Tyr740 or Tyr751 on PDGFβR. PI3K can phosphorylate phosphatidylinositol-4,5-bisphosphate (PIP2) leading to the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 recruits PH (Pleckstrin homology) domain containing proteins, such as Akt. This causes a conformational change leading to the activation of Akt by phosphorylation of two residues, first, Thr^{308} via PDK1 and second, Ser^{473} via mTORC2.

1.2.2. PLCγ pathway

Phospholipase C (PLCγ) is activated after binding to PDGFβR (Heldin et al., 1998). PLCγ produces two molecules by using $PI(4,5)P_2$ as a substrate; inositol (1,4,5) trisphosphate which increases the concentration of cytoplasmic Ca^{2+}; and diacylglycerol that leads to protein kinase C activation (Berridge, 1993) **(Figure 1.4**). There are two isoforms of PLCγ, PLCγ1 and PLCγ2 each with two SH2 domains and one SH3 domain (Kamat and Carpenter, 1997, Sultzman et al., 1991). PLCγ1 binds to the phosphorylated Tyr^{1009} and Tyr^{1021} on PDGFβR (Kashishian and Cooper, 1993, Valius et al., 1993). Upon binding with PDGFβR, PLCγ1 is phosphorylated on Tyr^{783} for activation (Kim et al., 1991).

PDGFβR has been shown to stimulate the differentiation of monocytes in response to PDGF-BB and this effect was diminished by the mutation of Tyr^{1021} to phenylalanine residue, suggesting that the PLCγ pathway is essential for the differentiation (Heldin et al., 1998). PLCγ is also involved in the activation of the Na^+/H^+ exchanger (NHE) in presence of Ca^{2+} and PKCs (Y. H. Ma et al., 1994). It has been demonstrated the importance of PLCγ in several physiological functions such as cytoskeletal reorganization, activation of ion channels and involved in PDGF-dependent cell growth and motility (Hansen et al., 1996, Ji et al., 2015, Kundra et al., 1994, Y. H. Ma et al., 1994). Additionally, PLCγ has been indicated to play a crucial role in differentiation of hematopoietic stem cells by regulating the activity of histone variant macroH2A2 (mH2A2) (Schnoder et al., 2015).

PLCγ is involved in the activation of phosphatidylcholine-specific phospholipase D (PLD) leading to generation of diacylglycerol (DAG) (Jones and Carpenter, 1993, Y. H. Lee et al., 1994, Yeo et al., 1994). DAG is essential for the activation of PKCs in

response to PDGF-BB (Plevin et al., 1991). Researchers have been reported that PKCδ can be activated by association to the diacylglycerol and phorbol ester (Castagna et al., 1982, Michell, 1975). Nevertheless, PKCδ can be phosphorylated on different residues and this leads to either activation or inactivation of catalytic activity of the enzyme (Okhrimenko et al., 2005, Zrachia et al., 2002). Src family kinases, c-Abl and Ras have shown to phosphorylate tyrosine sites and increase PKCδ activity in response to PDGF, EGF, and PMA. PKCδ promotes cell cycle arrest by downregulates cyclin D1 and cyclin E (Ashton et al., 1999, Fukumoto et al., 1997), suggesting that PKCδ decreases cell proliferation.

PKCδ has been reported to play an important role in regulation of insulin signalling in skeletal muscle (M. Li et al., 2015c). In addition, PKCδ can induces apoptosis by regulate the activity of TA isoforms of p63 (TAp63) and this occurs by phosphorylation of TAp63 at Thr^{157} (D. Li et al., 2015a). Recently, researchers described that knockout of PKCδ in mice caused a significant defect in fertility and embryonic development suggesting a fundamental role for PKCδ in regulation of reproductive system (W. Ma et al., 2015b). PKCδ can be phosphorylated and activated by c-Abl (Lu et al., 2007). c-Abl phosphorylates Tyr^{311} on PKCδ upon H_2O_2 induction leading to apoptosis (Lu et al., 2007).

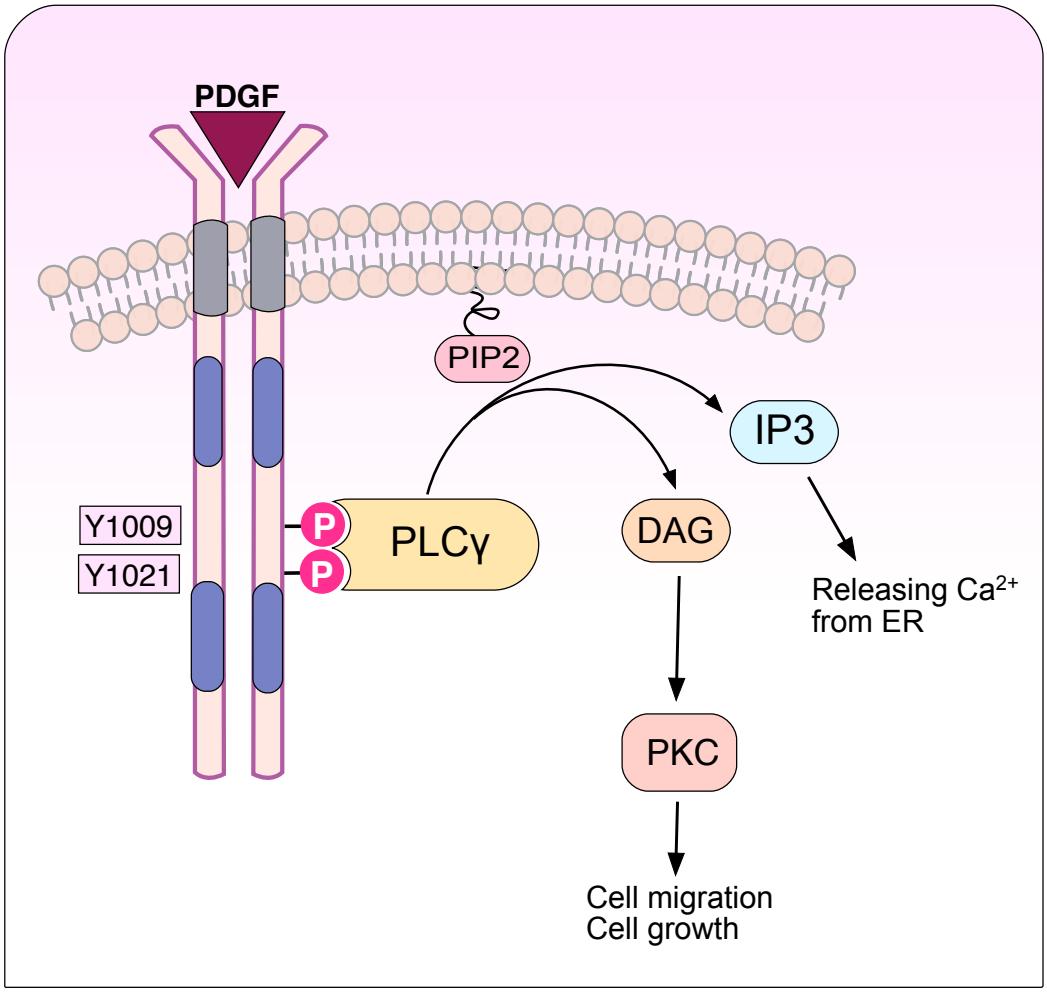

Figure 1.4. Schematic representation of the PLC-γ pathway. PLCγ binds to the phosphorylated Tyr1009 and Tyr1021 on PDGFβR. PLCγ produces two molecules by using $PI(4,5)P_2$ as a substrate; inositol (1,4,5) trisphosphate which increases the concentration of Ca^{2+}; and diacylglycerol that leads to protein kinase C activation.

1.2.3. Ras pathway

Grb2 is an adaptor molecule with one SH2 domain and two SH3 domains and has been shown to bind directly to the autophosphorylated Tyr^{716} on the PDGFβR (Arvidsson et al., 1994). Grb2 forms a complex with Sos1 in order to activate Ras by converting Ras from inactive GDP-bound form to its active GTP-bound form (**Figure 1.5**) (Schlessinger, 1993). The activation of Ras in response to PDGF stimulation leading to initiate a cascade of signalling transduction whereby Ras-GTP activates Raf-1 by binding with regulatory N-terminal part of Raf-1 (Nanberg and Westermark, 1993, Satoh et al., 1993). On the other hand, GAP can bind to the phosphorylated Tyr^{771} of the PDGFβR (Fantl et al., 1992, Kashishian et al., 1992, van der Geer et al., 1997) to converts Ras from active GTP-bound form to its inactive GDP-bound form in order to deactivates Ras signalling. Ras activation occurred either via direct binding of Grb/Sos1 to Tyr^{716} on the receptor or indirect binding of the complex Grb/Sos1 to the phosphorylated Shc (Heldin et al., 1998). Moreover, several studies have revealed that Ras act as anti-apoptotic factor by mediating PI3K activation (Kauffmann-Zeh et al., 1997).

Raf-1 phosphorylates and activates Mek which then phosphorylates and activates MAP kinases Erk1 and Erk2. Erk1/2 then translocated into the nucleus to regulate many transcription factors (Nanberg and Westermark, 1993, Satoh et al., 1993). Erk5 can also be activated via Mek and Mekk2 in response to PDGF stimulation (Tsioumpekou et al., 2016). Because of the Ras and MAP kinase activation caused by PDGF, PDGFβR has been shown to play important role in cell growth.

Grb2 has also been shown to bind indirectly to the PDGFβR via adaptor protein Shc and tyrosine phosphatase SHP-2 (Benjamin and Jones, 1994, W. Li et al., 1994a,

Yokote et al., 1994). Upon stimulation with PDGF, these proteins bind to the PDGFβR and become phosphorylated on tyrosine sites present within the pYXNX motifs. This phosphorylation then leads to recruitment of Grb2 and Sos1 for the activation of Ras signalling pathway.

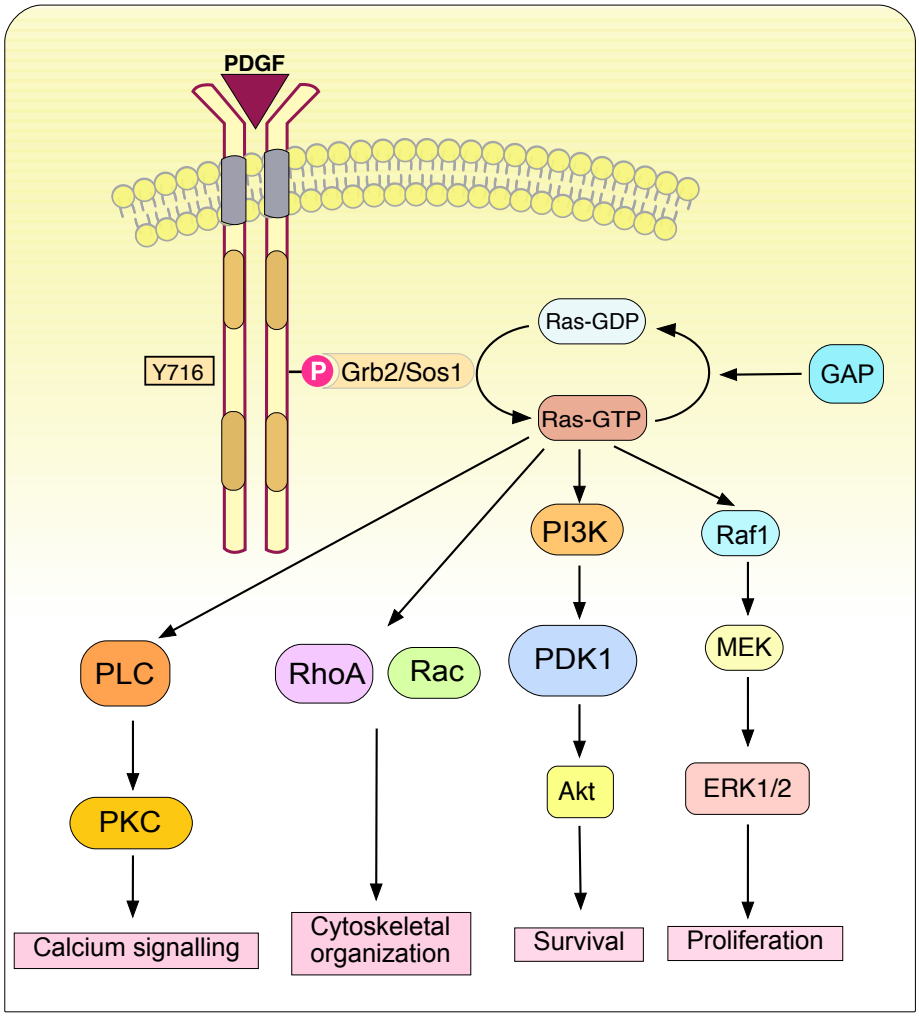

Figure 1.5. Schematic representation of the Ras pathway. Grb2/Sos1 complex binds to phosphorylated Tyr716 residue on PDGFβR in order to activate Ras by converting Ras from inactive GDP-bound form to its active GTP-bound form. This leads to initiating MAPK/ERK signalling pathway.

In addition, Ras can also activate the small GTP binding proteins Rho, Rac and Cdc42 demonstrating cross-talk between different signalling pathways (Khosravi-Far et al., 1995, Olson et al., 1995, Qiu et al., 1997, Qiu et al., 1995). The activation of MAP kinase cascade by Ras has been reported to play an important role in regulating cell growth, cell migration and differentiation (Fanger et al., 1997, Ruusala et al., 1998,

Seger and Krebs, 1995). Ras can also regulate focal adhesion via Src. Src is a member of a family of tyrosine kinases that has been shown to be activated after interacting with phosphorylated Tyr^{579} or Tyr^{581} on PDGFβR (Mori et al., 1993). Since the physiological response of the cell adhesions is mainly regulated by focal adhesion kinase (FAK), the association of ECM with integrin leads to autophosphorylation of FAK at Tyr^{397} to facilitate interaction with Src (Parsons, 2003). This interaction in turn results into another round of phosphorylation of FAK at Tyr^{407}, which results in phosphorylation of paxillin at Tyr^{118} (Bellis et al., 1995, Schaller and Parsons, 1995). Signalling initiated by FAK can then lead to the activation of several downstream proteins (Abbi and Guan, 2002, Schlaepfer et al., 1999).

Moreover, Ras can act as anti-apoptotic factor through the activation of Akt via PI3K and NF-κB (Downward, 1998, Kauffmann-Zeh et al., 1997). Ras can also signal to the JNK pathway via Tripartite Motif-containing 7 (Trim7) to regulate transcription and tumor progression (Chakraborty et al., 2015). Ras pathway has been found hyperactivated in cancers. For instance, Ras has been found upregulated in acute myeloid leukaemia (AML) suggesting Ras as one of the most enriched pathway responsible to initiates leukemogenesis (Z. Zhao et al., 2015b). In addition, researchers have been described that 32% of lung adenocarcinoma is driven by Ras (McCormick, 2015). Furthermore, 78% of patients with neuroblastoma showed upregulation of Ras pathway (Eleveld et al., 2015).

1.2.4. JNK pathway

C-Jun N-terminal kinase (JNK) is a serine/threonine kinase which is activated by a broad range of external stimuli including PDGF, transforming growth factor-β, and environmental stress (Kyosseva, 2004). There are three genes that encode for JNK; *Jnk1, Jnk2*, and *Jnk3* (Davis, 2000). Stimulation by PDGF leads to activation of JNK through Rac GTPase signalling (**Figure 1.6**) (Amagasaki et al., 2006, Bagrodia et al., 1995, Kyriakis, 1999). The JNK signalling pathway is involved in many cellular functions including inflammation, apoptosis and differentiation (Barr and Bogoyevitch, 2001, Y. T. Ip and Davis, 1998). For example, the inhibition of JNK activity significantly reduced inflammation in atherosclerosis-induced mice demonstrating a key role for JNK signalling pathway in preventing inflammation in adipose tissues (Kwok et al., 2015). JNK is also involved in regulating of migration and enhance chemotaxis in response to PDGF-BB stimulation (Amagasaki et al., 2006).

MKK4 and MKK7 activate JNK by the phosphorylation of tyrosine and threonine residues (Lawler et al., 1998). MKK4 phosphorylates Tyr^{185} whereas MKK7 phosphorylates Thr^{183} in response to cellular stress for full activation (Davis, 2000, Lawler et al., 1998). Genetic studies demonstrated that knockout of either *MKK4* or *MKK7* genes prevent the activation of JNK upon stimuli (Tournier et al., 2001). Upon activation, JNK can phosphorylate its downstream effector c-Jun on two sites Ser^{63} and Ser^{73}, within the activation loop leading to a transcriptional affect (Weston and Davis, 2002). Mutation of these residues to Alanine leads to disruption of the activation of c-Jun (Behrens et al., 2000, Behrens et al., 1999).

It has also been reported that JNK signalling is regulated by scaffold proteins such as filamin, β-arrestin and CrkII. For instance, JNK can interact with CrkII in order to be activated by Rac1 (Girardin and Yaniv, 2001). In addition, filamin can associate with MKK4 and MKK7 to contribute to the activation of JNK signalling (Marti et al., 1997). JNK can phosphorylate and activate the oncoprotein 18 (stathmin1) suggesting a key role of JNK in regulating microtubules (Ng et al., 2010).

Dual specificity phosphatases (DSP), a subfamily of PTPs have been shown to inactivate JNK signalling pathway by dephosphorylation of Tyr and Thr residues such as MAP kinase phosphatase 7 (MKP7) (Camps et al., 2000, Keyse, 2000, N. K. Tonks and Neel, 2001). MKP7 has been shown to suppress the activity of JNK (Masuda et al., 2001). In addition, protein phosphatase type 2C α (PP2Cα) has also been reported to dephosphorylates MAPKKs (MKK6 and MKK4) leading to the inhibition of JNK signalling (Takekawa et al., 1998). However, one of DSPs, Jnk Stimulatory Phosphatase 1 (JSP-1) activates JNK by enhancing the activity of JNK upstream protein, MKK4 (Shen et al., 2001).

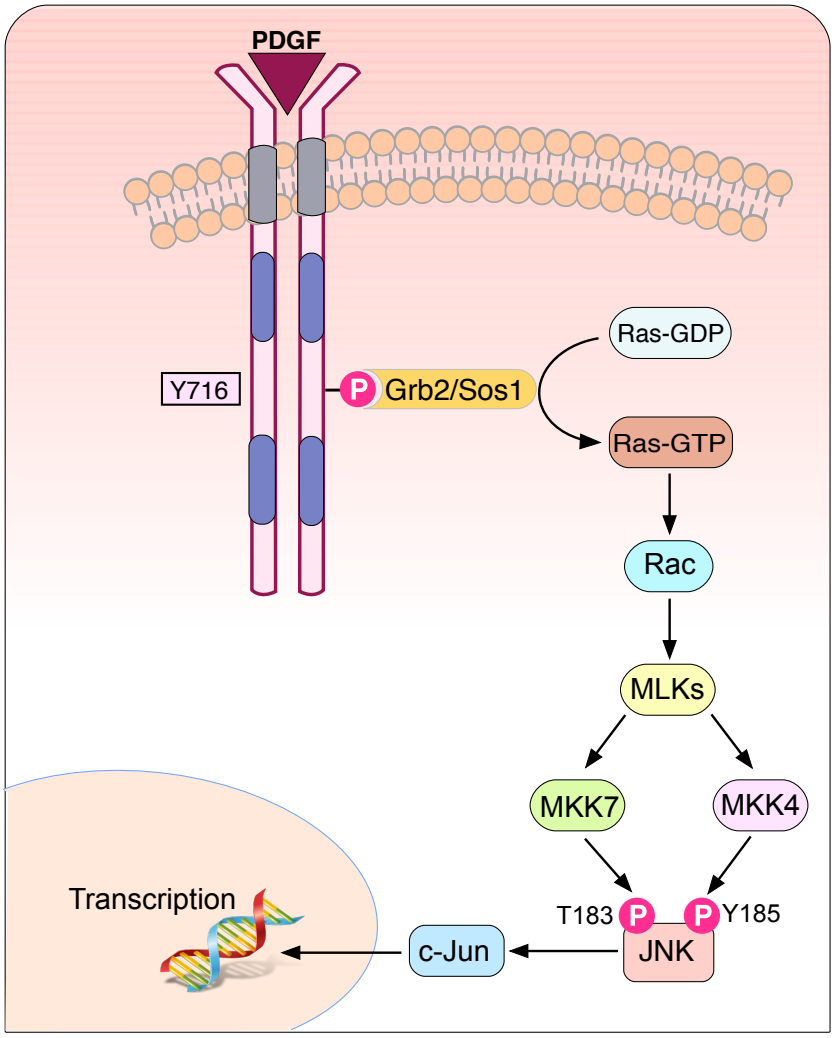

Figure 1.6. Schematic representation of the JNK pathway. Grb2/Sos1 complex binds to phosphorylated Tyr716 residue on PDGFβR in order to activate Ras by converting Ras from inactive GDP-bound form to its active GTP-bound form. This leads to initiating signal transduction cascade including Rac, MLKs, MKK4 and MKK7. MKK4 phosphorylates Tyr185 whereas MKK7 phosphorylates Thr183 JNK full activation. Upon activation, JNK can phosphorylate its downstream effector such as c-Jun leading to a transcriptional affect.

1.3. Protein Tyrosine Phosphatases

1.3.1. Introduction

Tyrosine phosphorylation is a fundamental mechanism in signal transduction for enzyme activity and play a crucial role for regulation a wide range of physiological processes in health and disease (T. Hunter, 1987, Mustelin et al., 2002). There is a large volume of published studies describing the role of protein tyrosine phosphatases (PTPs) in regulating cellular functions including cell migration, proliferation and differentiation. Protein tyrosine phosphatases are classified into four major classes (**Figure 1.7**); class I PTPs which include classical PTPs and dual specificity phosphatases (DSPs) (Alonso et al., 2004).

Classical PTPs are strictly tyrosine specific (Andersen et al., 2004) whereas DSPs are more diverse group and able to dephosphorylate serine, threonine, inositol phospholipids or mRNA (Alonso et al., 2004, Ostman et al., 2006). Class II PTP contains a single PTP called low molecular weight PTP (LM-PTP) which is tyrosine specific (X. D. Su et al., 1994, N. K. Tonks, 2006a). Class III PTPs which include CDC25 family members (CDC25A, CDC25B, CDC25C) PTPs and are tyrosine/threonine-specific phosphatases mainly involved in regulation of cell cycle (Fauman et al., 1998, McCain et al., 2002). Class IV PTPs include eyes absent homologue (EYA) proteins and act as serine/tyrosine affinity (Julien et al., 2011, Rayapureddi et al., 2003, Tootle et al., 2003).

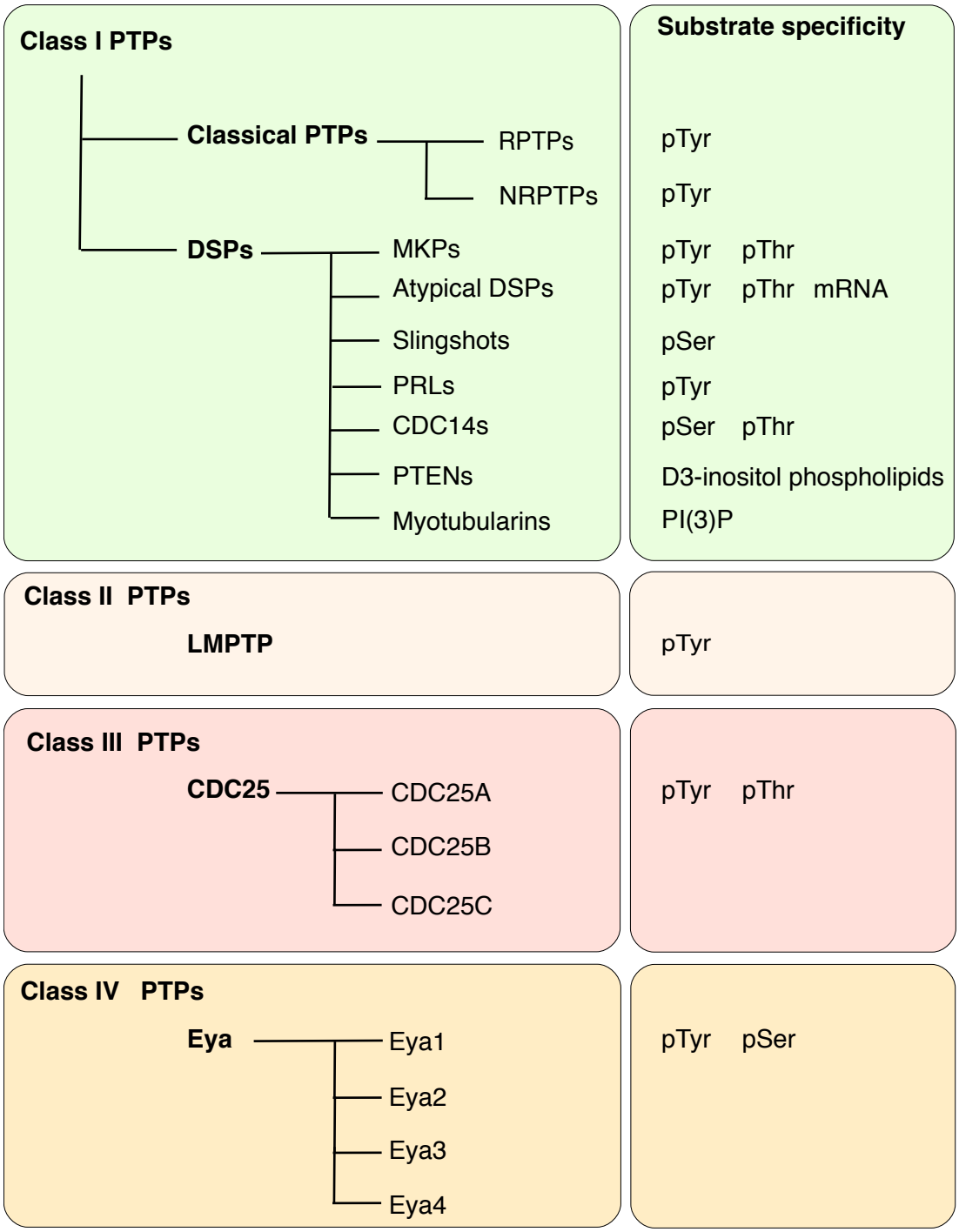

Figure 1.7. The classification of PTPs with their substrate specificity. Adapted from (Alonso et al., 2004).

Classical PTPs are divided into two subfamilies, receptor-like PTPs (RPTPs), including CD45, LAR, PTPα and non-receptor PTPs (NPTPs) such as SHP-1, TC-PTP (Andersen et al., 2001, N. K. Tonks, 2006a, N. K. Tonks and Neel, 2001). Structurally, RPTPs are composed of extracellular domains that have immunoglobulin and fibronectin type III domains, which are involved in cell-cell interaction. In addition, most RPTPs have intracellular regions with phosphatase activity domains (D1) and regulatory domains (D2) (N. K. Tonks, 2006a).

The majority of the PTPs, in particular the classical PTPs use the same mechanism to regulate the catalytic activity. PTPs contain a nucleophilic cysteinyl residue, which has been shown to recognize the phosphate group of the target substrates for dephosphorylation (Shi, 2009). PTPs either receptor-like or non-receptor have been shown to play a key role in regulating a wide range of signal transduction proteins (Gurzov et al., 2015).

1.3.2. LAR Protein Tyrosine Phosphatase

1.3.2.1. Structure

Leukocyte common antigen-related (LAR), also known as receptor type protein tyrosine phosphatase F (PTPRF), is a receptor-like protein tyrosine phosphatase (RPTP). LAR is composed of an extracellular region containing three N-terminal immunoglobulin-like domains and eight fibronectin type III repeats (FnIII) (Serra-Pages et al., 1994). The intracellular part of LAR is composed of a proximal phosphatase domain called D1 and a distal phosphatase domain called D2 (**Figure 1.8**) (Nam et al., 1999, Streuli et al., 1988). D1 has a phosphatase activity while D2 shows a regulatory function and gives the protein substrate specificity (Streuli et al., 1990, Tsujikawa et al., 2001).

Crystal structure analysis showed that D1 and D2 are very similar differing by only two amino acids in D2 domain (Leu-1644 and Glu-1779) (Nam et al., 1999). Interestingly, the substitution of Leu-1644 to Tyr and Glu-1779 to Asp in D2 convert the domain from regulatory to fully active as D1 (Nam et al., 1999). On the other hand, FnIII domains are composed of eight fibronectins type III repeats which are approximately 90 amino acids long and have suggested to be involved in cell adhesion (Beltran and Bixby, 2003). It has been shown that laminin-nidogen complex bind to FnIII domain 5 on LAR and act as a ligand (O'Grady et al., 1998). However, the physiological function for this binding has not been studied.

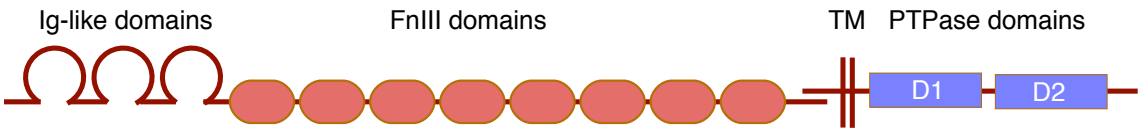

Figure 1.8. Schematic structure of LAR. Ig, immunoglobulin-like domain; FNIII, fibronectin type III repeat domain; TM, transmembrane; D1, phosphatase domain 1; D2, phosphatase domain 2.

1.3.2.2. Mechanism

Most PTPs are cysteine-based and share a common motif sequence (H/V)CX_5R(S/T) to form a phosphate binding loop (P-loop) where the catalytic reaction occurs (Cirri et al., 1993, Kolmodin and Aqvist, 2001). The hydrolysis reaction utilises two steps (**Figure 1.9**). First, nucleophilic attack by cysteine in the P-loop on the phosphorus atom of the substrate in order to break the phosphorus–oxygen bond. Simultaneously, the catalytic aspartate in the WPD (Trp-Pro-Asp) loop works as a general acid to donate a proton to the oxygen leaving group, resulting the formation of phospho-cysteine and releasing of dephosphorylated substrate (K. L. Guan and Dixon, 1991, Kolmodin and Aqvist, 2001, Pannifer et al., 1998). The second step is initiated by hydrolysis of the phospho-cysteine intermediate with the assistance of the aspartate in the WPD loop yielding free phosphate (Brandao et al., 2012, Kolmodin and Aqvist, 2001, S. Zhao et al., 2015a).

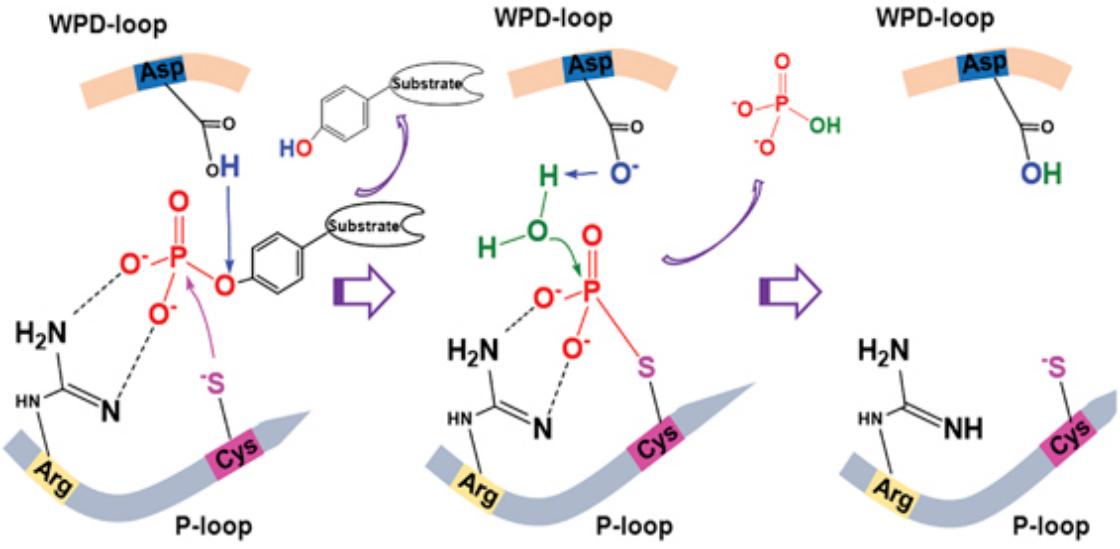

Figure 1.9. Schematic diagram representing the mechanism of action of PTPs. See LAR mechanism 2.2.2 section for more details (S. Zhao et al., 2015a).

1.3.2.3. Function

A number of studies have found that LAR plays an important role in regulating cell signalling and contributing to cellular functions such as neuronal growth and neuronal regeneration (Sethi et al., 2010). On the other hand, several phenotypic disorders have been recognized in LAR-deficient mice including defective development of mammary gland, neuron degeneration, axon guidance defects, reduction in the rate of hepatic glucose production, diabetes and cancer (Andersen et al., 2001, Bera et al., 2014, Du et al., 2013, Soulieres et al., 2015, Van Lieshout et al., 2001, Whitmore et al., 2012, Zabolotny et al., 2001). Mouse embryos lacking LAR (Ptprf) and RPTPσ (Ptprs) showed defects in embryos development such as small lower jaw, cleft palate and microglossia (small and deep tongue phenotype). This was due to the hyperactivation of Smad signalling and the downregulation of Wnt signalling in embryos and cells lacking LAR family phosphatase genes (Stewart et al., 2013).

Recently, quantitative proteomics study revealed that LAR is significantly expressed in lung cancer demonstrating LAR as potential biomarker for nonsmall cell lung cancer (P. J. Liu et al., 2015). Tumor promoter TPA induces cleavage of LAR into the extracellular subunit (E-subunit) and phosphatase subunit (P-subunit) in response to EGF (Ruhe et al., 2006). TPA also promotes the degradation of P-subunit and this proteolytic processes could be mediated by ADAM-17 (a disintegrin and metalloproteinase) (Ruhe et al., 2006). MicroRNA miR-24 increases EGFR phosphorylation and activation in breast carcinoma through the suppression of LAR expression resulting in promotion of tumor growth, cell invasion and metastasis (Du et al., 2013). Many studies in *Drosophila* demonstrated that Abl as substrate for *Drosophila* LAR (DLAR) and mediate several cellular functions (M. J. Chagnon et

al., 2004b). Enabled protein (Ena), a substrate of Abl, is active in its unphosphorylated state. Abl phosphorylates and inactivates Ena while LAR dephosphorylates Ena for activation demonstrating that Ena connecting Abl and LAR to control and maintain axon guidance (Sirvent et al., 2008, Wills et al., 1999).

In contrast to receptor tyrosine kinases which are activated by dimerization, receptor-like phosphatases (RPTPs) are negatively regulated via ligand-induced dimerization such as RPTPα, RPTPβ and CD45 (Majeti et al., 1998, N. K. Tonks, 2006a). This occurs due to interaction of one D1 domain with the active site of the adjacent D1 domain in the dimer leading to deactivation of both proteins (Bilwes et al., 1996, Blanchetot and den Hertog, 2000, Majeti et al., 1998, N. K. Tonks, 2006a). Homodimerization can also occur in the majority of RPTPs via the transmembrane domains (Chin et al., 2005). Later on researchers indicated that the induction of H_2O_2 can causes conformational changes in D1 and D2 of the cytoplasmic domains of LAR leading to promote the dimerization (Groen et al., 2008).

Data from several sources have identified a number of ligands, which bind to extracellular domains of LAR. For instance, laminin–nidogen complex has shown to bind to the fibronectin type III position 5 (O'Grady et al., 1998). Fox and Zinn described the heparan sulfate proteoglycan syndecan (Sdc) as a new ligand for *Drosophila* LAR (DLAR) and it has shown positively regulates DLAR signalling (Fox and Zinn, 2005). Loss of Sdc leads to impaired growth and axon guidance suggesting that Sdc positively regulates LAR *in vivo*. LARFN5C has also been identified as a ligand that associates with LAR to initiate cellular signalling pathways in neurons (T. Yang et al., 2003).

In 1995, Serra-Pages and co-workers demonstrated that LAR can binds to LAR-interacting protein 1 which now called Liprin 1 and localise to focal adhesion (Serra-Pages et al., 1995). Later on, researchers have found that Trio, which is a multidomain protein with two GEF domains and one kinase domain, binds with LAR cytoplasmic domains proposing a signalling pathway links LAR and Trio in regulation of cell adhesion and migration (Debant et al., 1996). In addition, LAR has been found associated with cadherin-catenin complex and mediates the dephosphorylation of tyrosine residues on β-catenin (Kypta et al., 1996). Up to now, however, the role of LAR in cell adhesion and mechanism of action remain unclear.

1.3.2.4. Role of LAR in Regulation of Receptor Tyrosine Kinases

Many protein tyrosine kinases have been identified as substrates for LAR such as the insulin receptor, EGF receptor and HGF receptor (Ahmad and Goldstein, 1997, Kulas et al., 1996). LAR associates with the insulin receptor and decreases the autophosphorylation of the receptor suggesting LAR as a pharmacological target for the treatment of diabetes and obesity (M. J. Chagnon et al., 2004b, Goldstein, 2002, Kulas et al., 1995, Tsujikawa et al., 2001). A method called "PTPs substrate trapping mutants" have been commonly used to identified physiological substrates. These mutations and name 'Substrate-trap' were firstly discovered by Tonks lab (Flint et al., 1997, Jia et al., 1995, Sun et al., 1993). To this end, when the PTP binds to phosphorylated tyrosine residue on the substrate for dephosphorylation, substrate can

trap in the catalytic pocket of the protein tyrosine phosphatase (Blanchetot et al., 2005, Flint et al., 1997).

LAR substrate trapping mutants can be generated by substitution of cysteine 1548 in the active site with serine (LAR C/S) or by substitution of aspartic acid 1516 with alanine (LAR D/A) (W. J. Wang et al., 2007). By using this approach, a number of substrates have been identified for LAR. For example, LAR can interact and dephosphorylate insulin receptor suggesting that insulin receptor is a substrate for LAR (Tsujikawa et al., 2001). Ephrin type-A receptor 2 (EphA2) has also been shown as LAR substrate by using LAR-substrate trapping mutants. LAR binds directly to EphA2 and dephosphorylates Tyr^{930} on the receptor where Nck1 is bound leading to reducing EphA2 mediated cell migration (H. Lee and Bennett, 2013).

Depletion of LAR in rat hepatoma cell line resulted in increases the autophosphorylation level of hepatocyte growth factor (HGF) demonstrating that LAR negatively regulates HGF signalling (Kulas et al., 1996). LAR can also bind with and deactivates the proto-oncogenic RET receptor by reducing the tyrosine autophosphorylation level (Qiao et al., 2001). In contrast, the autophosphorylation of FGF and EGF not affected by LAR, instead, LAR can mediate the dephosphorylation and deactivation of adaptor proteins downstream FGFR and EGFR such as Fibroblast growth factor receptor substrate 2 (FRS2) and Breast cancer anti-estrogen resistance protein 1 ($p130^{CAS}$) (X. Wang et al., 2000). Interestingly, LAR can inhibit FGF-induced MAPK signalling, while the activation of MAPK signalling induced by EGF is not affected indicating the specificity of LAR function in response to different growth factors (X. Wang et al., 2000). LAR can enhance PDGFβR activation and

signalling by supresses c-Abl activity (Zheng et al., 2011). However, the mechanism behind this inhibition is not understood.

1.4. Quantitative phosphoproteomics in studying cell signalling

Phosphorylation is one of the most common post-translational modifications that occurs in the cell and affects protein activity which in turn control the majority of cellular processes including cell proliferation, growth, survival and differentiation (T. Pawson, 2004). Protein phosphorylation also regulates protein-protein interactions and initiates signal transduction by add or remove phosphate groups, therefore switching "on" or "off" protein activity (T. Pawson and Scott, 1997). This function is modulated by kinases and phosphatases (Bauman and Scott, 2002). Over 400,000 phosphorylation site on >50,000 proteins for all phosphorylation types (Ser, Thr and Tyr) now list on the Phosphosite database (http://www.phosphosite.org) and this number is increasing because each phosphoproteomic large scale study identifies more novel phosphosites (Hornbeck et al., 2012).

Many of the biochemical methods have been widely used to study protein activity or protein-protein interaction on an individual basis. However, this kind of approaches can be arduous and time-consuming especially if the goal is to identified novel regulators or therapeutic targets. Therefore, mass spectrometry-based quantitative phosphoproteomics technique can be used for high-throughput experiments in order to monitoring the differential phosphorylation events and to study signalling pathways within the cell (Gygi et al., 1999, Harsha et al., 2008, Humphrey et al., 2015, Rigbolt

et al., 2011a, Rikova et al., 2007). The biochemical methods can then be used for the protein of interest for the validation.

One of the most commonly use technique for the quantitative phosphoproteomics is Stable isotope labelling by amino acids in cell culture (SILAC) (S. E. Ong et al., 2002). To this end, cells can be grown in culture medium that contains normal amino acid or medium where the normal amino acids, arginine and lysine are replaced with medium ($^{13}C_6$ L-Arginine; 4,4,5,5-D4 L-Lysine) or heavy ($^{13}C_6$ $^{15}N_4$ L-Arginine; $^{13}C_6$ $^{15}N_2$ L-Lysine) amino acid (Mann, 2006). To ensure that all amino acids are incorporated into the proteome, cells need to be grown in SILAC medium at least five doubling times (S. E. Ong and Mann, 2006). The incorporation of these amino acids into the proteome leading to known mass shift in the peptide compared with peptide that has light amino acid (Shao-En Ong and Mann, 2007). SILAC-based proteomics or phosphoproteomics have been widely used in cell biology field and several interesting findings have been obtained by using this approach (Humphrey et al., 2015, Humphrey et al., 2013, Leroy et al., 2009, Oppermann et al., 2009, Wilhelm et al., 2014a).

In this thesis, global SILAC-based phosphoproteomics was used to gain insight into the signal transduction pathways regulated by platelet-derived growth factor (PDGF) and Leukocyte common antigen-related protein (LAR).

Thesis Aims

1. To further investigate the tyrosine phosphorylation of PDGFβR in two cell lines WT and LARΔP MEFs in response to PDGF-BB stimulation.

2. Global SILAC-based mass spectrometry phosphoproteomics study to analyse differential phosphorylation in WT and LARΔP MEFs.

3. To test if the deletion of LAR phosphatase domains affects the PDGFβR downstream signalling including Akt, MAPK, JNK and mTOR signalling pathways.

4. To test if deletion of LAR phosphatase domains affects the cell adhesion to ECM.

5. To investigate the regulation of focal adhesions by LAR.

6. Global SILAC-based mass spectrometry phosphoproteomics study to identify novel PDGF-regulated proteins.

7. To investigate the regulation of PDGFβR downstream proteins by GRK2.

CHAPTER 2

MATERIALS AND METHODS

2.1. Antibodies, reagents and plasmid constructs

The primary antibodies used in this project are listed in **Table 2.1**. Secondary goat anti-mouse and goat anti-rabbit IgG IRDye conjugated antibodies were purchased from Licor Biosciences. Rabbit polyclonal antibody against the C-terminus of the PDGF β-receptor was a kind gift from Prof. Carl-Henrik Heldin (Ludwig Cancer Research, Uppsala, Sweden). Rabbit polyclonal antibody against Alix has been previously described (Karlsson et al., 2006, Lennartsson et al., 2006). Phalloidin Alexa 594 was from Invitrogen. Recombinant human PDGF-BB was purchased from Cell Signalling Technologies. The inhibitors, AG957 (c-Abl inhibitor) was purchased from Sigma-Aldrich; RO-3306 (CDK1 inhibitor) was from Millipore; InSolution Akt Inhibitor VIII, Okadaic Acid and SP600125 (JNK inhibitor) were purchased from Calbiochem. A plasmid expressing PDGFβR was a kind gift from Dr Neil Freedman (Duke University School of Medicine, Durham, USA). Plasmids expressing NHERF2 (plasmid #47801), cdc2-AF (plasmid #39872), cdc2 (Plasmid #1888), c-Abl (plasmid #52684) were purchased from Addgene. Full-length FLAG-tagged wild-type LAR (WT) and substrate-trapping mutants, CS (Cys1548 in the D1 domain was replaced with Ser1548) and DA (Asp1516 in the D1 domain was replaced by Ala1516), were kindly provided by Ruey-Hwa Chen (National Taiwan University, Taipei, Taiwan).

Table 2.1. Primary antibodies used in the study. IF: immunofluorescence, WB: Western blotting, IP: immunoprecipitation.

No.	Antibody	Supplier	Dilution
1	LAR	NeuroMab	10 µg/mL (WB)
2	c-Abl	Cell Signaling Technology	1:1000 (WB)-1:200 (IP)
3	NHERF-1	Cell Signaling Technology	1:1000 (WB)-1:200 (IP)
4	NHERF-2	Santa Cruz Biotechnology	1:2000 (WB)- 2µg/ml (IP)
5	SAPK/JNK	Cell Signaling Technology	1:1000 (WB)
6	pSAPK/JNK (Thr183/Tyr185)	Cell Signaling Technology	1:1000 (WB)-1:200 (IP)
7	Src	Cell Signaling Technology	1:1000 (WB)-1:100 (IP) (IF)
8	pSrc (Tyr416)	Cell Signaling Technology	1:1000 (WB)
9	p38 MAPK	Cell Signaling Technology	1:1000 (WB)-1:100 (IP)
10	pp38 MAPK (Thr180/Tyr182)	Cell Signaling Technology	1:1000 (WB)-1:50 (IP)
11	GRK2	Santa Cruz Biotechnology	1:1000 (WB)-2µg/500µg of WCL (IP)
12	GRK5	Santa Cruz Biotechnology	1:1000 (WB)-2µg/500µg of WCL (IP)
13	β-Actin	Sigma-Aldrich	0.5µg/ml (WB)
14	c-Jun	Cell Signaling Technology	1:1000 (WB)-1:50 (IP)
15	pc-Jun (Ser63)	Cell Signaling Technology	1:1000 (WB)-1:50 (IP)
16	MKK7	Cell Signaling Technology	1:1000 (WB)-1:50 (IP)
17	pMKK7 (Ser271/Thr275)	Cell Signaling Technology	1:1000 (WB)
18	PKCδ	Cell Signaling Technology	1:1000 (WB)-1:25 (IP)
19	SEK/MKK4	Cell Signaling Technology	1:1000 (WB)-1:50 (IP)
20	pSEK/MKK4 (Thr261)	Cell Signaling Technology	1:1000 (WB)
21	Akt	Cell Signaling Technology	1:1000 (WB)
22	pAkt (Thr308)	Cell Signaling Technology	1:1000 (WB)-1:100 (IP)
24	FAK	BD Biosciences	1:1000 (WB)
25	pFAK (Tyr407)	Santa Cruz Biotechnology	1:1000 (WB)-2µg/500µg of WCL (IP)
26	pFAK (Tyr397)	BD Biosciences	1:1000 (WB)-1:100 (IF)
27	Erk1/2	Santa Cruz Biotechnology	1:1000 (WB)-2µg/500µg of WCL (IP)
28	pErk (Thr202/Tyr204)	Santa Cruz Biotechnology	1:1000 (WB)-1:50 (IP)
29	pATF-2 (Thr69/71)	Cell Signaling Technology	1:1000 (WB)
30	mTOR	Cell Signaling Technology	1:1000 (WB)
31	pmTOR (Ser2448)	Cell Signaling Technology	1:1000 (WB)
32	pp70 S6 Kinase (Thr389)	Cell Signaling Technology	1:1000 (WB)
33	Paxillin	Cell Signaling Technology	1:1000 (WB)
34	pPaxillin (Tyr118)	Cell Signaling Technology	1:1000 (WB)-1:50 (IF)
35	CDK1	Thermo Fisher	1-2 µg/ml (WB)-2 µg/ml (IP)-1:25 (IF)
36	pCDK1 (Thr161)	Cell Signaling Technology	1:1000 (WB)
37	cyclin B1	Santa Cruz Biotechnology	1:1000 (WB)-2µg/500µg of WCL (IP)
38	p-Tyr Antibody (PY99)	Santa Cruz Biotechnology	1:1000 (WB)-2µg/500µg of WCL (IP)
39	Rac1	BD Biosciences	1:1000 (WB)
40	RhoA	Santa Cruz Biotechnology	1:1000 (WB)-2µg/500µg of WCL (IP)
41	FLAG M2	Sigma-Aldrich	10 mg/mL (WB)-20 mg/mL (IF)
42	eps8	Santa Cruz Biotechnology	1:1000 (WB)-2µg/500µg of WCL (IP)
43	CRMP2	Cell Signaling Technology	1:1000 (WB)-1:50 (IP)
44	pCRMP2 (Thr514)	Cell Signaling Technology	1:1000 (WB)
45	pThr antibody	Cell Signaling Technology	1:1000 (WB)-1:50 (IP)

2.2. Cell Culture

Mouse embryonic fibroblasts (MEFs) from mice where the LAR phosphatase domains had been deleted (LARΔP) and wild-type (WT). MEFs are isolated from embryos (single clone) and allowed to grow in culture medium as described in (Schaapveld et al., 1997). MEFs and Human embryonic kidney (HEK 293T) were grown in DMEM supplemented with 10% foetal bovine serum (FBS), 100 U/mL Penicillin, 10 mg/mL Streptomycin, and 250 μg/mL Amphotericin B.

2.3. Cell stimulation, cell lysis, immunoprecipitation and immunoblotting

The cells were counted and seeded into the indicated plate for each experiment. Cells were then grown in DMEM medium with FBS (complete medium) to approximately 80% confluency and starved for 16 hours in DMEM medium without FBS (starvation medium). Where applicable, cells were incubated with 10 μM c-Abl inhibitor (AG957) for 2 hours, or 10 μM CDK1 inhibitor (RO-3306) for 1 hour, or 10 μM InSolution Akt Inhibitor VIII overnight, or 20 μM JNK inhibitor (SP600125) for 1 hour, or 0.01 μM, 1 μM, 1.5 μM okadaic acid for 30 min.

Where appropriate, the starvation media was removed and the cells were stimulated with 20 ng/mL PDGF-BB for the indicated times at 37°C. Cells were then placed on ice, rinsed twice with cold phosphate buffered saline PBS (135 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 8 mM K_2HPO_4, pH 7.4), and then lysed in lysis buffer (20 mM Tris–HCl, pH 7.5, 0.5% Triton X-100, 0.5% deoxycholate, 150 mM NaCl, 10 mM EDTA, 0.5 mM Na_3VO_4, and 1% Trasylol) for 15 minutes on ice. The lysed cells

were scraped down, collected by centrifugation (15000g/ 15 minutes), and the supernatant transferred to an eppendorf tube.

The protein concentration in each sample was determined using the Coomassie blue protein assay (Thermo Fisher Scientific) as described in the manufacturer's protocol. Immunoprecipitation of PDGFβR and the indicated proteins was carried out using Dynabeads protein G (Thermo Fisher Scientific) or Anti-Flag beads (Sigma) according to manufacturer's protocol. Equal volumes of 2X sample buffer (1.0M Tris-HCl pH 8.8, 0.5% Bromophenol blue, 43.5% glycerol, 10% SDS, 1.3% β-mercaptoethanol) was added to the beads or whole cell lysate (WCL), boiled at 95 C° for 5 minutes. Samples from immunoprecipitation or WCL were loaded into SDS-PAGE gel and then transferred to Nitrocellulose membranes using Trans-Blot Turbo Transfer System (BIO-RAD).

The membranes were blocked with 5% Bovine Serum Albumin BSA (Sigma Aldrich) at room temperature for one hour on the shaker. The membranes were then put into primary antibody solution (TBS-T: 1M Tris pH 7.5, 5M NaCl) containing 5% BSA and incubated at 4°C overnight on rotation. The membranes were then washed 3 times for 10 minutes with TBS-T and the secondary antibody solution in TBS-T was added at room temperature for 1 hour. The membranes were washed a further 3 times in TBS-T prior to visualisation using the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.4. Adhesion assay

96-well plates were coated with serially diluted fibronectin (10 μg/mL to 0.009 μg/mL) in starvation medium overnight in 4 ˚C. The next day, the plates were washed three times with PBS and coated with 0.1% BSA, followed by incubation for 1 hour at 37 C°. 50,000 cells were added to each well and incubated for 30 minutes at 37 C°. Plates were gently flicked off to discard cells that did not adhere on fibronectin and washed three times with PBS. 5 mg/mL of Thiazolyl Blue tetrazolium bromide (MTT) was added to each well and plates were incubated for 1 hour at 37 C° followed by addition of 100 μL of isopropanol. Plates were incubated for 15 minutes at room temperature with gentle shaking and the number of adherent cells were calculated by measuring absorbance at 570 nm.

2.5. Immunofluorescence

WT and LARΔP MEFs cells were seeded on coverslips and incubated at 37 C° overnight. The following day, cells were starved overnight. Where indicated, seeded cells were incubated with either 10 μM RO-3306 for one hour, 10 μM InSolution Akt Inhibitor VIII or DMSO overnight. Cells were stimulated with 20 ng/mL PDGF-BB for 15 minutes and incubated on ice for 5 minutes to terminate the stimulation. Cells were washed three times with PBS and fixed in 4 % paraformaldehyde for 10 minutes, washed with PBS three times and then permeabilized in ice cold 0.2 % Triton-X-100 for 3 minutes. Cells were washes three times with PBS and blocked using 5% BSA in PBS at 37 C° for 1 hour and cells washed once with PBS and incubated with primary antibodies and Phalloidin Alexa 594 for 1 hour. Cells were then washed with PBS and

incubated with appropriate secondary antibodies. Cells were visualized using confocal/ TIRF Nikon A1R with NIS-Elements Software and images were quantified using ImageJ 1.48.

2.6. Quantitative phosphoproteomics study

2.6.1. Cell culture

For Stable Isotope Labelling of amino acids in Cell culture (SILAC) labelling, WT and LARΔP cells were cultured in SILAC DMEM (Thermo Fisher Scientific) supplemented with either "light" isotopically normal L-Lysine (Cat# L8662) and L-Arginine (Cat# A8094) (R0K0) (Sigma), "medium" $^{13}C_6$ L-Arginine (Cat# CLM-2265-H) and 4,4,5,5-D4 L-Lysine (Cat# DLM-2640) (R6K4), or "heavy" $^{13}C_6$ $^{15}N_4$ L-Arginine (Cat# CNLM-539-H) and $^{13}C_6$ $^{15}N_2$ L-Lysine (Cat# CNLM-291) (R10K8) (Goss Scientific), with 0.5 mg/mL proline (Sigma, Cat# P5607), 0.1mg/mL streptomycin, 100 U/mL penicillin, 250 µg/mL amphotericin B and 10% v/v dialysed fetal bovine serum (Labtech International) (**Figure 2.1**).

2.6.2. Tryptic digestion

15 mg of light, medium and heavy lysates were pooled prior to trypsin digestion. Ammonium bicarbonate was added to give a final concentration of 50 mM. Proteins were then reduced with 8 mM Dithiothreitol (DTT) and incubated at 56 C° for 45 minutes. Samples were alkylated with 20 mM Iodoacetamide in 50 mM Ammonium bicarbonate and incubated in dark for 45 minutes and then digested with trypsin Gold (Promega) (1 μg of enzyme/ 100 μg of protein) at 37 C° overnight. Digested samples were acidified by addition of 0.5% Trifluoroacetic acid (TFA).

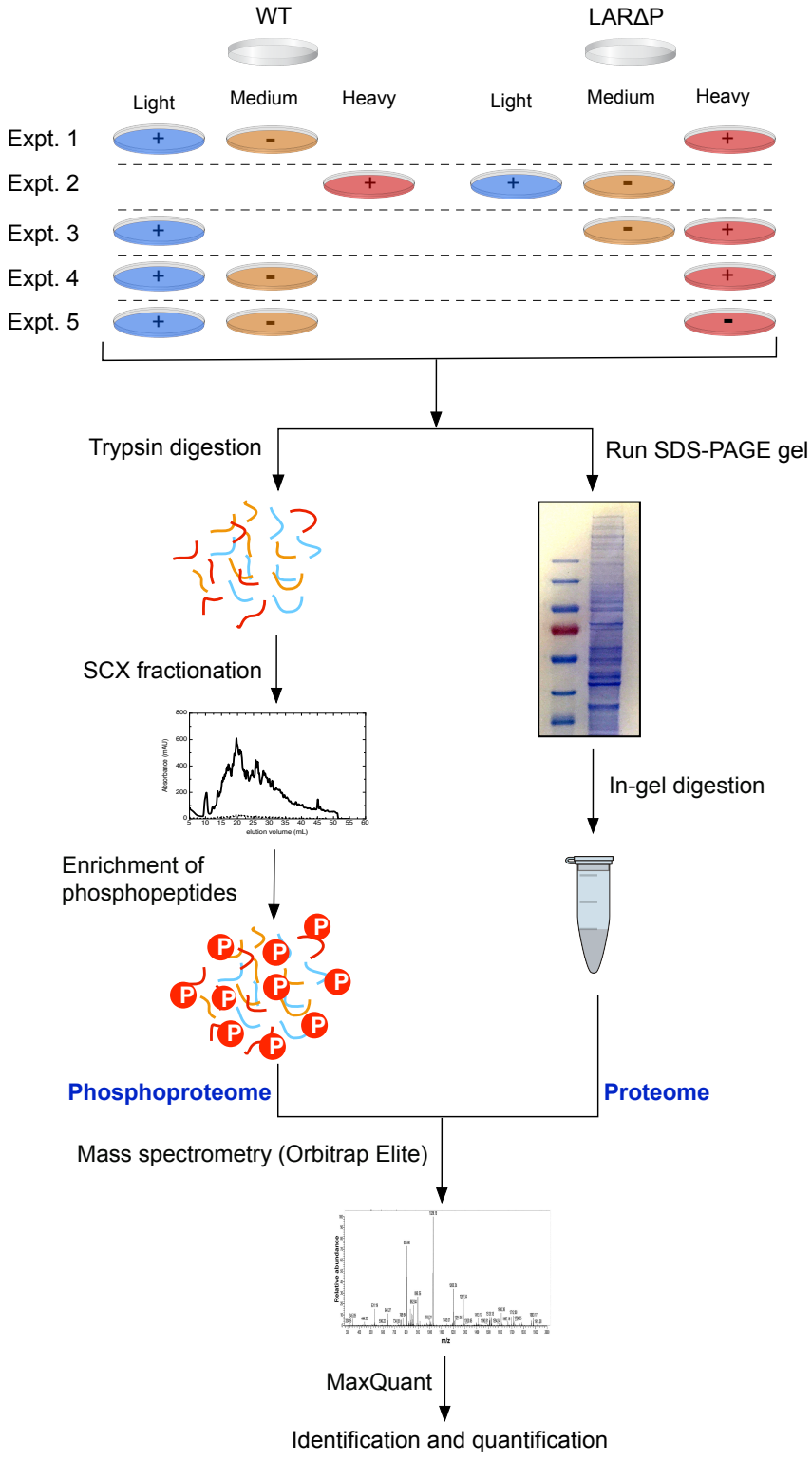

Figure 2.1. Schematic overview of experimental design, phosphopeptide purification, and proteome analysis. (+) with PDGF-BB, (-) without PDGF-BB.

2.6.3. Desalting using Sep-Pak

Peptides were desalted using SEP-PAK C18 Cartridges (Waters) according to manufacturer's instructions. Briefly, each cartridge was washed with 100% Acetonitrile (ACN), conditioned with 50% ACN, 0.5% acetic acid, and equilibrated in 0.1% TFA. The sample was loaded in 0.1% TFA. The cartridge was then washed with 0.5% acetic acid, and the sample eluted in 50% ACN, 0.5% acetic acid. The desalted samples were then dried using vacuum centrifugation.

2.6.4. Strong Cation Exchange (SCX) chromatography

Desalted and dried peptides were resuspended in 1 mL mobile phase A (10 mM KH_3PO_4, 20 % acetonitrile, pH 3) and loaded onto a SCX Large (100×4.6 mm polysulfoethyl, 5 μm particle size, 200 nm pore size, PolyLC) column. Peptides were separated using a gradient elution profile that started with 100% mobile phase A, increased from 0 to 50% mobile phase B (10 mM KH_3PO_4, 20% acetonitrile, 500 mM KCl, pH 3) over 60 min, increased to 100% mobile phase B over 10 min, and then returned to 100% A. The column flow rate was 1 mL/ minute.

2.6.5. Desalting and concentrating peptides using Macrotrap

Fractions collected from the SCX column were combined to give 20 fractions for each experiment. Samples were dried and desalted using a C8 Macrotrap cartridge with 50 μL bed volume (Michrom BioResources) according to manufacturer's protocol. Briefly, the cartridge was cleaned/ regenerated with 10 volumes 70% formic acid/ 30%

isopropanol and washed with 5-10 volume 90% ACN/ 0.1% TFA. It was then equilibrated in 5-10 volume 2% ACN, 0.1% TFA and the samples were loaded in 1mL 2%, 0.1 TFA. Peptides were washed with 5 volumes 2% ACN, 0.1% TFA to remove the salts and eluted with 300 μL of 90% ACN, 0.1% TFA. Peptides were dried using vacuum centrifugation.

2.6.6. Enrichment of phosphopeptides

Phosphopeptides were enriched using TiO2 tips (TitansphereTM Phos-TiO kit, GL Sciences). Tips were washed in buffer A (0.5% TFA, 80% ACN) and equilibrated in buffer B (0.38% TFA, 60% ACN, 25% lactic acid). Phosphopeptides were resuspended in buffer B and loaded onto the tips, washed once in buffer B and twice in buffer A before being eluted sequentially in 5% ammonia solution followed by 5% pyrrolidine. Phosphopeptide-enriched samples were desalted using ZipTips (Millipore) according to manufacturer's protocol. Peptides were eluted in 50% ACN, 0.1% TFA, dried and resuspended in 0.1% formic acid.

2.6.7. In-gel Trypsin Digestion

For the proteome study, in-gel trypsin digestion was done as previously described (Cunningham et al., 2010, Shevchenko et al., 2006). Briefly, bands were excised from the gel and cut into small pieces, before being destained in 30% acetonitrile for 15 min incubation with agitation. Then, 50% acetonitrile, 25 mM ammonium bicarbonate was added for 15 min. The gel pieces were dehydrated in a vacuum centrifuge for 5 min

followed by rehydration in 10 mM DTT, 25 mM ammonium bicarbonate at 56 C° for 45 min. Gels were then alkylated with 55 mM iodoacetamide, 25 mM ammonium bicarbonate at room temperature, in the dark for 45 min. The gel pieces were then washed with 25 mM ammonium bicarbonate once for 10 min and 50% acetonitrile, 25 mM ammonium bicarbonate twice for 5 min. Trypsin was first resuspended in 20 µL of 50 mM acetic acid and then diluted in 25 mM ammonium bicarbonate to give a final concentration of 12.5 ng/µL. Trypsin was added to gel pieces and incubated overnight at 37 C°. Samples were concentrated using vacuum centrifugation and resuspended in 10 µL 0.1% formic acid.

2.6.8. Mass spectrometry (work performed by The Proteomics and Metabolomics Facility, University of Birmingham)

All resulting peptide mixtures were analysed in duplicate by liquid chromatography tandem mass spectrometry (LC-MS/MS). On-line liquid chromatography was performed by use of a Dionex Ultimate 3000 LC system (Thermo Fisher Scientific). Peptides were loaded onto an Acclaim PepMap 100 C18 resolving column (15 cm length; 75 µm internal diameter; LC Packings, USA) and separated over a 40 min gradient from 3.2 % to 44 % acetonitrile (Baker, Holland). Peptides were eluted directly (350 nL/min) via a Triversa nanospray source (Advion Biosciences, NY, USA) into a LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). The mass spectrometer alternated between a full FT-MS scan (m/z 380-1600) and subsequent CID MS/MS scans of the twenty most abundant ions. Survey scans were

acquired in the Orbitrap cell with a resolution of 60,000 at m/z 200. Precursor ions were isolated and subjected to CID in the linear ion trap. Isolation width was 2 Th. Only multiply charged precursor ions were selected for MS/MS. CID was performed with helium gas at normalized collision energy of 35 %. Precursor ions were activated for 10 ms. Data acquisition was controlled by Xcalibur 3.0.63 software.

2.6.9. Peptide identification using MaxQuant/ Andromeda

Mass spectra were processed using the MaxQuant software (version 1.5.3.8) (Cox and Mann, 2008). Data were searched, using the Andromeda search engine within MaxQuant (Cox et al., 2011), against the mouse Swiss-Prot database (downloaded 6.10.15). The mouse database contained 16,719 reviewed protein entries. The search parameters were: minimum peptide length 7, peptide tolerance 20 ppm (first search) and 6 ppm (second search), mass tolerance 0.5 Da, cleavage enzyme trypsin/P, and 2 missed cleavages were allowed. Carbamidomethyl (C) was set as a fixed modification. Oxidation (M), acetylation (Protein N-term), and phospho (STY) were set as variable modifications. The appropriate SILAC labels were selected and the maximum labelled amino acids was set to 3. All experiments were filtered to have a peptide and protein false-discovery rate (FDR) below 1 %. Within the MaxQuant output, phosphorylation sites were considered to be localised correctly if the localisation probability was at least 0.75 (75 %) and the score difference at least 5. Bioinformatics analysis was performed in the Perseus software environment, which is part of MaxQuant (Perseus version 1.5.0.15; www.perseus.framework.org). Significance testing was carried out using a Student's t-test on log 2 transformed ratios and controlled with a Benjamini-

Hochberg FDR threshold of 0.05. Peptides quantified in three or more experimental repeats were deemed significantly changed and regulated by LAR phosphatase activity if they had a p-value of < 0.05 and a ratio of < 0.667 or > 1.5 (at least a 1.5-fold change in abundance). The mass spectrometry proteomics data, including the MaxQuant output, have been deposited to the Proteome Xchange (Vizcaino et al., 2013) Consortium via the PRIDE partner repository with the dataset identifier PXD002545.

2.6.10. Cluster and Kinase motif analysis

GProX software (Rigbolt et al., 2011b) was used to perform clustering of $\log_2$ transformed ratios from MaxQuant output. Unsupervised fuzzy *c*-means clustering was used with an upper regulation threshold of 1 and a lower regulation threshold of -1. Phosphopeptides containing well localised phosphosites were analysed using the Group-based Prediction System (GPS; version 2.1.2) (Xue et al., 2008) for kinase motifs in order to predict upstream kinases. To minimize false positives, the highest threshold was applied. Kinases upstream of the identified PDGF-regulated phosphopeptides were predicted using Kinase Enrichment Analysis (KEA) (Lachmann and Ma'ayan, 2009).

2.6.11. Functional enrichment analysis

DAVID (Database for Annotation, Visualization and Integrated Discovery) (Huang da et al., 2009) was used to identify over-represented GO terms and KEGG pathways. The background list comprised of all the proteins identified across our experiments. The threshold count and EASE score were set to 2 and 0.05 respectively. REVIGO (Supek et al., 2011) was also used for identified and visualised over-presented GO terms.

2.6.12. Protein-protein interaction network analysis

Protein-protein interaction (PPI) network analyses were performed using Cytoscape (version 3.3.0) (Shannon et al., 2003) with GLay plugin for network clustering and structured visualization (G. Su et al., 2010) and BiNGO plugin to assess over-representation of GO terms within the network (Maere et al., 2005). Indicated PPI networks were also built using interaction information obtained from a public database using the PSICQUIC client in Cytoscape (Aranda et al., 2011).

2.6.13. Reprocessing proteomics public data and protein expression analysis

Data from human proteome project (Wilhelm et al., 2014b) was reanalyzed using PRIDE Reshake function in PeptideShaker software (Vaudel et al., 2015). For the analysis of protein expression (protein abundance), the number of peptide spectrum matches (PSM) were normalized based on the total sum of all PSMs to be able to

compare protein abundances across multiple samples (Kuntumalla et al., 2009, Wilhelm et al., 2014b). Protein abundances were obtained by dividing normalized PSMs by its molecular weight. Protein abundance was also calculated by using the iBAQ option (intensity based absolute protein quantification) in MaxQuant (Schwanhausser et al., 2011). iBAQ is the summation of peptide intensities for the protein in a sample divided by the number of observable peptides. iBAQ values were then normalized and $\log_{10}$ transformed.

2.7. RNA interference

Cells were transfected with 80 pmol of ON-TARGET plus Mouse siRNA SMARTpool siRNA for LAR (CGAAUUACGUGGAUGAAGA; GCUUUGAGGUAAUUGAGUU; GGACUUCUGGCGCAUGUUA; GAAUAUGCUUUCCGUGUGU), GRK2 (GAUCCCUGCUGGAAGGUUU; GAUCUUUGACUCCUAUAUU; CCAGAUCUCUUCCAGCCAU; GCAAGUGUCUCCUGCUUAA), or CRMP2 (GUUGAGAAGAGGCGGGUUU; GUGCUCAGAUUGACGACAA; CGGCUGAAGUCAUCGCUCA; GGGAAUGACAUCCGCUGAU) (Dharmacon). Non-Targeting siRNA was used as a control for each preformed experiment. Lipofectamine RNAiMAX (Invitrogen, Life Technologies) was using as transfection reagent and the siRNA transfection was done for 48 hours according to the manufacturer's protocol.

2.8. Transformation and DNA transfection

For the amplification of plasmids, 5 μL of each plasmid was mixed with 50 μL of either *E. coli* DH5α strain or MC1061/P3 strain and incubated on ice for 20 minutes. The mixture was heat shock in 42°C for 30-60 seconds followed by 3 min incubation on ice. The mixture was added to 950 μL of LB media (without antibiotics) and incubated for 45min in 37°C with shaking. 200 μL of the transformed cells was plated onto a 10 cm LB agar plate containing the appropriate antibiotic and incubated overnight at 37 C°. One colony was picked and added to 250 mL of LB medium with the appropriate antibiotic and incubated overnight at 37 C° with agitation. Plasmids were purified using the PureYield Plasmid Maxiprep System (Promega) according to manufacturer's instructions. Cell lines were transiently transfected with the indicated constructs using Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer's protocol.

2.9. Measurement of mitochondrial respiration

The Seahorse XF24 (Seahorse Biosciences) was used to determine the mitochondrial respiration by measuring oxygen consumption rate and extracellular acidification rates as previously described (Rogers et al., 2011, Salabei et al., 2014). WT and LARΔP cells were seeded to 40,000 cells/ well in XF24 cell culture plates and incubate in either complete or starvation media for 24 h prior to the assays. Oxygen consumption rate (OCR) was measured under basal state followed by the sequential injection of oligomycin (Oligo; 1 μM), carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP; 1

μM), and Rotenone/ Antimycin A (0.5 μM). The assay was run in 3 technical replicates for each independent biological replicate (n = 3). Cells were counted in each well following an assay to normalize OCR raw data.

2.11. Scratch wound assay

Cells were plated in 96-well cell culture plates and cultured to confluency. Scratch wounds were made using an automated wound maker (Essen BioScience, Hertfordshire, UK) and images collected at hourly intervals post-wounding using an IncuCyte ZOOM imaging system (Essen BioScience, Hertfordshire, UK).

2.10. Statistics

Statistical analysis performed in PRISM (GraphPad Software) using either two-way ANOVA with Sidak's multiple comparison test or Student's t-test.

CHAPTER 3

Quantitative Phosphoproteomics Reveals a Key Role for LAR in Regulation of PDGF-Dependent mTOR and JNK Signalling Pathways

3.1. Introduction

Post-translational modifications (PTM) are involved in the regulation of almost all aspect of cell function (Jun Wang and Schwartz, 2016). Therefore, characterizing and understanding how PTM regulated is substantial in biology of the cell in health and disease. One of the PTM is the phosphorylation, which control most of the biological processes and dysregulation is implicated in many diseases (Bode and Dong, 2004). Kinases and phosphatases have opposing roles in regulating the activity of proteins, and play a vital role in maintain cellular homeostasis through their regulation of signal transduction pathways (Fontanillo and Köhn, 2016, Tony Hunter, 1995). Whilst the role of kinases has been extensively studied, however, less is known about the contribution of phosphatases in regulating cell signalling pathways.

Quantitative phosphoproteomics is a powerful technique used to identify large numbers of phosphorylation sites coupled to biologically informative results regarding regulation of these specific events. In this study, global phosphoproteomics and proteomics were carried out to identify and understand the cellular function which regulated by LAR. An additional aim was to characterise those LAR-regulated phosphorylation events that were PDGF dependent. We applied stable isotope labelling by amino acids in cell culture (SILAC) (Cunningham et al., 2010, S. E. Ong

et al., 2002) to characterized the phosphorylation events between mouse embryo fibroblasts (MEFs) obtained from mice in which the LAR cytoplasmic phosphatase domains had been deleted (LARΔP) and littermate wild-type (WT) mice (Schaapveld et al., 1997) in the absence and presence of PDGF. This type of comprehensive analysis of the LAR regulated phosphoproteome has not been done before. In this study, we identified 270 LAR-dependent phosphorylation events on 205 proteins which are significantly changed, including known LAR interactors, adaptor proteins, kinases, phosphatases, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs).

Gene ontology analysis revealed an enrichment of LAR-mediated phosphorylation events on proteins involved in many signalling transduction pathways including actin cytoskeleton, adhesion, endocytosis and cell metabolism. Kinase prediction tools and western blot analysis have revealed a key role for LAR in regulating mTOR and JNK signalling pathways. Collectively, these results significantly expand the range of proteins implicated downstream of LAR and provide an extensive dataset for further scrutiny.

3. 2. Results

3.2.1. Evaluation of the SILAC-based phosphoproteomics

First, the efficiency of SILAC labelling was analysed in WT and LARΔP MEFs. WT and LARΔP cells were grown separately in both SILAC 'heavy' R10 K8 and SILAC 'medium' R6K4 media for 7 doubling times. Cell lysates from each of the four cell populations were prepared as described in Materials and Methods chapter. Amino acid incorporation of both SILAC labelling media were very efficient with the average labelling efficiency of 96% for WT and 98% for LARΔP cells (**Figure 3.1**).

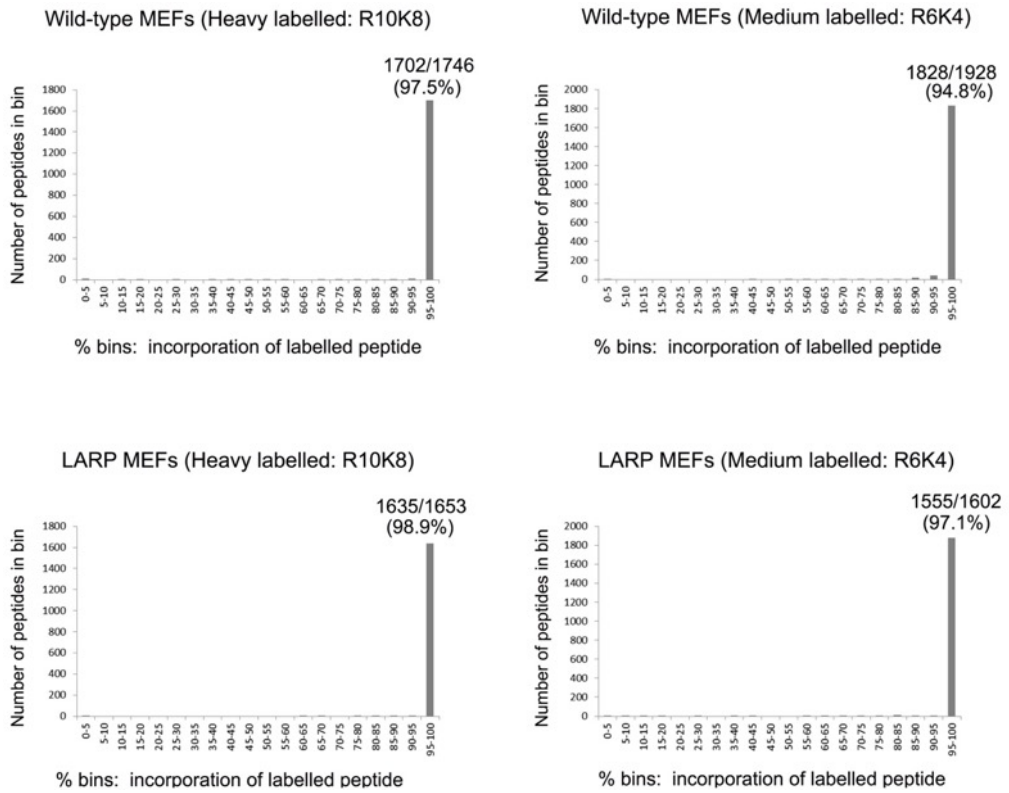

Figure 3.1. SILAC label incorporation into WT and LARΔP MEFs. Each of the four raw files were analysed separately using MaxQuant but without selecting match between runs and re-quantify. Using the peptide.txt output, reverse and contaminants were removed and the % incorporation was calculated using the following equation: % Incorporation = (Ratio H/L or M/L x 100)/ (Ratio H/L or M/L + 1).

After the identification of SILAC labelling technique, next we have designed the experimental conditions for our global proteomics and phosphoproteomics analysis. Three populations of WT and LARΔP cells were SILAC-labelled by culturing them in light R0K0, medium R6K4, or heavy R10K8 SILAC media. Cells were left unstimulated or stimulated with PDGF-BB for 7 min (**Figure 3.2**).

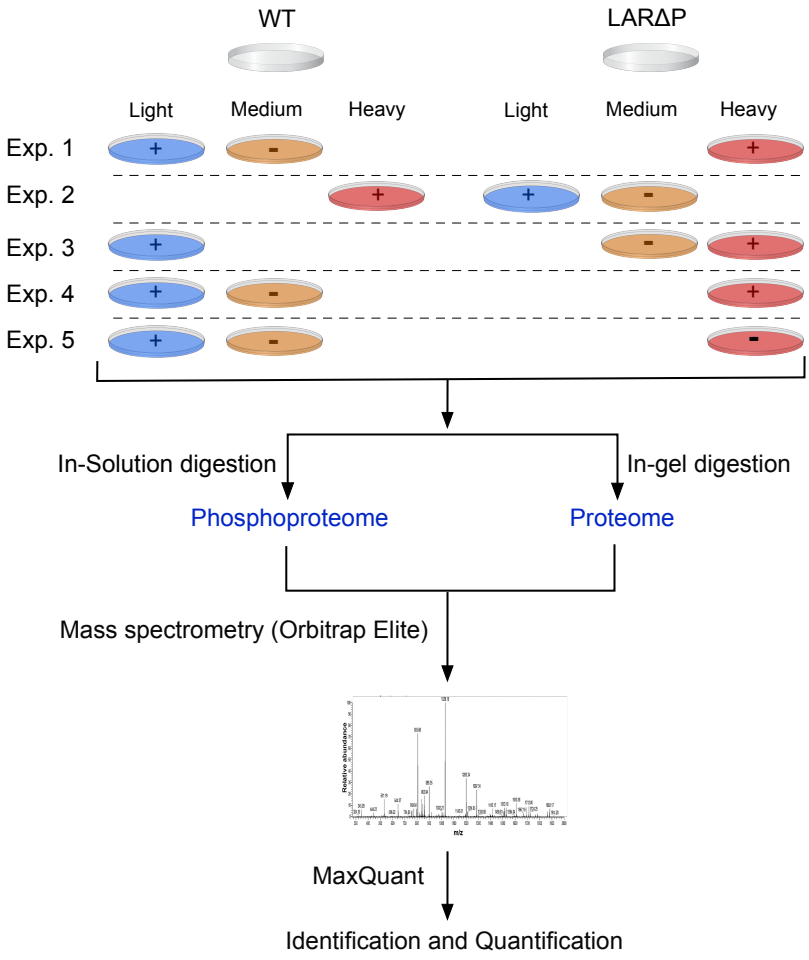

Figure 3.2. Schematic overview of experimental design. Plus (+) stimulated with PDGF for 7 min, minus (-) nonstimulated. In experiment 2 the labels were reversed.

A total of 30 mg of digested peptides from each experiment were divided into three parts and fractionated using Strong cation exchange (SCX). The elution of each part was combined correspondingly, resulting 20 fractions for each experiment. The SCX profile for representative experiment (Expt. 2) was plotted in **Figure 3.3A**. Biological replicates 3, 4 and 5 were relatively similar with around 700 mAU, except experiment 1 which showed the lowest UV absorbance whereas experiment 2 was the highest (**Figure 3.3A**). After desalting, the resulting 20 fractions from the SCX were phosphoenriched using Titanium dioxide (TiO_2) tips. One of the experiment is shown in **Figure 3.3B**, we found that phosphopeptides enrichment was very efficient. Fraction 1 to 18 showed the best overall enrichment with average efficiency of 88%. In fact, it is noteworthy that fraction 19 and 20 were showed the lowest phosphoenrichment efficiency with more unmodified peptides (**Figure 3.3B**).

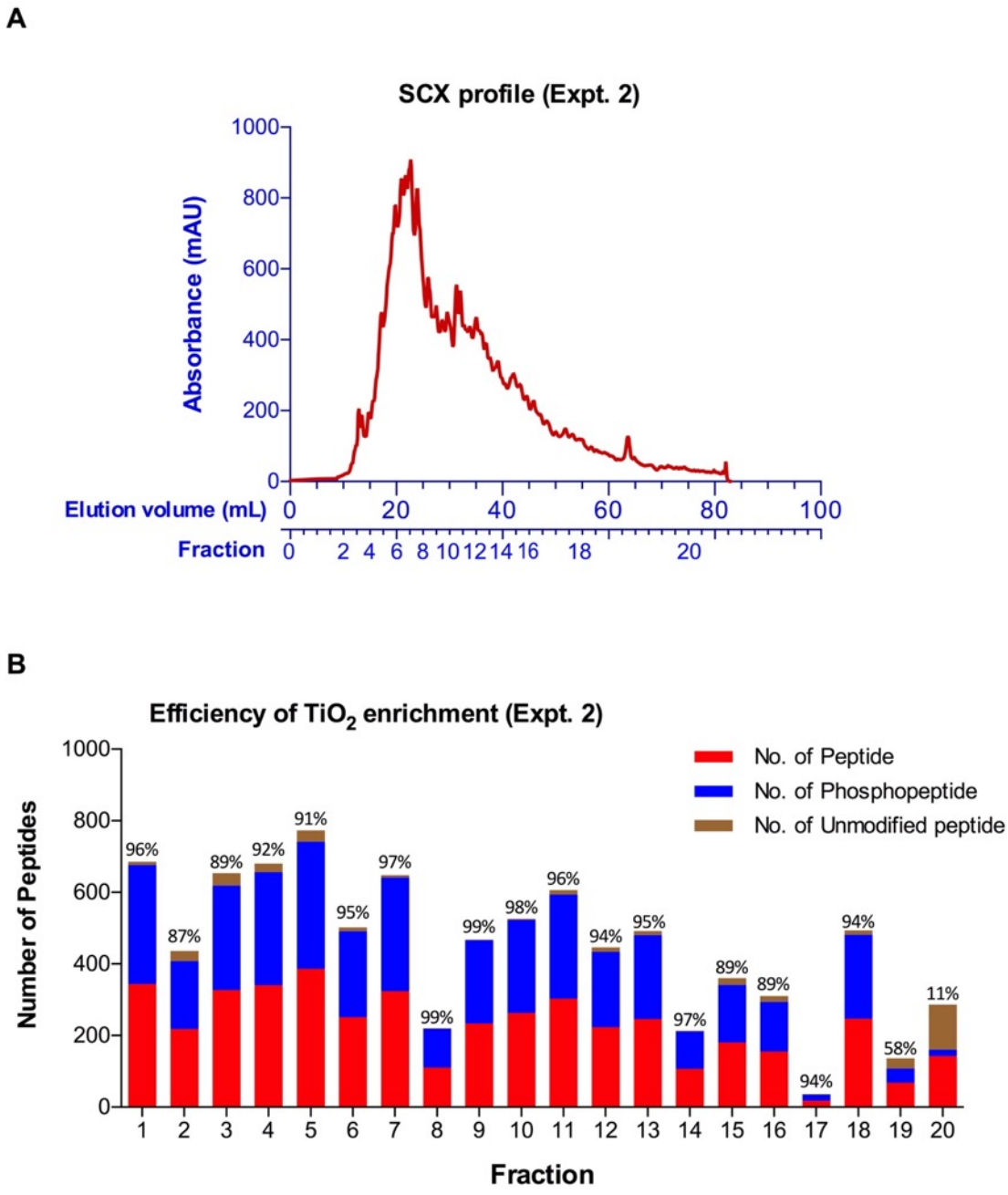

Figure 3.3. SCX profile and the efficiency of phosphopeptides. A, SCX fractionation of the phosphopeptides in experiment 2. **B**, represents the number of peptides, phosphopeptides and unmodified peptides being identified in each fraction of the experiment 2. The percentage of TiO_2 enrichment efficiency was calculated by dividing the number of phosphorylation sites by all peptides in each fraction.

A total of 29,734 phosphopeptides were identified across the four biological replicates including redundant and non-redundant peptides, giving a total of 2,958 non-redundant peptides. The overlap between all experiments is plotted as a Four-way Venn diagram as shown in **Figure 3.4A**. 54% of the phosphopeptides were identified in two or more replicates, whereas 27% were identified in three or more replicates. Correlation coefficients for the peptide ratios measured across the biological and technical replicates including the label swap experiment, ranged from 0.58 to 0.93 (**Figure 3.4B**), indicating good biological reproducibility.

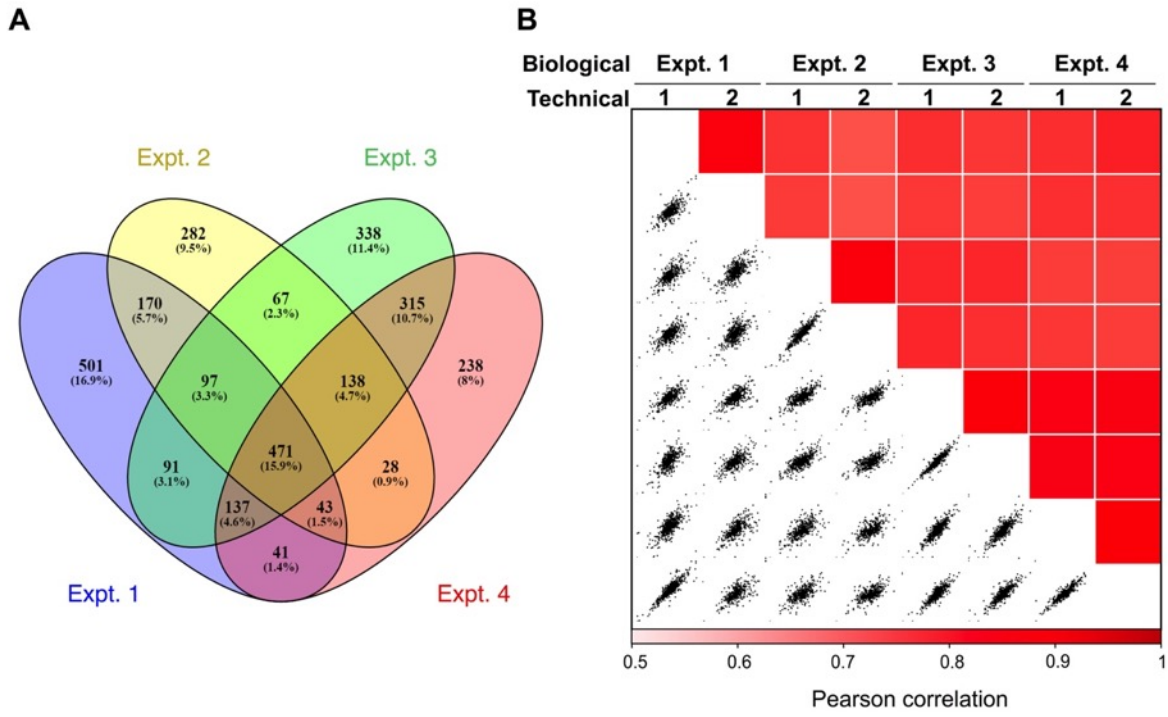

Figure 3.4. Overlap and correlation of phosphopeptide identifications between four biological repeats. A, the overlap between the four replicate experiments is plotted as a Four-way Venn diagram. **B,** Pearson correlation matrix and multi scatter plots demonstrating reproducibility between the phosphoproteome of four biological and technical replicates.

3.2.2. Global phosphoproteome in LARΔP cells

LAR is known to contribute to PDGF activation in fibroblasts through attenuation of the c-Abl activity (Zheng et al., 2011). However, the signalling consequences of this regulation have not been fully studied. To gain insight into the signal transduction pathways downstream of LAR, we utilised SILAC-based mass spectrometry (S. E. Ong et al., 2002) to quantitatively compare both levels of protein expression (**Chapter**

6) and phosphorylation in WT and LARΔP MEFs in response to PDGF stimulation (**Figure 3.2**). Within the phosphoproteome data set, 2559 unique phosphosites from 1311 proteins were identified with high localization scores (localization probability >0.75; score difference >5) in one or more biological replicates (**Figure 3.5**).

Of these, the majority of phosphopeptides were singly phosphorylated with percentage of 79% and the remainder of phosphopeptides were either doubly (16%), triply (3%), or above (2%) phosphorylated peptides (**Figure 3.6A**). The phosphorylation sites were comprised of serine (83%), threonine (10%), and 7% were tyrosine phosphorylated sites (**Figure 6B**). Of these, 266 phosphosites (10%) are not listed in PhosphoSitePlus database (Hornbeck et al., 2012) and are considered novel.

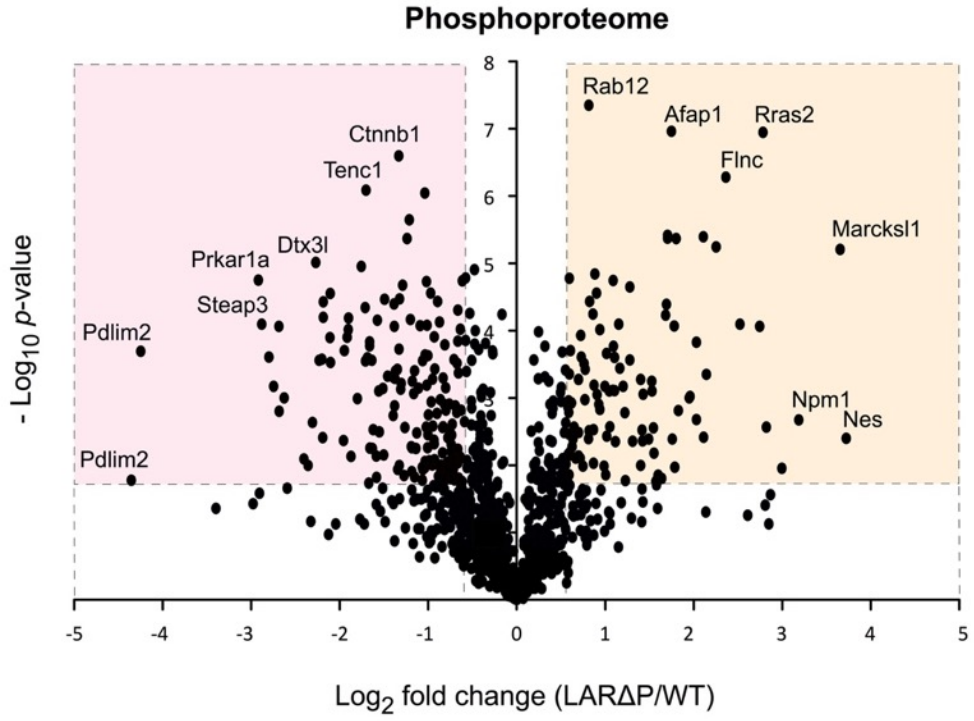

Figure 3.5. Global phosphoproteomics to measure contribution of LAR to PDGF signalling. Volcano plots showing the ratio (log2 fold change) and significance ($-\log_{10}$ Benjamini-Hochberg adjusted p-value) of the phosphoproteome upon PDGF stimulation. Significantly upregulated phosphopeptides are marked in light brown box and the downregulated phosphopeptides are marked in pink box (adjusted p value <0.05; >1.5 fold-change).

In LARΔP cells, a total of 270 phosphopeptides from 205 proteins showed a significant change in abundance ($p<0.05$; >1.5-fold change). Of these, 255 (95%) are serine phosphorylation sites, 9 (3%) threonine, and 6 (2%) were tyrosine phosphorylation sites indicating that LAR could contribute to the regulation of these phosphorylation sites via modulation of the activity of specific kinases, or via direct dephosphorylation for the tyrosine site. A total of 103 phosphosites were upregulated and 167 downregulated.

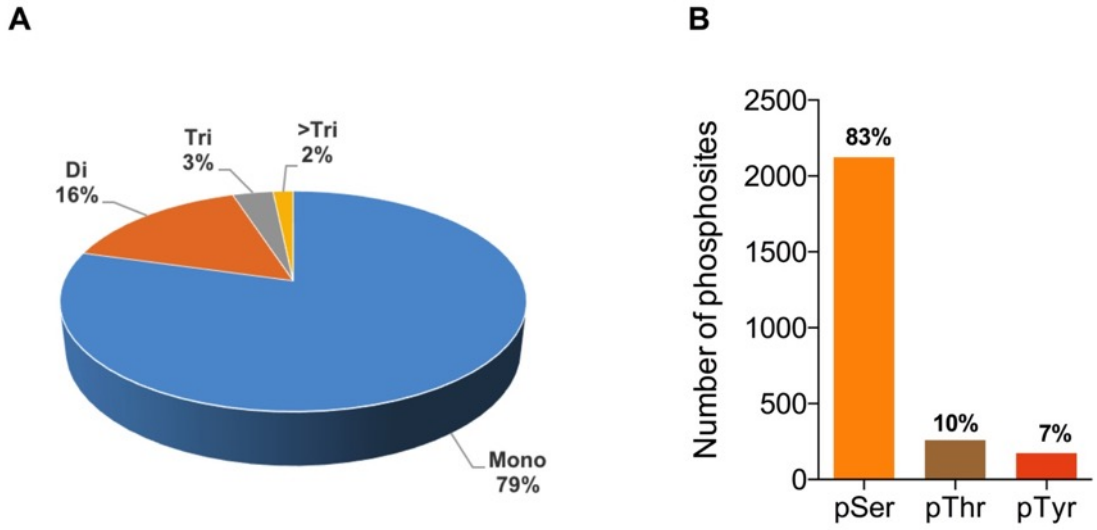

Figure 3. 6. Type and classification of phosphorylated peptides within phosphoproteome data set. **A**, Percentage of phosphorylation sites within phosphoproteome containing either one, two, three or more than three phosphorylation sites. **B**, Numbers of serine, threonine and tyrosine phosphorylation sites identified within our phosphoproteome data set are presented.

3.2.3. Regulation of tyrosine phosphorylation by LAR

Loss of LAR phosphatase activity would lead to an increase in phosphorylation of tyrosine residues and can be consider as a LAR potential direct substrates. Whereas, the downregulation tyrosine sites could be indirectly regulated by LAR. Within phosphoproteomics data set we found only one tyrosine phosphorylated peptide belonging to the Lymphocyte cytosolic protein 2 (also known as SH2 domain containing leukocyte protein of 76kDa; SLP76) and showed increased in abundance in the absence of LAR activity. SLP76 is a scaffold protein, mainly studied in T cells

and has been shown to enhancing T cell development in response to T cell receptors (J. N. Wu and Koretzky, 2004, Zeng et al., 2003). We have identified an increase in Tyr^{465} phosphorylation which is a tyrosine residue located in the SH2 domain of SLP76 and this protein could be direct substrate for LAR (**Figure 3.7**).

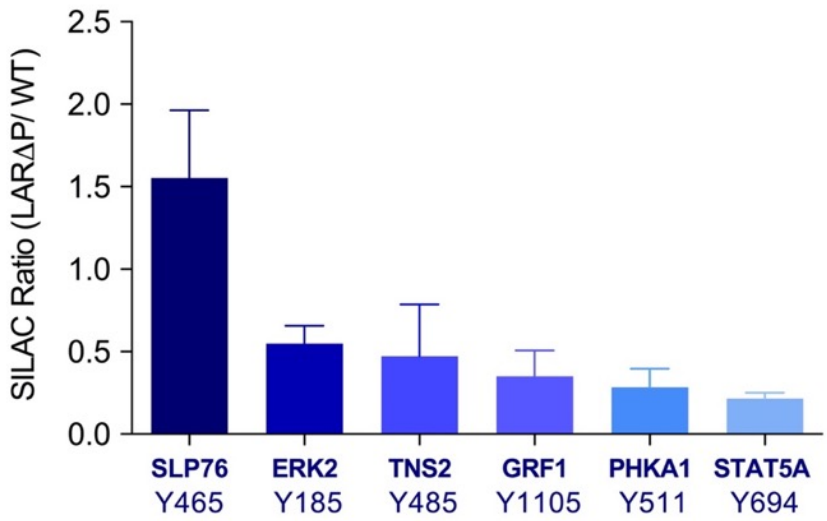

Figure 3.7. Tyrosine phosphorylation regulated by LAR. Shows proteins and their phosphorylated tyrosine sites that regulated by LAR. The quantitative ratios for each site has been identified in three or more biological replicates.

However, we identified tyrosine sites that were significantly decreased in LARΔP cells including Tensin-2 (TNS2), Rho GTPase activating protein 35 (Arhgap35; known as GRF1), Phosphorylase b kinase regulatory subunit alpha (PHKA1) and Signal transducer and activator of transcription 5A (STAT5A) (**Figure 3.7**). All these sites were downregulated supporting the notion that these proteins indirectly regulated by LAR. For example, we identified decreased in phosphorylation of Tyr^{185} on ERK2 (**Figure 3.7**). The phosphorylation of this site by MEK2 induced the kinase activity which in turn initiating MAPK signalling pathway leading to the promotion of many cellular functions such as cell proliferation (Roskoski, 2012).

To validate the phosphoproteomic data, western blotting analyses was performed using phospho-specific antibody targeting phosphorylation of ERK1/2. Western blotting analyses revealed that ERK1/2 phosphorylation is significantly reduced in LARΔP cells when compared with WT cells upon PDGF stimulation (**Figure 3.8A and B**). Re-expression of WT LAR in LARΔP cells restored the phosphorylation of ERK1/2, confirming that LAR phosphatase activity is required for ERK1/2 activation and can be a positive regulator for MAPK/ERK signalling pathway (**Figure 3.8C and D).**

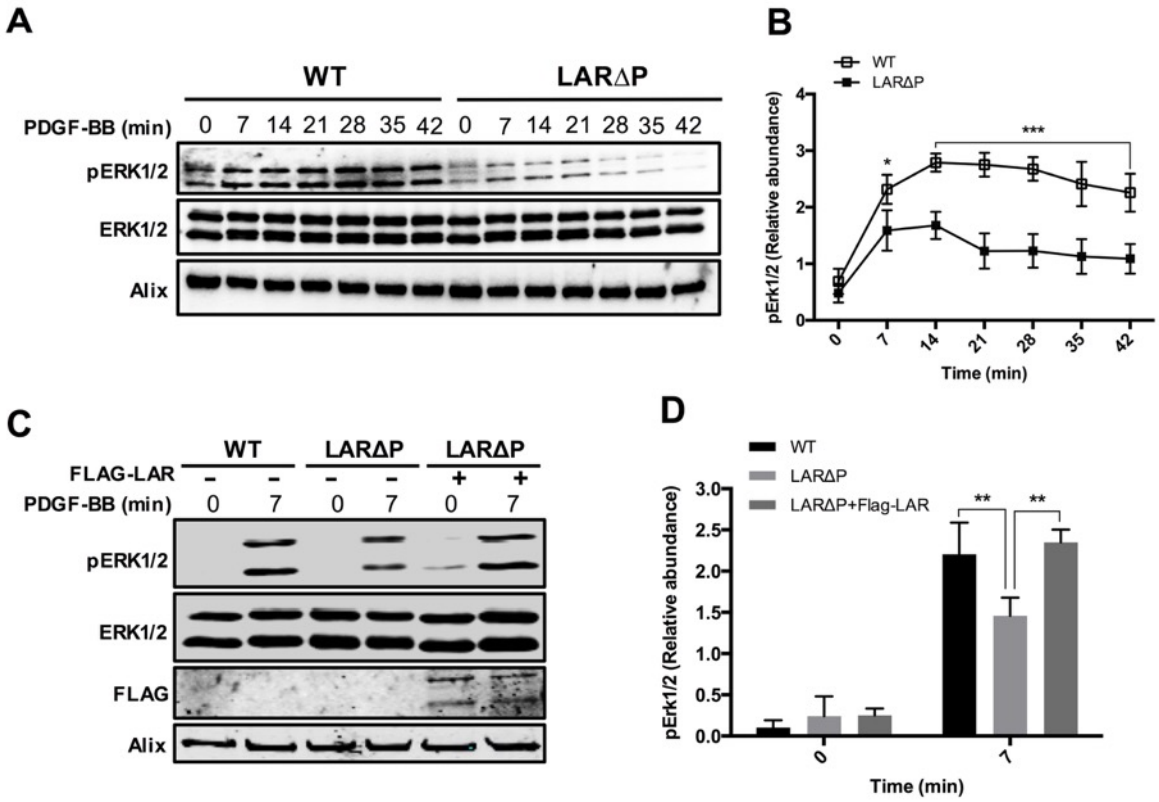

Figure 3.8. LAR Regulates the ERK signalling pathway. **A**, WT and LARΔP cells were stimulated with 20 ng/mL PDGF for the indicated time. Levels of pERK1/2 (Thr202/Tyr204), total ERK1/2 and Alix in whole cell lysates were analyzed by Western blotting. **B**, Western blots (n=3) were quantified and ratios of pERK to Alix were plotted over time using two-way ANOVA with Sidak's test (*** $p<0.001$; * $p<0.05$). More samples have been loaded into the gel in time point 0 causing decrease the ratio of pERK/Alix in quantification. **C**, WT, LARΔP, and LARΔP cells transfected with Flag-WT-LAR, were stimulated with 20 ng/mL PDGF for the indicated time. Levels of pERK 1/2, total ERK and Alix in whole cell lysates were analyzed by Western blotting. **D**, Western blots (n=3) from the rescue experiments were quantified and ratios of pERK to Alix were plotted and statistical significance was calculated using two-way ANOVA with Sidak's test (** $p<0.01$).

3.2.4. Enriched GO terms regulated by LAR

To investigate the biological processes that may be regulated by LAR activity, Gene Ontology (GO) enrichment analysis was performed. Analysis of the phosphoproteome regulated by LAR indicated a number of enriched biological processes, molecular functions, and cellular components (**Figure 3.9**). The cytoskeletal organization and cell adhesion were over-presented GO terms. LAR has been previously linked to cell adhesions via its interaction with β-catenin (Ctnnb1) (Aicher et al., 1997). Within our phosphoproteomic dataset we have identified changes in abundance of specific phosphorylation events on α-catenin (Ser^{641}), β-catenin (Ser^{191}; Ser^{675}), and δ-catenin (Ser^{864}), and also several proteins involved in the cell adhesion protein interaction network, due to the absence of LAR phosphatase activity (**Figure 3.10**).

β-catenin is a substrate for LAR, resulting in a negative regulation of tyrosine phosphorylation and inhibition of epithelial cell migration (T. Muller et al., 1999). Within our data set, we have not detected any tyrosine phosphorylation sites, instead, serine and threonine residues were identified such as Ser^{191}, which is significantly reduced in LARΔP cells. This site has been shown to be essential for nuclear accumulation of β-catenin in response to Wnt and is regulated by JNK2 (X. Wu et al., 2008). Therefore, it is possible that LAR is capable of regulating β-catenin phosphorylation on two levels in response to cellular signals; either by directly dephosphorylating specific tyrosine residues or regulation of upstream kinase activity such as JNK2.

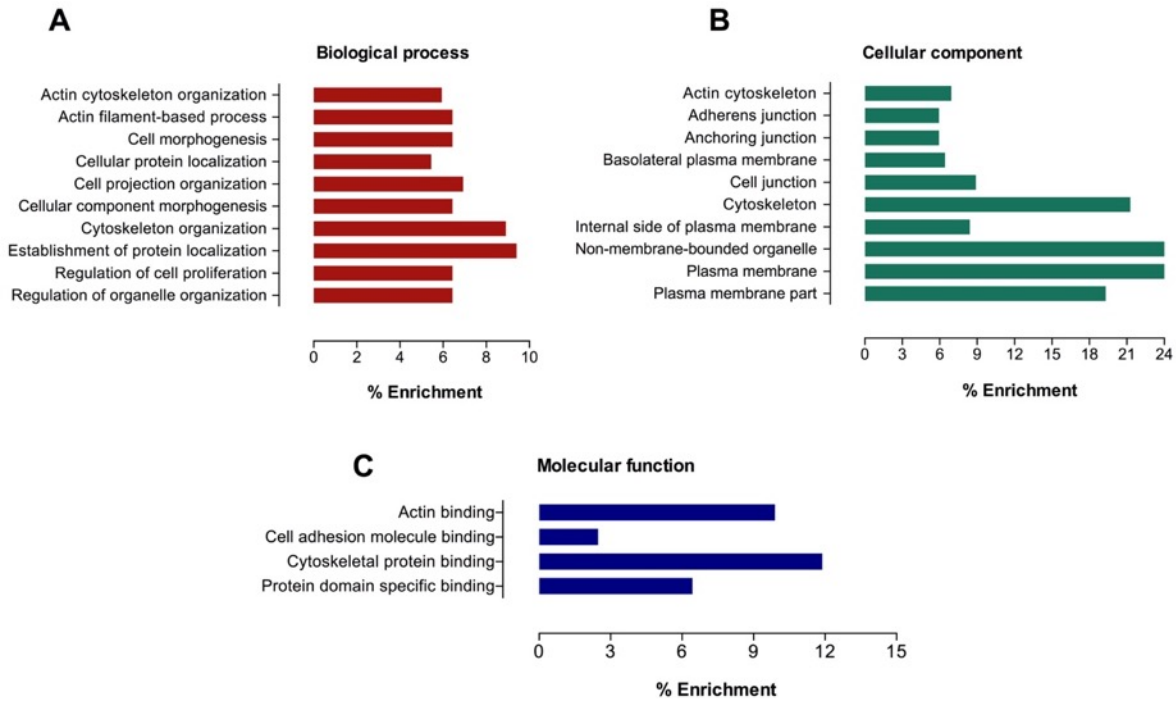

Figure 3.9. LAR regulated distinct cellular functions. Proteins containing the 270 LAR regulated phosphosites were analyzed using DAVID to identify enriched GO Terms. The top (10) enriched categories for Biological Processes (**A**), Cellular Component (**B**) and Molecular Function (**C**) are plotted as bar charts.

LAR has also been linked to regulation of the cytoskeleton via its interaction with TRIO (Debant et al., 1996). TRIO has one serine/threonine kinase domain and two guanine nucleotide exchange factor (GEF) domains which are involved in regulation of the small GTPases (Debant et al., 1996). Two TRIO phosphosites, Ser^{2458} and Ser^{2462} have increased in abundance in LARΔP cells indicating that in addition to interaction with TRIO, LAR can also regulate its phosphorylation status. Network and functional analysis revealed that phosphorylation for the large number of cytoskeletal proteins are modulated by LAR (**Figure 3.10**).

In addition, we have identified a decrease in the phosphorylation of cadherin-11 (Ser^{714}), plakoglobin (Ser^{665}), ZO-1 (Ser^{1614}), ZO-2 (Ser^{107}; Ser^{239}; Ser^{1136}), providing an evidence that LAR regulates cell-cell adhesion and tight junctions.

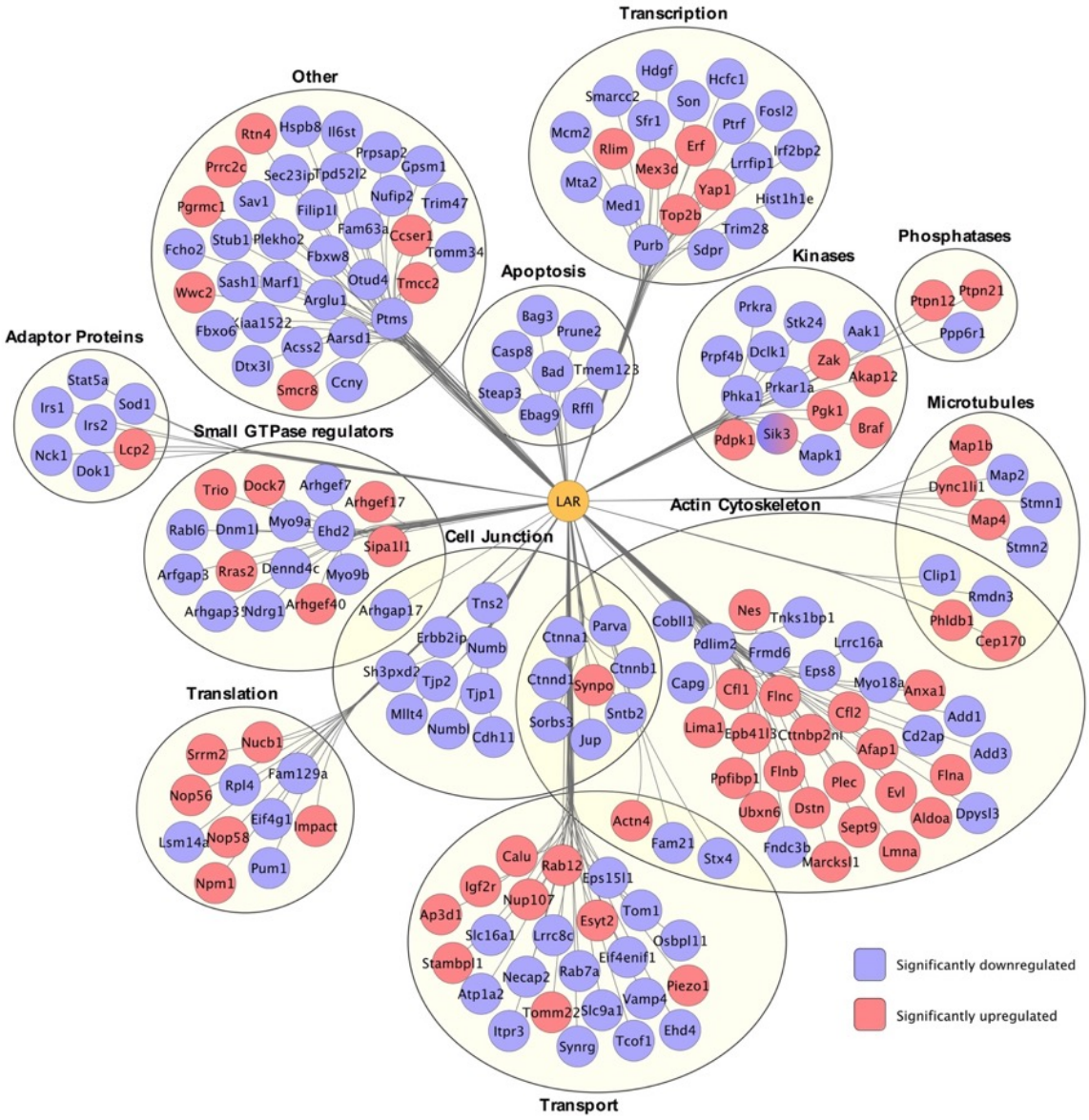

Figure 3.10. A network of proteins regulated by LAR. Proteins were clustered according to their functional keywords. Blue indicates that the protein contained a phosphosite(s) that was down-regulated in LARΔP cells and red indicates up-regulation.

3.2.5. PDGF-dependent signalling events regulated by LAR

The comparison of the phosphoproteome of WT and LARΔP cells stimulated with PDGF provides information regarding the proteins whose phosphorylation is regulated by LAR in response to PDGF stimulation. In order to specifically analyse the influence of PDGF on these events, phosphopeptides were clustered according to their response to PDGF stimulation versus unstimulated cells using GProX software (Rigbolt et al., 2011b). We carried out two comparisons, first, PDGF-stimulated WT and LARΔP cells versus unstimulated WT cells for the evaluation of the comparative end point of phosphopeptide abundance. The second comparison is between PDGF-stimulated LARΔP cells versus unstimulated LARΔP cells. In fact, our experimental design included three biological replicates for the ratios between PDGF-stimulated and unstimulated WT cells and two biological replicates for the ratios between PDGF-stimulated LARΔP cells and unstimulated WT or LARΔP cells.

We have generated six clusters as shown in **Figure 3.11A**. Clusters 2, 3, 4, and 6 contained those phosphopeptides that showed a LAR-dependent events when compared with basal levels in WT cells. However, similar levels were observed in clusters 1 and 5 in both PDGF-stimulated WT and LARΔP cells when compared with unstimulated WT cells. In clusters 2 and 3, the phosphopeptides showed no differences in abundance upon PDGF stimulation in WT and LARΔP cells compared with their basal levels. In contrast, PDGF stimulated LARΔP cells was significantly changed compared with unstimulated WT cells. Moreover, a similar fold change was observed in clusters 4 and 6 in LARΔP cells upon PDGF stimulation when compared with either unstimulated WT or LARΔP cells.

GO terms enrichment analysis for these clusters showed a discrete between the cellular component and molecular functions regulated by these groups of phosphoproteins (**Figure 3.11B and C**). The majority of over-presented GO terms in cellular components are cytoskeletal, focal adhesion, endosomes and vesicular compartments. Cluster 4 is shows the most interesting because it contains phosphorylation sites that are significantly increased in response to PDGF (**Figure 3.11C**). Cytoskeletal proteins were enriched in cluster 4 including proteins play a role in regulation of actin reorganisation and stress fiber formation such as Vinexin (Sorbs3) (Kioka et al., 1999). This cluster also contains γ-adducin (Add3), a protein involved in regulation of tight junctions (Naydenov and Ivanov, 2011). We detected 3-fold reduction in phosphorylation of Ser^{669} on Sorbs3 and Ser^{423} on Add3 in LARΔP cells upon PDGF stimulation. In addition, we observed enriched cellular component in cluster 6 including lysosomal membrane, endosome and microtubule (**Figure 3.11B**). This is supporting the notion that LAR involved in regulation of distinct biological processes and highlight the cooperation between PDGF and LAR in modulation of the cytoskeleton and protein transport.

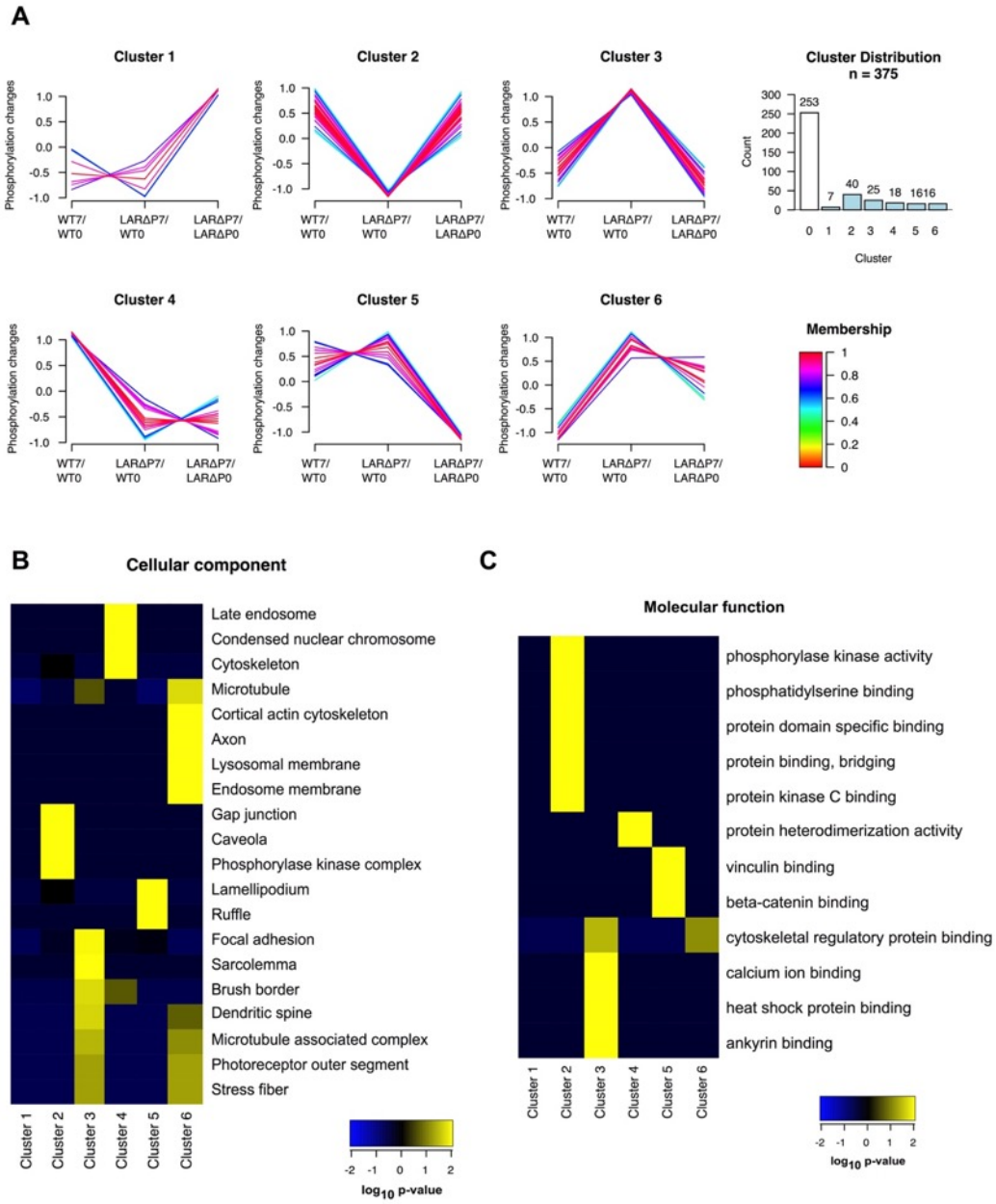

Figure 3.11. Phosphorylation events in WT and LARΔP cells show clusters of regulation correlated to distinct biological processes. **A**, GProX clustering of phosphopeptide abundance changes. Ratios of PDGF-stimulated WT and LARΔP cells over WT unstimulated cells and PDGF-stimulated LARΔP cells over LARΔP unstimulated cells were subjected to unsupervised clustering using the fuzzy c means algorithm. The number of phosphopeptides in each cluster is indicated. Overrepresentation of cellular components (**B**) and molecular function (**C**) in the clusters are represented as a heat map. GO terms were performed within GProX using a binomial statistical test with a Benjamini-Hochberg p-value adjustment (p-value threshold 0.05).

3.2.6. Kinase predication analysis revealed LAR regulates distinct kinase nodes

Kinases are key regulators of phosphorylation events in signalling pathways, and those kinases whose activity is regulated by LAR phosphatase activity would have altered regulation in LARΔP cells. In order to identify key nodes of regulation within our phosphoproteome dataset, the kinase prediction tool GPS (Xue et al., 2008) was used to identify predicted upstream kinases of those phosphorylation peptides that showed significant changes (270 phosphopeptides). GPS predicts upstream kinases based on substrate sequence specificity, protein-protein interactions and experimentally determined protein-protein interactions, combined with the high stringency setting.

Prior to analysis, by taking an advantage of our proteome data set, any of the proteins showed similar fold change in abundance in expression to the change in phosphorylation were removed in order to assured that the change in phosphorylation was not a result of change in protein expression. We found 223 phosphopeptides as putative substrates for a particular kinase. Members of the CMGC family (includes Cyclin-dependent kinases, Mitogen-activated protein kinases, Glycogen synthase kinases and CDK-like kinases) were predicted to phosphorylate the majority of phosphosites (**Figure 3.12**). The most predominant predicted kinase was the CMGC/CDK family and CMGC/MAPK subfamily, including ERK, JNK, and p38 kinases. Another predominant kinase was mTOR (**Figure 3.12**). Therefore, we decided to investigate if LAR regulates mTOR and JNK signalling pathways.

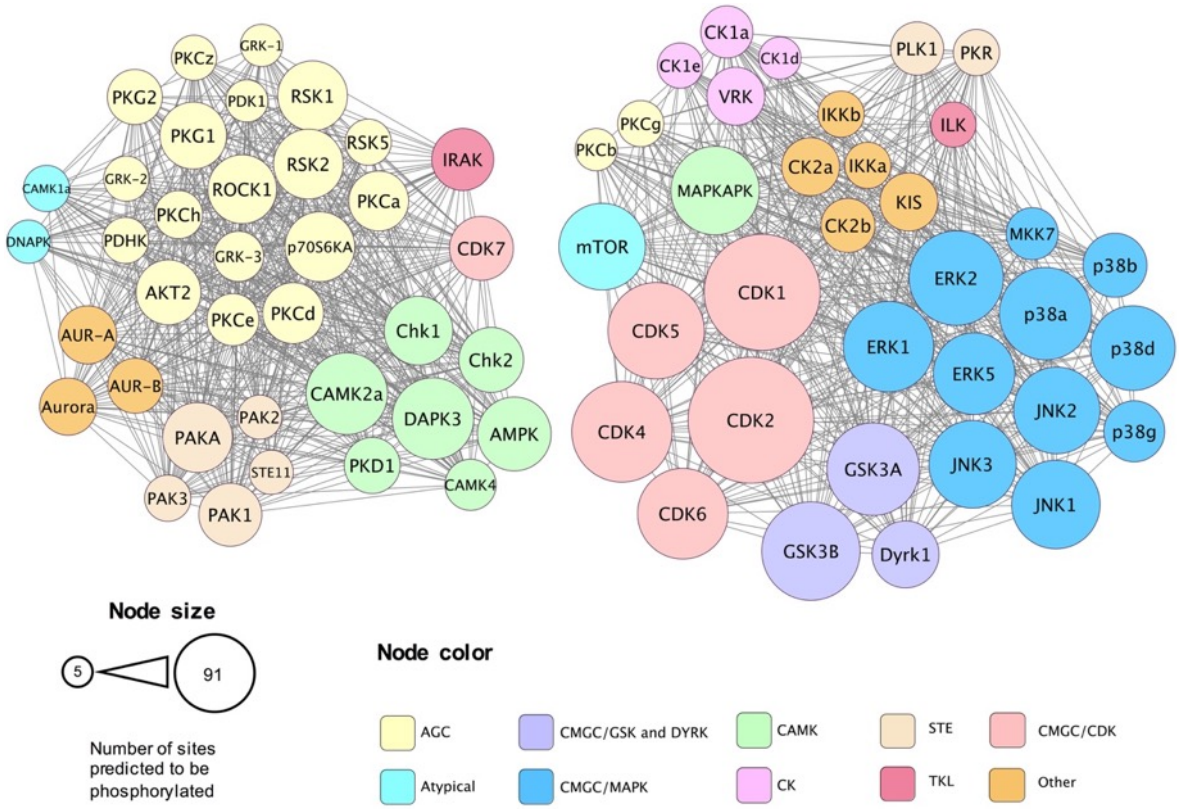

Figure 3.12. LAR Regulates Distinct Kinase Nodes. Phosphorylation sites regulated by LAR were searched using the kinase prediction tool GPS. All kinases predicted to phosphorylate at least five identified phosphorylation sites are displayed. Each node represents an individual kinase or group/family of kinases and colored according to kinase family (see key for details). An edge (line) connecting two nodes indicates that the corresponding kinase groups were predicted to phosphorylate at least one common residue (clustered nodes were predicted to phosphorylate similar residues). Node size corresponds to the total number of LAR regulated phosphorylation sites that were predicted to be phosphorylated by the corresponding kinase.

3.2.7. JNK signalling pathway is regulated by LAR

By using GPS tool, JNK1 was predicted for regulation of 53 motifs in our phosphoproteome data set (**Figure 3.12**). The predicted JNK substrates (53 motifs) were then submitted to Motif-x (Schwartz and Gygi, 2005) to evaluate these potential substrates. We found proline-directed phosphorylation motif [(P)-X-S/T-P] was enriched (**Figure 3.13**). This consensus motif has been found in most JNK substrates and can also be phosphorylated by MAPKs and CDKs (Bogoyevitch and Kobe, 2006, Davis, 2000).

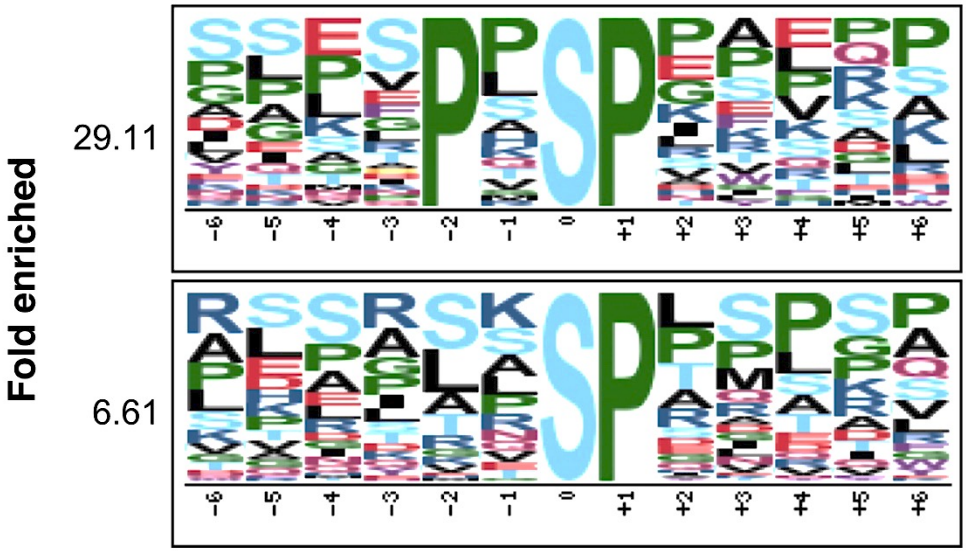

Figure 3.13. Identification of JNK consensus motif. Phosphorylation motifs that were enriched above the mouse proteome using Motif-x.

Number of interesting proteins belonging to these motifs were predicted to be regulated by JNK such as Stathmin1 (Stmn2), microtubule protein involved in regulation of destabilizing microtubule filament (Wittmann et al., 2004). JNK is known to phosphorylate Stmn2 at Ser^{62} and inhibit its affinity towards tubulin (Tararuk et al., 2006). Within our phosphoproteome data set, we found this site significantly reduced in LARΔP cells compared with WT. Taken together, these finding prompted us to investigate whether LAR regulates JNK signalling pathway.

Immunoblotting analysis showed a significant reduction in the activity of JNK in LARΔP cells when compared with WT in response to PDGF stimulation (**Figure 3.14**). MKK4 and MKK7 act as upstream of JNK and can activate JNK by the phosphorylation of Tyr^{185} and Thr^{183} residues, respectively (Davis, 2000, Lawler et al., 1998). The activity of MKK7 was also reduced in LARΔP cells (**Figure 3.14**). Active JNK leads to control the activity of many transcription factors including c-Jun, JunB, JunD, ATF2, Stat1 and Stat3 (Bogoyevitch and Kobe, 2006). For instance, JNK can activates c-Jun by the phosphorylation of Ser^{63} residue (Derijard et al., 1994). Therefore, we decided to investigate the activity of c-Jun in WT and LARΔP cells. In line with MKK7 and JNK data, a significant decreased was observed in the phosphorylation of c-Jun at Ser^{63} in LARΔP cells (**Figure 3.14**).

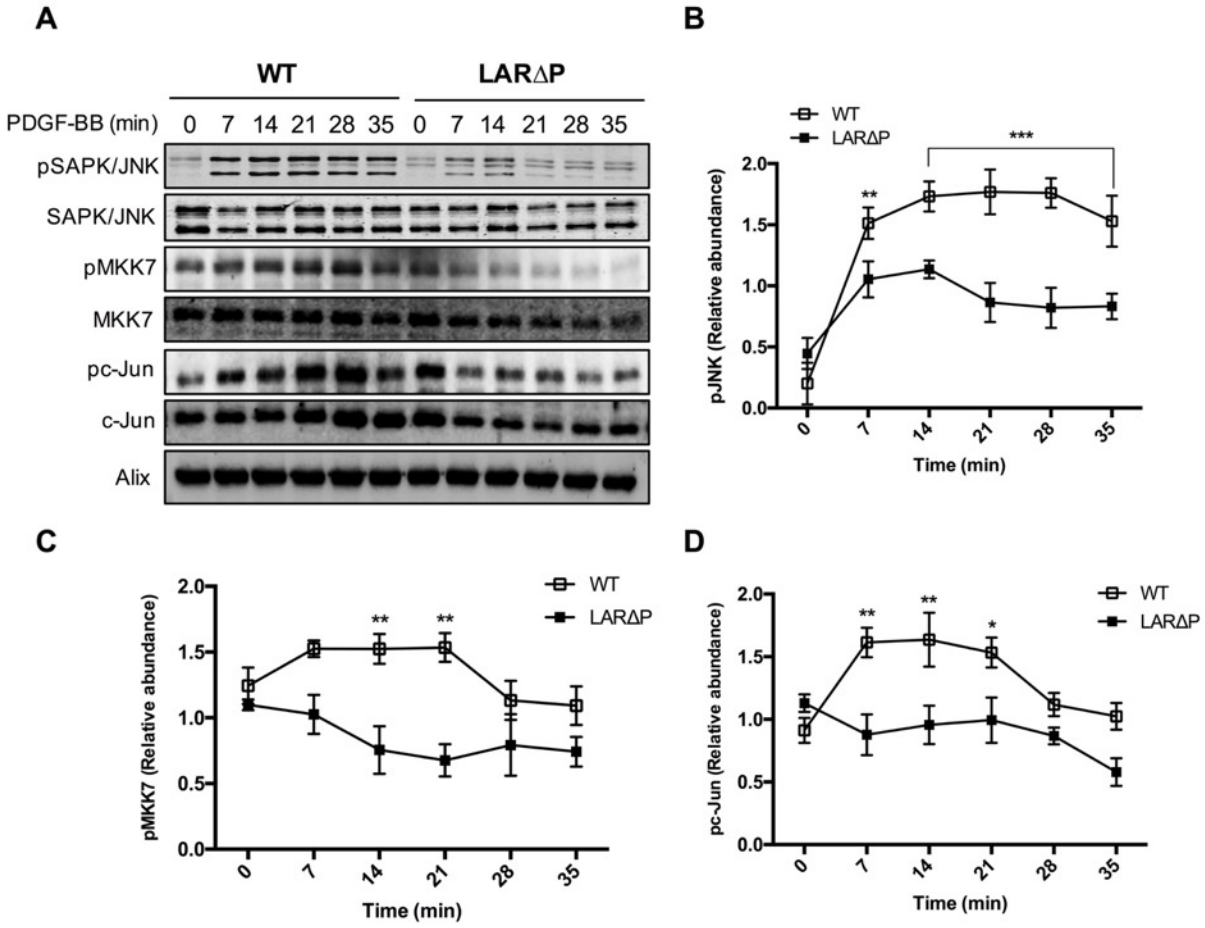

Figure 3.14. LAR Regulates the JNK signalling pathway. **A**, WT and LARΔP cells were stimulated with 20 ng/mL PDGF for differing periods of time as indicated. Levels of JNK Thr183/Tyr185, total JNK, MKK7 Ser271/Thr275, total MKK7, c-Jun Ser63, c-Jun and Alix in whole cell lysates were analyzed by Western blotting. **B**–**D**, Western blots (n=3) were quantified and ratios of phospho-protein to total protein were plotted over time and statistical significance was calculated using two-way ANOVA with Sidak's test (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).

Re-expression of WT LAR in LARΔP cells restored JNK activity (**Figure 3.15**). These data indicating that LAR is required for JNK activity and plays a role in regulating PDGF-mediated JNK signalling pathway.

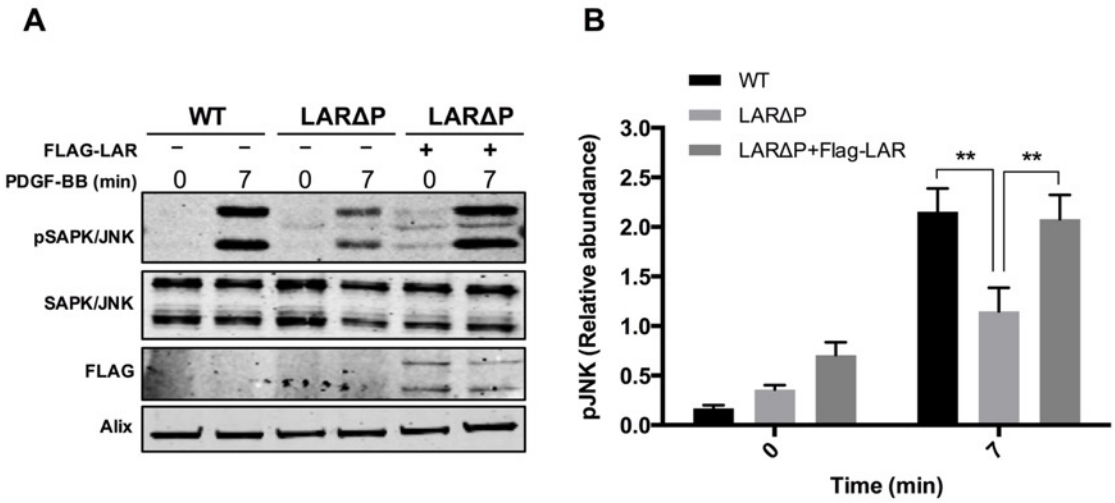

Figure 3.15. LAR is required for JNK activity. A, WT, LARΔP, and LARΔP cells transfected with Flag-WT-LAR were stimulated with 20 ng/mL PDGF for 7 min (this time point was used because the SILAC experiment was done at this time point and higher PDGFR phosphorylation was also recorded at 7 min). Levels of JNK Thr183/Tyr185, total JNK, Flag-LAR and Alix in whole cell lysates were analyzed by Western blotting. **B**, Western blots (n=3) from the rescue experiments were quantified and ratios of JNK Thr183/Tyr185 to total JNK were plotted and statistical significance was calculated using two-way ANOVA with Sidak's test (** $p<0.01$).

3.2.8. LAR regulates mTOR signalling

mTOR is an atypical serine/threonine protein kinase and can binds to several proteins to form two complexes, mTORC1 and mTORC2 to regulate a diverse cellular functions including protein synthesis, energy metabolism and cytoskeletal organisation (Laplante and Sabatini, 2012). The mTOR signalling pathway is tightly controlled by upstream regulators. It has been reported that Ras homolog enriched in brain (Rheb) GTPase can interacts with mTORC1 to promotes its kinase activity (Inoki et al., 2003). While tuberous sclerosis 1 (TSC1) and TSC2 act as a negative regulator for mTOR activity by converting the GTP-Rheb active form to GDP-Rheb inactive form (Tee et al., 2003).

In addition, mTOR is also regulated by Akt through directly phosphorylate TSC2 at Ser^{939} and Thr^{1462} thus promoting mTOR activation which in turn enhance Ribosomal protein S6 kinase (p70S6K) and Eukaryotic translation initiation factor 4E (eIF4E) activity (Inoki et al., 2005, Q. Yang and Guan, 2007).

Kinase prediction analysis revealed mTOR as a prominent node of regulation by LAR (**Figure 3.12**). In order to validate the bioinformatics data and to investigate whether LAR is regulating mTOR activity, we used antibodies targeting phosphorylated Ser^{2448} on mTOR and phosphorylated Thr^{389} on p70S6K, an mTOR downstream effector. The phosphorylation of mTOR on Ser^{2448} significantly increased upon stimulation with PDGF (**Figure 3.16A and B**). However, the absence of LAR phosphatase activity resulted in a significant reduction in the phosphorylation of this site (**Figure 3.16A and B**). In addition, western blotting analysis of the phosphorylation of p70S6K at Thr^{389} showed a similar response to that seen with mTOR phosphorylation establishing a noel role for LAR in regulation of mTOR activity (**Figure 3.16A and C**).

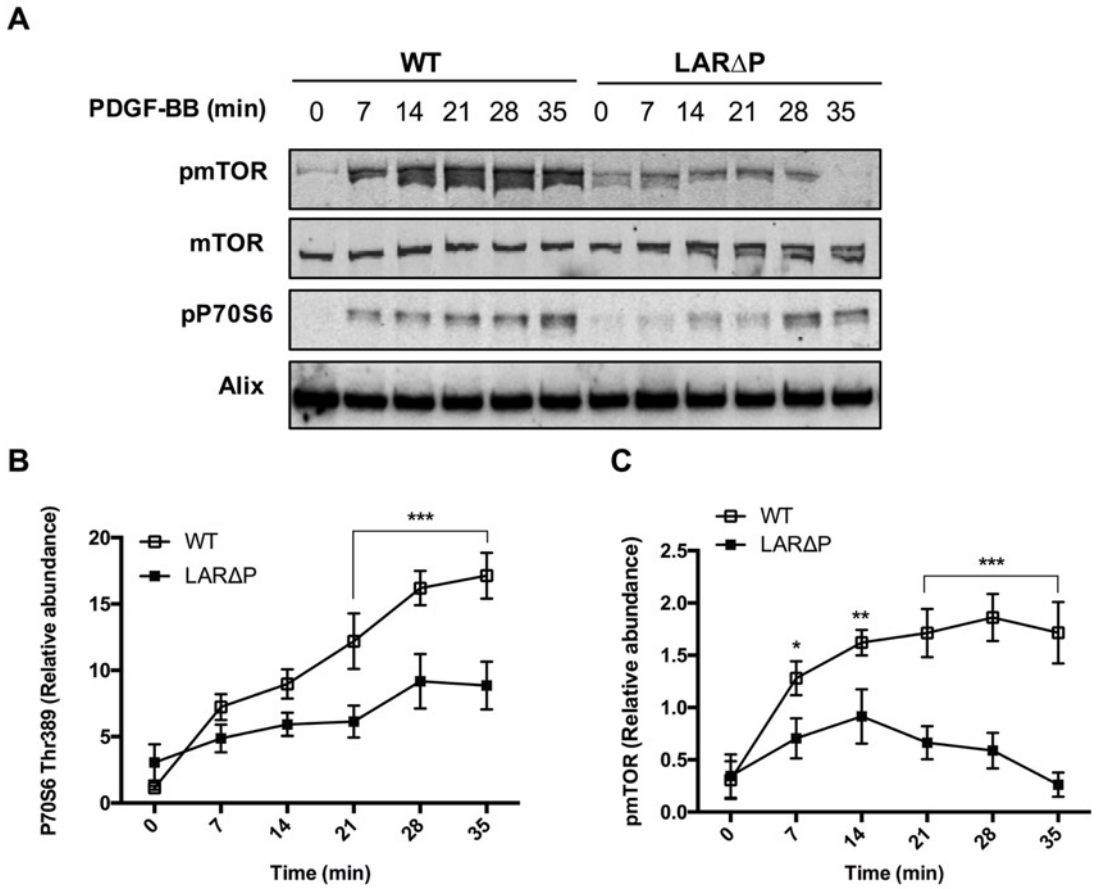

Figure 3.16. LAR regulates the mTOR signalling pathway. **A**, WT and LARΔP cells were stimulated with 20 ng/mL PDGF for indicated time periods. Levels of mTOR Ser2448, total mTOR, P70S6 Thr389, and Alix in whole cell lysates were analyzed by Western blotting. **B**, **C**, Western blots (n=3) were quantified and ratios of phospho-proteins to Alix were plotted over time and statistical significance was calculated using two-way ANOVA with Sidak's test (*** $p<0.001$; ** $p<0.01$; *$p<0.05$).

The phosphorylation of mTOR at Ser^{2448} was restored by re-expression of WT LAR in LARΔP cells (**Figure 3.17**) confirming a novel role for LAR in the regulation of mTOR signalling. It's noteworthy that the expression of WT LAR can also enhance the phosphorylation of mTOR at time point 0 indicating that LAR could regulate mTOR phosphorylation in PDGF-independent manner.

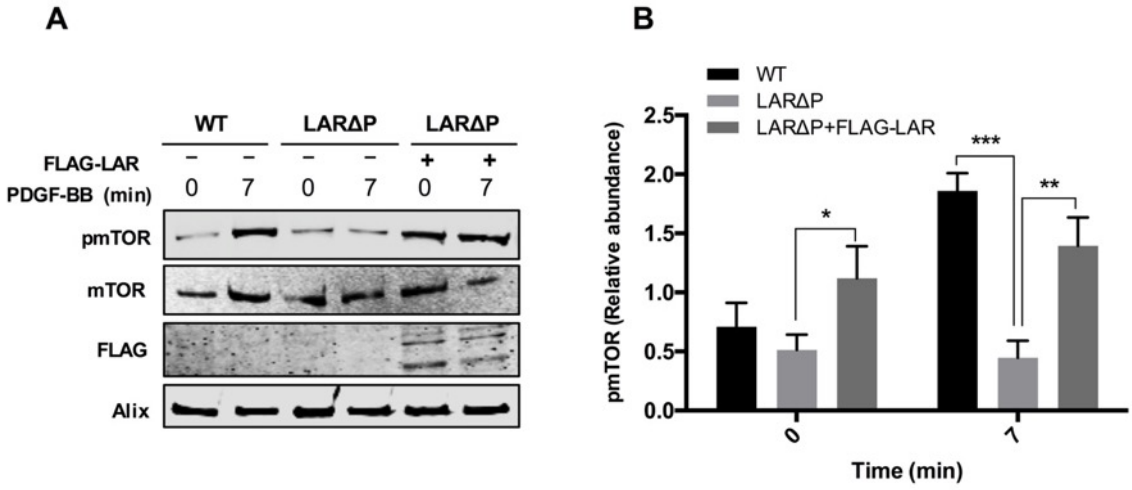

Figure 3.17. LAR is required for mTOR activity. A, WT, LARΔP, and LARΔP cells transfected with Flag-WT-LAR were stimulated with 20 ng/mL PDGF for 7 min. Levels of mTOR Ser2448, total mTOR, FLAG-LAR and Alix in whole cell lysates were analyzed by Western blotting. **B**, Western blots (n=3) from the rescue experiments were quantified and ratios of mTOR Ser2448 to Alix were plotted and statistical significance was calculated using two-way ANOVA with Sidak's test (*** $p<0.001$; ** $p<0.01$; * $p<0.05$).

It has been shown that JNK promotes mTORC1 activity by phosphorylation of Raptor, one of the mTORC1 component, at Ser^{863} (Fujishita et al., 2011). However, the underling mechanism on how JNK crosstalk with mTOR signalling remain unstudied. Therefore, we examined whether JNK control the activity of mTOR by applying JNK inhibitor (SP600125). SP600125 has been widely used to study JNK signalling (Bennett et al., 2001, Q. H. Guan et al., 2005, W. Wang et al., 2004). WT MEFs were treated with SP600125 or DMSO and the phosphorylation of mTOR at Ser^{2448} in response to PDGF stimulation was examined. The phosphorylated Ser^{2448} was reduced upon SP600125 treatment (**Figure 3.18**). SP600125 could inhibit JNK signalling as the phosphorylation of JNK and the downstream effector c-Jun were also decreased (**Figure 3.18)**. These finding demonstrate that JNK may involve in regulation of mTOR activity.

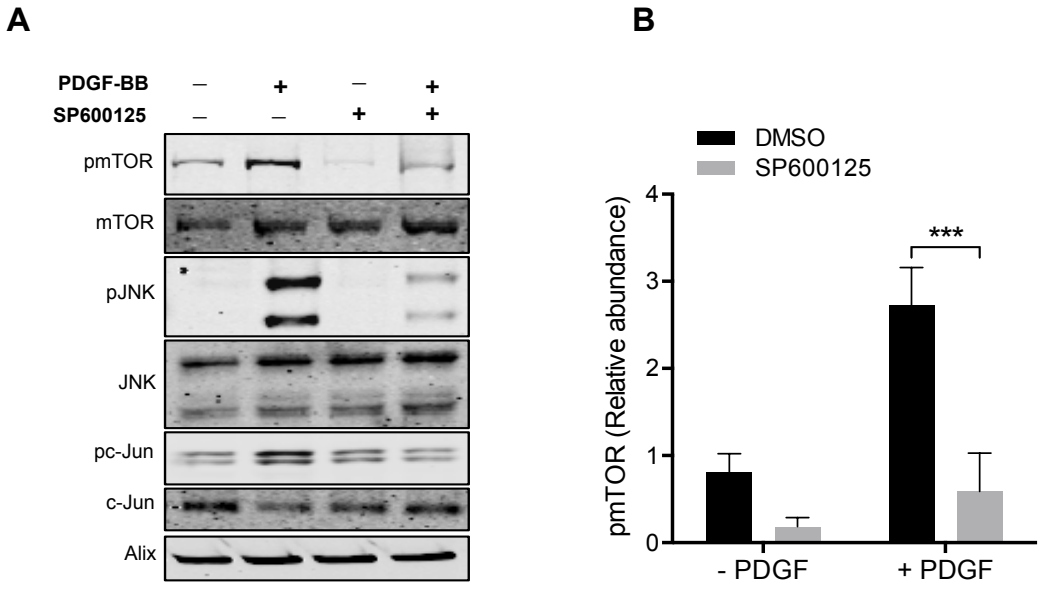

Figure 3.18. JNK regulates mTOR activity. A, WT MEFs were treated with an JNK inhibitor (SP600125) or DMSO (control) for 2 hrs before being stimulated with 20 ng/mL PDGF-BB for 14 minutes. Levels of pmTOR Ser2448, total mTOR, pJNK Thr183/Tyr185, total JNK, pc-Jun Ser63, total c-Jun and Alix in whole cell lysates were analyzed by immunoblotting. **B**, Western blots (n=3) were quantified and ratios of pmTOR to Alix were plotted and statistical significance was calculated using two-way ANOVA with Sidak's test (*** $p<0.001$).

3.3. Discussion

Post-translational modifications are involved in the regulation of multiple cellular functions (Jun Wang and Schwartz, 2016). Therefore, characterizing and understanding on how PTM regulated is substantial in biology of the cell in health and disease (Deribe et al., 2010). Signal transduction pathways that regulated by LAR have not been studied, therefore global quantitative phosphoproteomics and proteomics were carried out in order to provide an extensive study of signalling events regulated by LAR.

Within the phosphoproteome data set, 2559 unique phosphosites from 1311 proteins were identified with high localization scores (localization probability >0.75; score difference >5) in one or more biological replicates. A total of 270 phosphopeptides from 205 proteins showed a significant change in abundance ($p<0.05$; >1.5-fold change) in LARΔP cells upon PDGF stimulation. Of these, 255 (95%) are serine phosphorylation sites, 9 (3%) threonine, and 6 (2%) were tyrosine phosphorylation sites. In addition, a total of 103 phosphosites were significantly upregulated and 167 downregulated. It is possible that LAR contributes to the regulation of these phosphorylation sites via modulation of the activity of specific kinases, or via direct dephosphorylation of the tyrosine sites.

Most of the previous studies were focused on the role of LAR in the regulation of nervous system including neuronal development, growth, neuronal regeneration and axon guidance (Mélanie J Chagnon et al., 2004a, Sethi et al., 2010, Fang Wang et al., 2012, Julie S Zhang and Longo, 1995). However, GO terms analysis revealed an enrichment of LAR-mediated phosphorylation events on proteins which are involved

in many signal transduction pathways including actin cytoskeleton, adhesion, endocytosis and metabolism. Within phosphoproteome data set, TRIO, and β-catenin were identified. These proteins have been shown as a direct substrate for LAR (Debant et al., 1996, Dunah et al., 2005, Kypta et al., 1996, T. Muller et al., 1999). TRIO is an adaptor protein which acts as a GEF for Rac and Rho small GTPases (Debant et al., 1996). β-catenin a protein involved in regulation of cell-cell adhesions (Harris and Tepass, 2010). β-catenin is a substrate for LAR, resulting in a negative regulation of tyrosine phosphorylation and inhibition of epithelial cell migration (T. Muller et al., 1999). Within our data set, the phosphorylation of β-catenin at Ser^{191} was significantly reduced in LARΔP cells. This site has been shown to be essential for nuclear accumulation of β-catenin in response to Wnt and is regulated by JNK2 (X. Wu et al., 2008).

Kinase prediction and motifs analysis have identified a key role for LAR in regulating ERK, mTOR and JNK signalling pathways in response to PDGF stimulation. These data have been confirmed by immunoblotting. Interestingly, the re-expression of WT LAR in LARΔP MEFs rescued the activity of these signalling pathways providing further support that PDGF-induced mTOR, ERK and JNK pathways is LAR-dependent phenotype. Collectively, these results significantly expand the range of proteins implicated downstream of LAR and provide a comprehensive dataset for further investigation.

CHAPTER 4

LAR Protein Tyrosine Phosphatase Regulates Focal Adhesions via CDK1

4.1. Introduction

Cell adhesion to the extracellular matrix (ECM) components is essential for several fundamental cellular processes including cell signalling, differentiation, proliferation, motility and survival. Focal adhesions (FA) are composed of a number of protein molecules and mediate transfer of signals from ECM to the cytoplasm as well as nucleus and further regulate biological functions. For instance, integrins can bind to the extracellular molecules leading to autophosphorylation of focal adhesion kinase (FAK) resulting in activation of cascade of downstream signalling proteins (Guo and Giancotti, 2004). In addition, the role of receptor tyrosine kinase (RTK) such as platelet-derived growth factor receptor (PDGFR) in cell adhesion mediated via FAK has also been established (Sieg et al., 2000).

Many studies have found that *Drosophila* LAR (DLAR) play a role in remodelling synaptic actin (Johnson et al., 2006, Sethi et al., 2010). It has also been demonstrated that the LAR-interacting protein 1 (LIP.1) binds to LAR phosphatase domain (D2) and recruits it to focal adhesions (Serra-Pages et al., 1995), indicating that LAR could play a crucial role in cell adhesion. However, no evidence for the role of LAR in regulating cell adhesion is currently available. Therefore, we have investigated the role of LAR in cell adhesion.

The non-receptor tyrosine kinase Abelson murine leukaemia viral oncogene (c-Abl) is non-receptor tyrosine kinase which involved in regulating many signalling pathways such as proliferation, migration, morphogenesis and apoptosis (Hernandez et al., 2004, Keshet et al., 2015, Levy et al., 2008). It has shown to localized in cytoplasm, cell membrane, nucleus as well as actin cytoskeleton region (Van Etten, 1999). c-Abl is highly controlled and regulated by intramolecular interactions to prevent oncogenic activity of the protein. In 2004 Hantschel and Superti-Furga demonstrated that these interactions keep the kinases domain closed and inactive (Hantschel and Superti-Furga, 2004). The phosphorylation of Tyr^{245} and Tyr^{412} can cause conformation in order to activate c-Abl (Hantschel and Superti-Furga, 2004, Harrison, 2003). Nevertheless, any disruption in these interactions leads to transforming and oncogenic activity in c-Abl. C-Abl can interact and activate by PDGFβR in response to PDGF stimulation and it has been shown to play a crucial role in PDGFβR downstream signalling pathways (Plattner et al., 1999). Researchers indicated that c-Abl is phosphorylated and activated directly by binding to the PDGFβR via SH2 domain or indirectly by Src family kinases, which in turn phosphorylate Tyr^{245} and Tyr^{412} in activation loop of c-Abl protein (Plattner et al., 1999).

Cyclin-dependent kinase 1 (CDK1) is a serine/threonine kinase encoded by *cdc2* gene and a key regulator in cell cycle progression (Enserink and Kolodner, 2010). CDK1 activity is highly maintained and regulated by multistep process involving the association with cyclin B1, phosphorylation of Thr^{161} by a CDK activating kinase (CAK) and finally dephosphorylation of Thr^{14} and Tyr^{15} by CDC25 (Rhind and Russell, 2012).

Our results demonstrate that the deletion of LAR phosphatase domains leads to a significant reduction of focal adhesion complexes and cell adhesion to fibronectin compared with WT cells. To establish how LAR regulates cell adhesion, we used our phosphoproteome data set to identified a target regulated by LAR. As indicated in **Figure 3.12 (Chapter 3)**, CDK1 was predicated to phosphorylate the majority of phosphosites. Closer inspection of the phosphoproteome data revealed that loss of LAR phosphatase activity was associated with altered phosphorylation of CDK1 at Thr^{161}. Immunoblotting and microscopy analysis established that c-Abl acts downstream of LAR to regulate CDK1 activity via Akt.

4.2. Results

4.2.1. Cell adhesion and focal adhesion formation require LAR phosphatase activity

To analyse whether LAR might play a role in cell adhesion, the formation of adhesion complexes and cell adhesion to ECM were investigated. WT or LARΔP cells were serum-starved overnight before being stimulated with 20 ng/mL PDGF-BB for 14 minutes and stained using an anti-phospho-paxillin (Tyr^{118}) antibody to visualise adhesion complexes. Treatment of WT cells with PDGF resulted in increased in focal adhesions compared to untreated cells (**Figure 4.1A and B**). Since LARΔP cells can spread and could make focal adhesions under normal condition, however, treatment of LARΔP cells with PDGF resulted in significantly fewer focal adhesions compared with WT cells (**Figure 4.1A and B**).

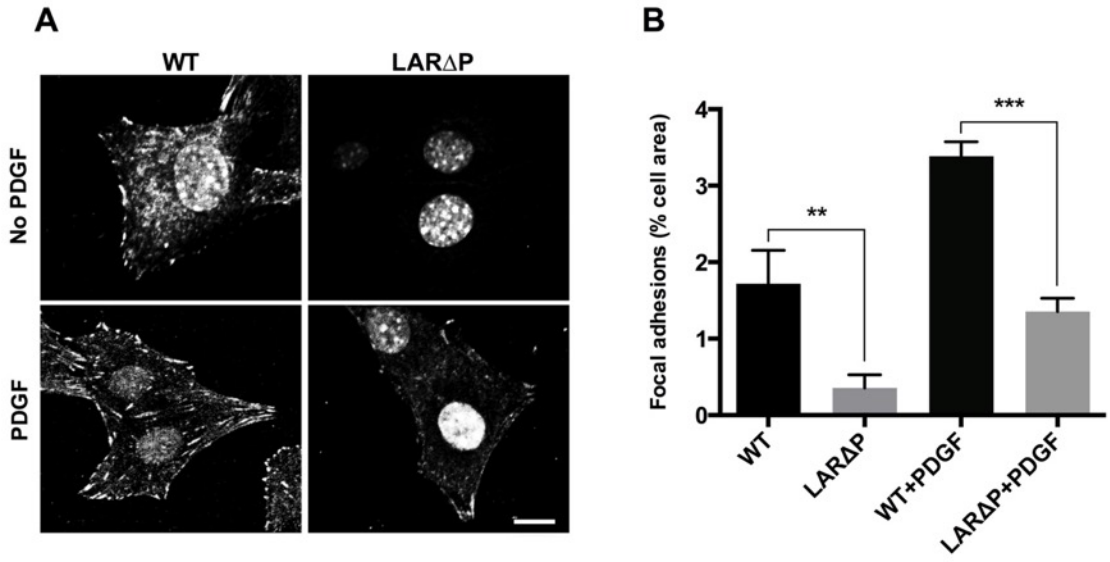

Figure 4.1. Loss of LAR phosphatase activity is associated with decreased adhesion complex formation. **A**, WT and LARΔP MEFs were serum-starved overnight before being treated with 20 ng/mL PDGF-BB for 14 minutes. Cells were fixed and permeabilised and then stained using an antibody against phosphorylated paxillin Tyr118 to visualise adhesion complexes. Nuclei were visualised using DAPI. Scale bar represents 20 μm. **B**, the cell area occupied by adhesion complexes was quantified using ImageJ and expressed as a percentage of the total cell area (one-way ANOVA with post hoc Tukey's test; ** $p<0.01$; *** $p<0.001$).

Having observed decreased adhesion complex formation in LARΔP cells, next we analysed whether LAR phosphatase activity contributes to cell attachment to ECM protein such as fibronectin. WT and LARΔP cells were allowed to attach to fibronectin in the presence or absence of 20 ng/mL PDGF-BB for 30 minutes (**Figure 4.2A**). LARΔP cells were significantly impaired in their ability to attach to fibronectin when compared to WT cells (**Figure 4.2A**). In the presence of PDGF a slight but not

significant increase in attachment to fibronectin was observed in WT cells but treatment with PDGF had no effect on LARΔP cells (**Figure 4.2A**).

To confirm the adhesion phenotype was specific for LAR, we used RNA interference to knockdown expression of LAR in WT cells. Transfection of WT cells with LAR siRNA oligos resulted in a significant decrease in cell attachment to fibronectin (**Figure 4.2B** and **C**). We also examined whether LAR regulates cell migration using wound healing assay and we found no significant changes between WT and LARΔP cells (**Figure 4.2D**). These findings indicated that LAR regulates the formation of adhesion complexes and cell attachment to fibronectin but not cell migration in MEFs.

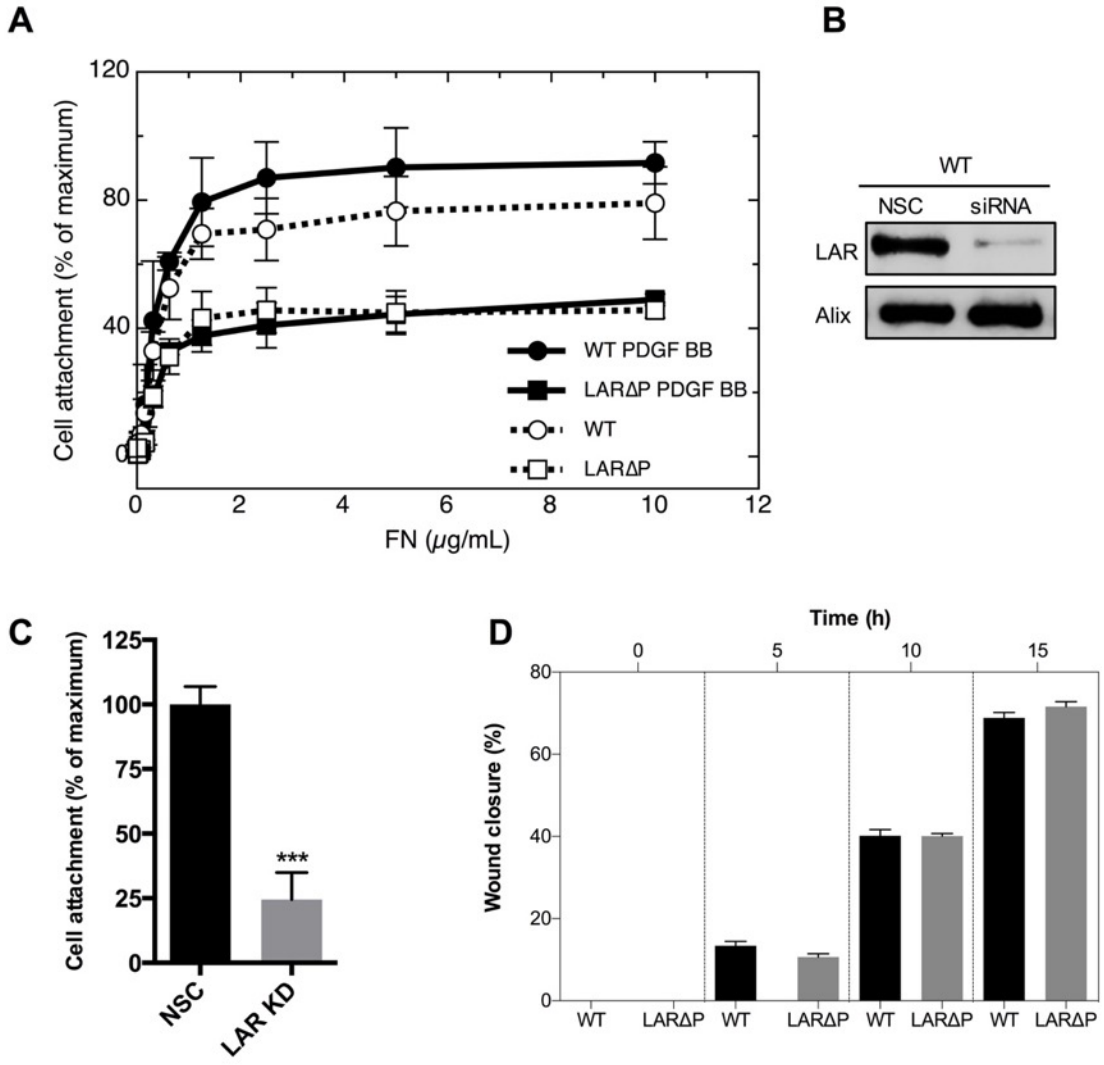

Figure 4. 2. LAR phosphatase is required for optimal cell adhesion to fibronectin. **A**, WT and LARΔP MEFs were allowed to attach to fibronectin for 30 minutes in the presence or absence of 20 ng/mL PDGF-BB. **B**, **C**, WT MEFs were transiently transfected with siRNA oligos targeted against LAR or with control non-silencing oligos (NSC). 48 hours after transfection cells were serum-starved overnight and either (**B**) cell lysates were prepared for immunoblotting with antibodies against LAR and Alix or (**C**) cells were allowed to attach to 10 μg/mL fibronectin as described in **A**. Statistical significance (n=3) was calculated using Student's T test (*** $p<0.001$).

4.2.2. Focal adhesion kinase is not affected by LAR

Focal Adhesion Kinase (FAK) is a non-receptor tyrosine kinase frequently linked to adhesion complex formation and cell adhesion (Mitra et al., 2005, Seong et al., 2011). However, recent study showed that the inhibition of FAK or Src is not affected adhesion complex composition (Horton et al., 2016). As FAK is also known to play a role in integration of integrin and growth factor signalling and can also phosphorylate paxillin on Tyr^{118} (Sieg et al., 2000), we asked whether LAR was regulating adhesion complex formation through FAK. To do this we used phospho-specific antibodies raised against Tyr^{397} and Tyr^{407} of FAK. Tyr^{397} is an autophosphorylation site, which when phosphorylated recruits c-Src which then further phosphorylates other tyrosine residues to which src-homology 2- (SH2) domain containing proteins can bind (Calalb et al., 1995). Phosphorylation of Tyr^{407} results in optimal FAK catalytic activity (Boivin et al., 2013). Consistent with immunostaining of adhesion complexes (**Figure 4.1**) we observe significantly reduced paxillin Tyr^{118} phosphorylation in both serum-starved and PDGF-stimulated LARΔP cells (**Figure 4.3A**). However, when we analysed FAK activity in LARΔP cells we observed no significant difference in phosphorylation of either Tyr^{397} or Tyr^{407} when compared to WT cells (**Figure 4.3B-D**). This would indicate that LAR does not regulate activity of FAK and would be consistent with previous observation that activation of c-Src is not regulated by LAR (Zheng et al., 2011). In summary, these data demonstrated that the regulation of adhesion complexes by LAR is independent of FAK activity.

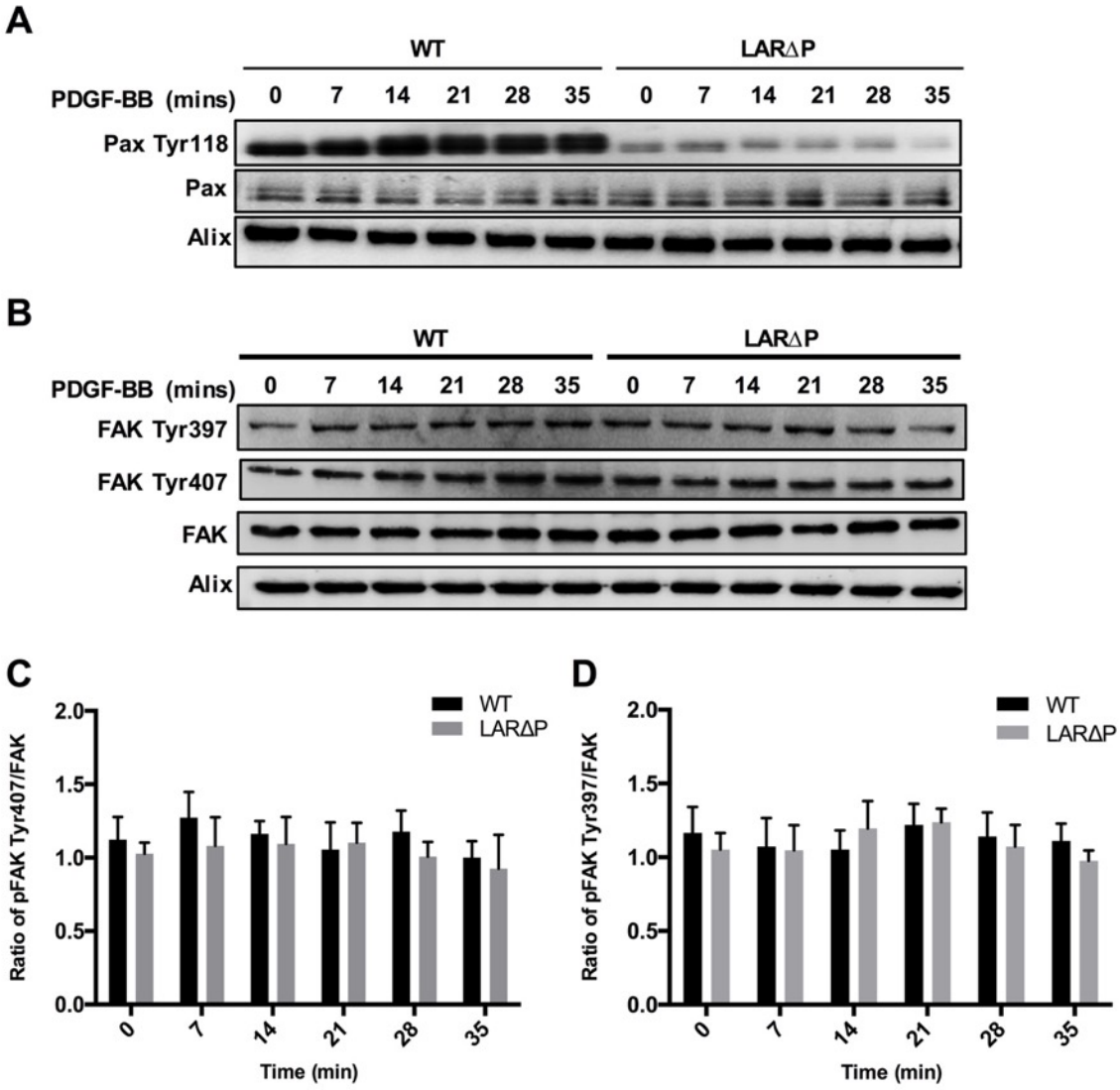

Figure 4. 3. FAK tyrosine phosphorylation is unaffected by loss of LAR activity. **A**, **B**, Serum-starved WT and LARΔP MEFs were stimulated with 20 ng/mL PDGF-BB for indicated time periods before protein lysates were prepared and immunoblotted using antibodies against (**A**) paxillin and phospho-paxillin Tyr118 or (**B**) FAK, phospho-FAK Tyr397 and phospho-FAK Tyr407. **C**, **D**, the ratio of either phosphorylated FAK Tyr407 (**C**) or phosphorylated FAK Tyr397 (**D**) to total FAK was calculated using densitometric analysis of immunoblots (n=3).

4.2.3. Phosphoproteomic analysis identifies a role for CDK1 in regulation of focal adhesions

To establish how LAR regulates cell adhesion, we used kinase predication tool (GPS) for our phosphoproteome data set to identified a target regulated by LAR. CDK1 was predominant and predicated to phosphorylate the majority of phosphosites as shown in **Figure 3.12 (Chapter 3).** We have identified 38 putative substrates for CDK1 and 50% of the 38 predicated substrates have been found in adhesome (Horton et al., 2015). CDK1 putative substrates are also including adhesion related-proteins including: Tenc1, a phosphoprotein that localized to focal adhesions (Lo, 2004, Moon et al., 2012); Tjp2, a protein involved in the regulation of adherent and tight junctions (Itoh et al., 1999) and Mllt4, a cell adhesion binding protein (Ikeda et al., 1999) (**Figure 4.4**).

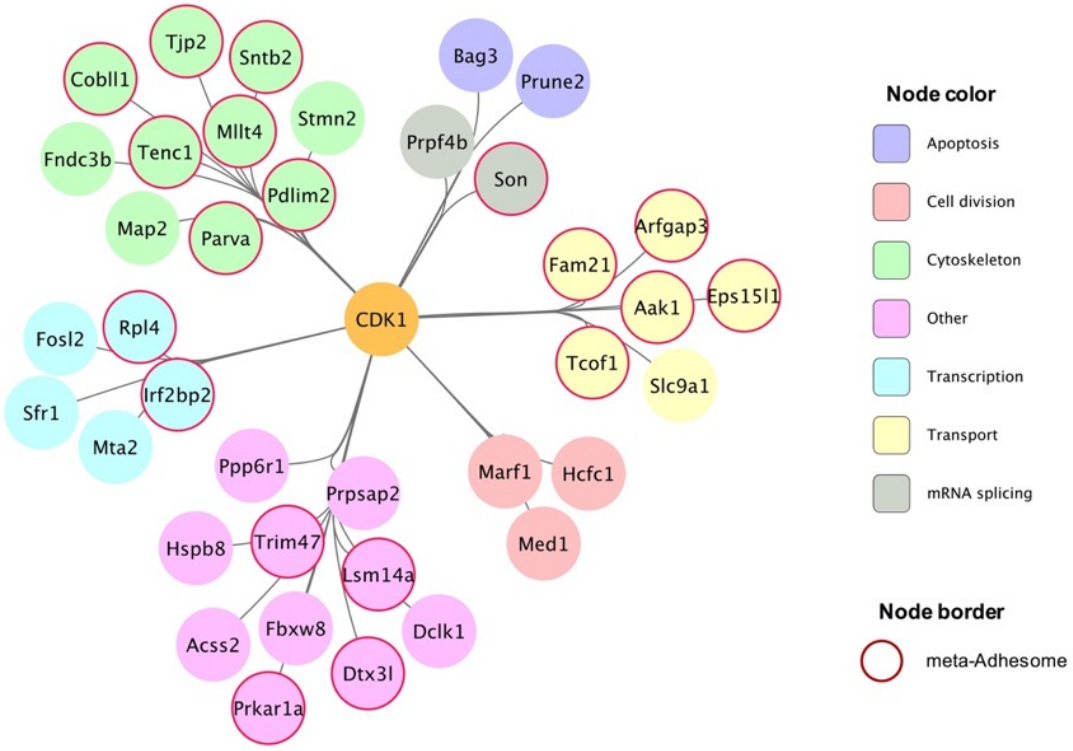

Figure 4. 4. Network of CDK1 putative substrates. CDK1 substrates were analysed using the Group-based Prediction System (GPS). Of 270 phosphopeptides, 38 were identified as potential CDK1 substrates. Protein-protein interaction network analyses were performed using Cytoscape. Nodes are clustered according to functional group and nodes identified as being part of the meta-adhesome are highlighted with a red border.

In addition, phosphoproteomics analysis identified Thr^{161} was down-regulated in LARΔP cells suggesting LAR is required for phosphorylation of this key regulatory residue in the activation loop of CDK1 (Solomon et al., 1992, Ubersax et al., 2003). Therefore, the role of CDK1 in cell adhesion was investigated and asked whether LAR regulates cell adhesion via CDK1. To confirm the phosphoproteomics data, we used a

phospho-specific antibody to detect Thr^{161} phosphorylation and hence CDK1 activation status in WT and LARΔP cells. The phosphorylation of CDK1 at Thr^{161} was higher in WT compared with LARΔP cells upon PDGF stimulation (**Figure 4.5A**). CDK activity also requires association with cyclin B1 during G2/M (Malumbres, 2014), we asked whether LAR might regulates this interaction. In fact, there were no differences in expression of cyclin B1 or the interaction with CDK1 (**Figure 4.5B**). Thus, our data indicate LAR phosphatase activity is required for phosphorylation of CDK1 Thr^{161} but not affecting the association with cyclin B1.

Having observed phosphorylation events in LARΔP cells consistent and identified CDK1 potential substrates which are adhesion-related proteins, we examined whether CDK1 might also regulate adhesion complex formation in WT MEFs. To visualise adhesion complexes, we immunostained cells using an antibody to phosphorylated Tyr^{118} on paxillin. Treatment of WT cells with RO-3306, a specific inhibitor of CDK1, resulted in significantly fewer focal adhesions (**Figure 4.5C**). To confirm that this phenotype is via CDK1 downstream of LAR, rescue experiment was done in LAR cells by transiently expressed a constitutively active form of CDK1 (T14A; Y15F; cannot be phosphorylated at Thr^{14} and Tyr^{15}) (Hagting et al., 1998). The expression of CDK-AF resulted in a significant increase in focal adhesions (**Figure 4.5D**). These finding indicate CDK1 act as a downstream of LAR phosphatase to regulate the formation of focal adhesion in MEFs.

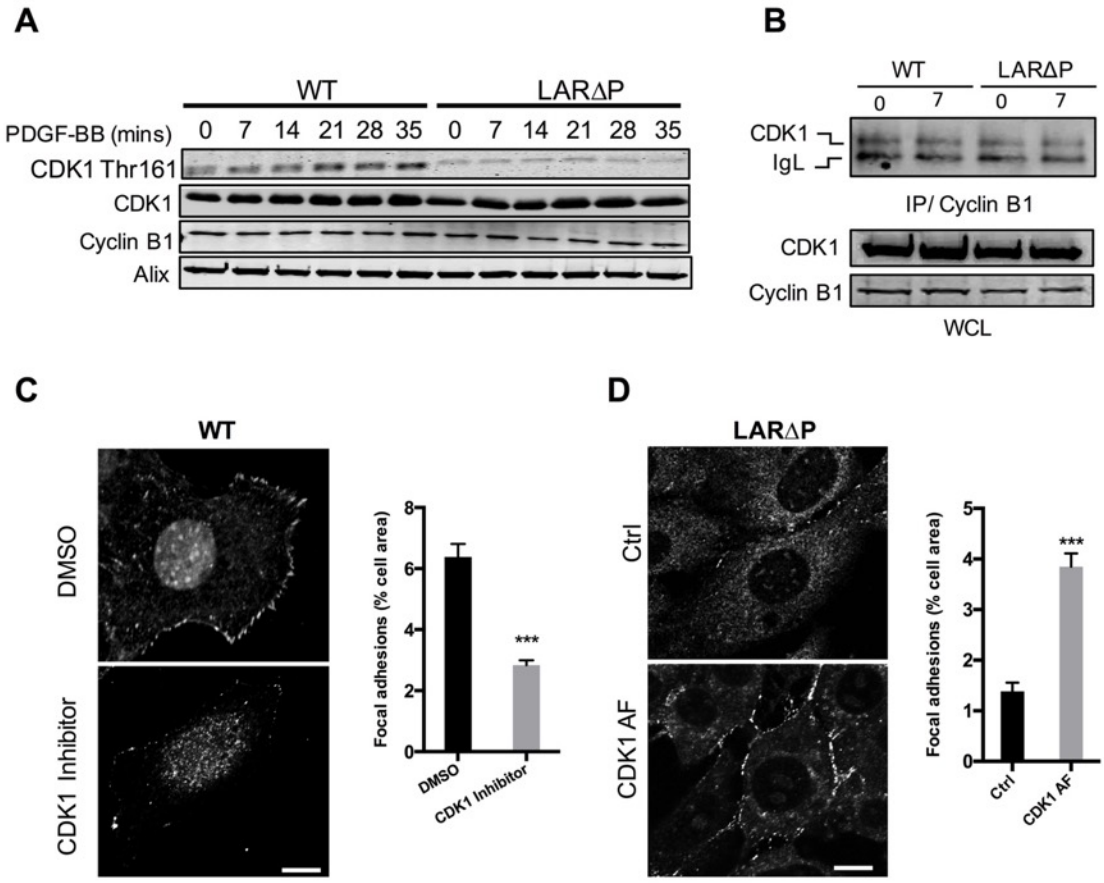

Figure 4. 5. CDK1 functions downstream of LAR to regulate focal adhesion complex formation. **A**, Serum-starved WT and LARΔP cells were stimulated with 20 ng/mL PDGF-BB for increasing periods of time before protein lysates were prepared and immunoblotted using antibodies against CDK1, CDK1 phospho-Thr161, cyclin B1 and Alix. **B**, WT and LARΔP MEFs were stimulated with 20 ng/mL PDGF-BB for 7 min, Cyclin B1 immunoprecipitated and immunoblotted with CDK1 or Cyclin B1. **C**, WT MEFs were treated with a CDK1 inhibitor (RO-3306) or DMSO (control) for 60 minutes before being stimulated with 20 ng/mL PDGF-BB for 14 minutes, fixed and stained with a phospho-paxillin (Tyr118) antibody to visualise focal adhesions. Scale bar represents 20 μm. **D**, LARΔP cells were transfected with constitutively active form of CDK1 (CDK1-AF) before being stimulated with 20 ng/mL PDGF-BB for 14 minutes and stained with a phospho-paxillin (Y118) antibody. The cell area occupied by adhesion complexes was calculated using ImageJ and expressed as a percentage of the total cell area (Student's T test; *** $p<0.001$). Scale bar represents 20 μm.

4.2.4. LAR phosphatase signals to CDK1 through Akt

Activation of the serine/threonine kinase Akt downstream of PI3K is a well-established PDGF-regulated pathway, and previous work has established that LAR regulates PDGF-mediated activation of Akt (Zheng et al., 2011). Activation of Akt requires phosphorylation of Thr^{308} in the activation loop (Alessi et al., 1996). Therefore, to confirm that LAR regulates Akt downstream of PDGFβR, phospho-specific antibody raised against Thr^{308} was used. In control, serum-starved WT cells, the stimulation with PDGF resulted in a rapid increase in phosphorylation of Akt at Thr^{308} whereas in LARΔP cells phosphorylation of Thr^{308} was significantly reduced (**Figure 4.6A** and **B**).

Regulation of CDK1 activity is complex but Akt1 has previously been reported to be required for phosphorylation of CDK1 at Thr^{161} (Nogueira et al., 2012) suggesting Akt may be part of the pathway downstream of LAR and PDGFβR leading to activation of CDK1 and regulation of adhesion complex formation. To identify whether LAR regulates adhesion complex formation via Akt and CDK1, we first analysed activity of CDK1 in WT cells treated with a specific Akt inhibitor (InSolution Akt Inhibitor VIII). Treatment of PDGF-stimulated WT cells with this inhibitor resulted in a reduction of Thr^{161} phosphorylation on CDK1 suggesting Akt-dependent activation of CDK1 (**Figure 4.6C**). To analyse whether Akt was also playing a role in LAR-dependent cell adhesion we analysed adhesion complex formation in WT cells treated with the Akt inhibitor (**Figure 4.6D**). We observed a decrease in adhesion complex formation compared to control, vehicle (DMSO) treated cells providing further evidence of a novel role for LAR in regulation of cell adhesion via CDK1.

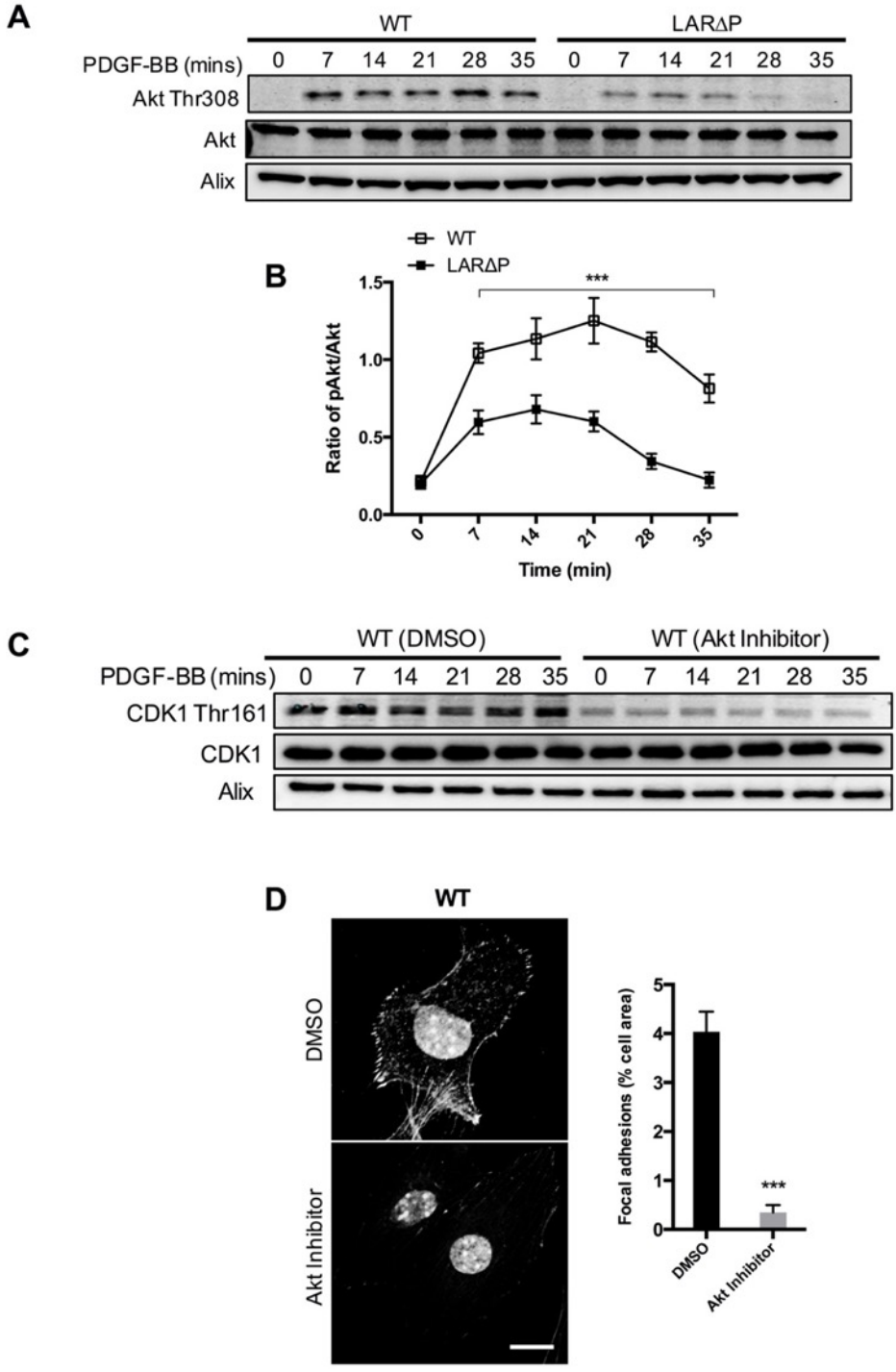

Figure 4. 6. Akt is required for LAR-mediated regulation of CDK1 and adhesion complex formation. **A**, Serum-starved WT and LARΔP MEFs were stimulated with 20 ng/mL PDGF-BB for increasing periods of time before protein lysates were prepared and immunoblotted using antibodies against Akt, phospho-Akt Thr308 and Alix. **B**, Densitometric analysis of data

from 3 independent experiments using ImageJ and statistical significance was calculated using two-way ANOVA with Sidak's test (*** $p<0.001$). **C**, Serum-starved WT MEFs were stimulated with 20 ng/mL PDGF-BB for increasing periods of time in the presence of Akt inhibitor (InSolution Akt Inhibitor VIII) or DMSO (control) before protein lysates were prepared and immunoblotted using antibodies against CDK1, phospho-CDK1 Thr^{161} and Alix. **D**, WT MEFs were treated with an Akt inhibitor (InSolution Akt Inhibitor VIII) or DMSO (control) overnight before being stimulated with 20 ng/mL PDGF-BB for 14 minutes and stained with a phospho-paxillin (Tyr118) antibody. The cell area occupied by adhesion complexes was calculated using ImageJ and expressed as a percentage of the total cell area (Student's T test; *** $p<0.001$). Scale bar represents 20 μm.

4.2.5. c-Abl is a mediator connecting LAR and Akt/ CDK1

It has been previously shown that LAR-mediated inhibition of the protein tyrosine kinase c-Abl and was required for maximal PDGF receptor signalling (Zheng et al., 2011). C-Abl is also involved in regulation of integrin-mediated cell adhesion and actin cytoskeletal reorganisation (Lewis and Schwartz, 1998, J. Y. Wang, 2014, Woodring et al., 2003). Therefore, we decided to examine whether c-Abl is a substrate for LAR. HEK 293T cells were transiently transfected with either LAR WT or LAR substrate trapping mutants LAR C/S (C1548S); LAR D/A (D1516A) (**Figure 4.7A**) (W. J. Wang et al., 2007). Protein tyrosine phosphatases (PTP) substrate trapping mutants have been commonly used to identified physiological substrates. These mutations and name 'Substrate-trap' were firstly discovered by Tonks lab (Flint et al., 1997, Jia et al., 1995, Sun et al., 1993).

To this end, the when the PTP binds to phosphorylated tyrosine residue on the substrate for dephosphorylation, substrate can trap in the catalytic pocket of the protein tyrosine phosphatase (Blanchetot et al., 2005, Flint et al., 1997). By using this method, we identified c-Abl as direct substrate for LAR and can binds to LAR WT and LAR substrate trapping mutants (**Figure 4.7B**). Interestingly, a reduction was observed in total phosphotyrosine on Abl when compared to cells expressing Abl only (**Figure 4.7B**). These data clearly demonstrate that c-Abl is a direct substrate for LAR in mammalian cells.

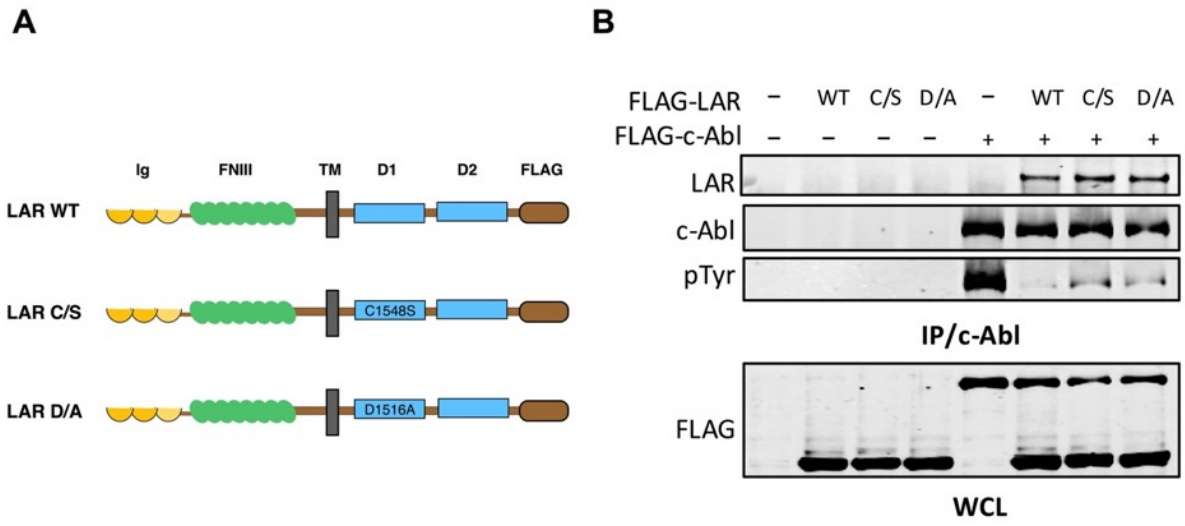

Figure 4. 7. Substrate-trapping mutants approach identified c-Abl as a substrate for LAR. A, Schematic structure of LAR substrate-trapping mutants. Full-length FLAG-tagged wild-type LAR (WT), substrate-trapping mutants C/S (Cys1548 in D1 domain replaced by Ser1548) and D/A (Asp1516 in D1 domain replaced by Ala1516). Ig, immunoglobulin-like domain; FNIII, fibronectin type III repeat domain; TM, transmembrane; D1, protein tyrosine phosphatase domain 1; D2, protein tyrosine phosphatase domain 2. **B**, FLAG-tagged wild-type LAR (WT) and substrate-trapping mutants of LAR along with WT c-Abl were either expressed alone or co-expressed in HEK 293T cells. c-Abl was immunoprecipitated using a c-Abl antibody and immunoprecipitates were immunoblotted with either LAR, c-Abl or total phosphotyrosine (PY99) antibodies. To control for expression levels, whole cell lysates (WCL) were immunoblotted with FLAG antibody.

After identified LAR interacted with c-Abl, next we tested whether c-Abl involved in regulation of CDK1 or Akt. To do so, LARΔP cells were treated with c-Abl inhibitor (AG957) and the activity of Akt and CDK1 were assessed. Inhibition of c-Abl resulted in increased in activity of Akt and CDK1 (**Figure 4.8**). These finding suggest that c-Abl could be a mediator linking LAR with Akt/CDK1 and establishing a signalling pathway connecting LAR and cell adhesion.

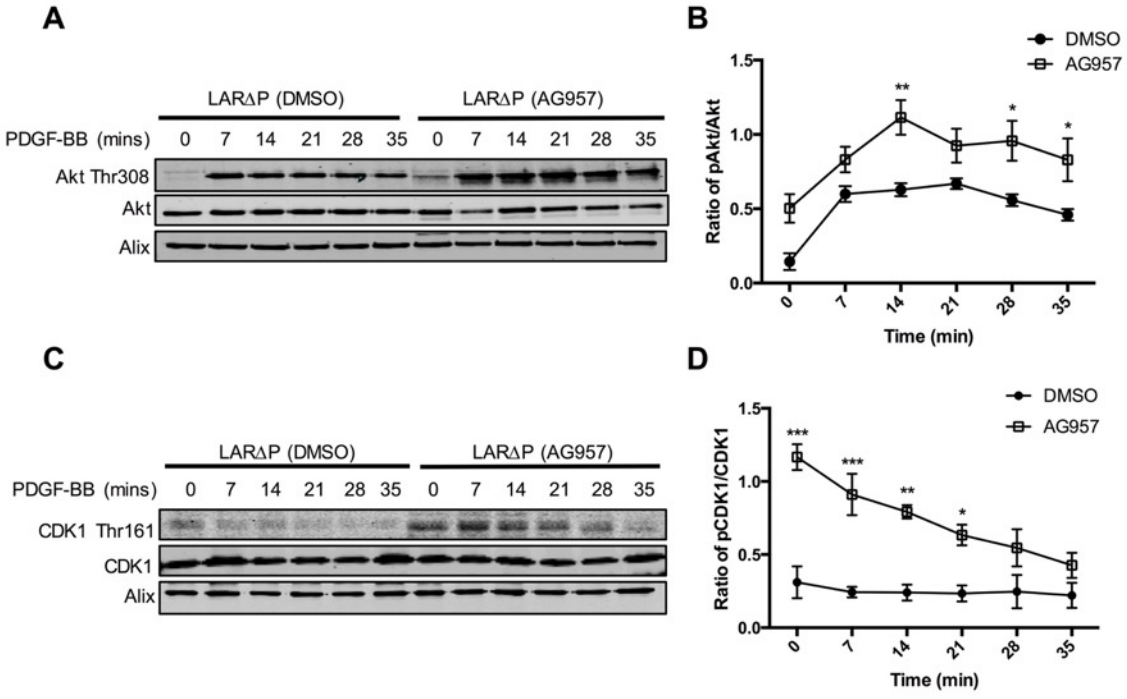

Figure 4. 8. Inhibition of c-Abl restored the activity of Akt and CDK1. **A**, Serum-starved LARΔP cells were stimulated with 20 ng/mL PDGF-BB for increasing periods of time in the presence of a c-Abl inhibitor (AG957) or DMSO (control) before protein lysates were prepared and immunoblotted using antibodies against Akt, phospho-Akt Thr308 and Alix, or CDK1, phospho-CDK1 Thr161 and Alix (**C**). **B**, **D**, both CDK1 and Akt blots from 3 independent experiments were analysed using ImageJ and statistical significance was calculated using two-way ANOVA with Sidak's test (** $p < 0.01$; * $p < 0.05$).

4.3. Discussion

Most of the previous studies were focused on the role of LAR in the regulation of nervous system including neuronal development, growth, neuronal regeneration, axon guidance and have been mostly done on the *Drosophila* ortholog, DLAR (Mélanie J Chagnon et al., 2004a, Sethi et al., 2010, Fang Wang et al., 2012, Julie S Zhang and Longo, 1995). DLAR has been shown to localize to synaptic junctions for the regulation of axonal guidance through Rho family GTPases (Bateman et al., 2000, Kaufmann et al., 2002, C. Pawson et al., 2008). Here, a significant decreased in cell adhesion and the formation of focal adhesion were observed in the absence of LAR activity demonstrating a key role for LAR in regulation of cell adhesion to fibronectin in mammalian cells.

kinase predication analysis of our phosphoproteome data set was used to identified a target regulated by LAR. CDK1 was predominant and predicated to phosphorylate the majority of phosphosites. It was identified 38 putative substrates for CDK1 and 50% have been found in adhesome (Horton et al., 2015). CDK1 is a serine/threonine kinase which is a key regulator of cell cycle progression (Enserink and Kolodner, 2010). CDK1 activity is highly maintained and regulated, one of which is the phosphorylation of Thr^{161} by a CDK7 (Rhind and Russell, 2012). However, phosphoproteome analysis showed no significant change in phosphorylation of either Ser^{164} or Thr^{170} on CDK7, the phosphorylation of these sites are required for activation of CDK7 (Larochelle et al., 2001) suggesting that LAR is regulating the activity of CDK1 through an alternative mechanism.

CDK1 has been reported to localize to focal adhesions and plays a role in the regulation of integrin-based cell adhesion and motility (Manes et al., 2003, Robertson et al., 2015). Therefore, the role of CDK1 in cell adhesion was investigated. Phosphoproteomics and western blotting analysis revealed that LAR is required for the activation of CDK1. Interestingly, the regulation of focal adhesion complexes by LAR was through Akt and CDK1 pathway. Surprisingly, however, no significantly differences in the activity of FAK in both cell lines (**Figure 4.1 and 4.2**), suggesting that LAR could regulate cell adhesion in PDGF-independent manner. By using LAR substrate-tapping mutants, this phenotype was mainly regulated by c-Abl demonstrating a key role for c-Abl in mediating LAR-induced the phosphorylation of CDK1. Taken together, these findings reveal a novel pathway downstream of LAR that regulates cell adhesion in c-Abl, Akt and CDK1 dependent manner.

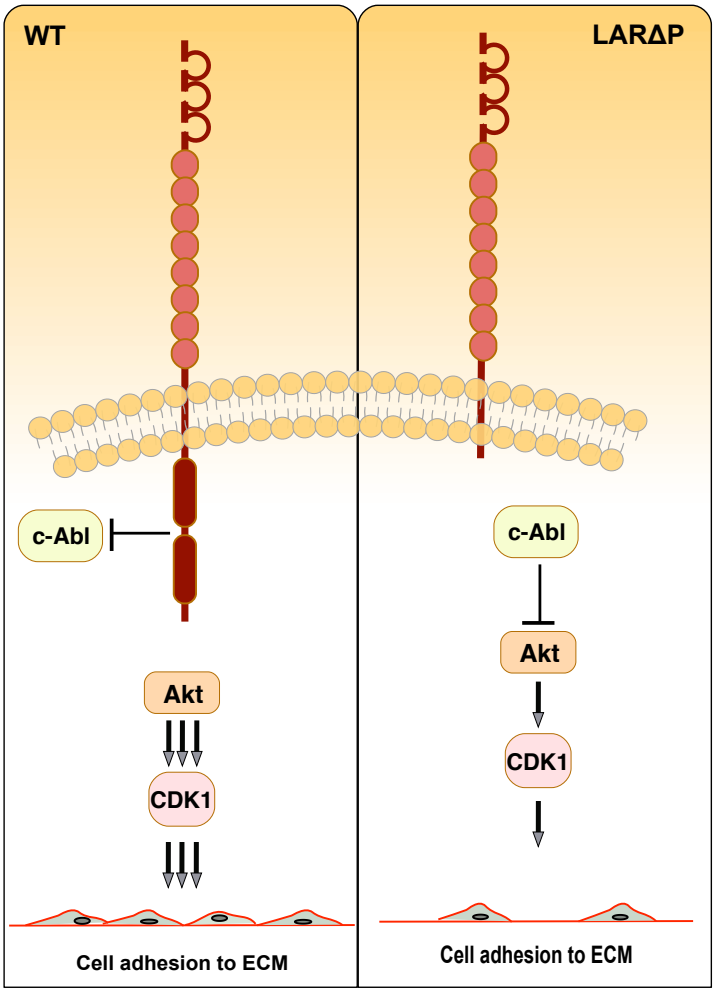

Figure 4. 9. Model showing how LAR phosphatase regulates cell adhesion via CDK1. In wild type MEFs, LAR dephosphorylates and inactivates c-Abl resulting in enhanced Akt and CDK1 signalling, leading to increased cell adhesion to extracellular matrix (ECM). In the absence of LAR phosphatase activity, c-Abl inhibits Akt, resulting in decreased CDK1 activity and decreased cell adhesion to extracellular matrix.

CHAPTER 5

Quantitative Phosphoproteomics Reveals a Role for Collapsin Response Mediator Protein 2 in PDGF-Induced Cell Migration

5.1. Introduction

Platelet Derived Growth Factor (PDGF) was first identified in platelet extracts as a potent mitogen for fibroblasts and endothelial cells (Waterfield et al., 1983). PDGF receptors are expressed by cells of mesenchymal origin and the cognate PDGF ligands are expressed by overlying epithelial cells indicative of paracrine signalling from epithelium to the underlying mesenchyme. For example, in the lung PDGFR alpha is expressed in the lung mesenchyme and PDGFA is expressed in the epithelium (Ataliotis and Mercola, 1997). This pattern is seen in multiple organs and tissues such as gut, kidney, gonads, somites and nervous system (Lindahl et al., 1998). These data show that the principle cellular targets for PDGF action are cells of mesenchymal origin. The PDGF signalling axis is implicated in regulation of tumour/stromal cell interactions (Cao, 2013, Heldin, 2013).

PDGF-BB produced by tumours of epithelial origin acts upon adjacent connective tissues cells, pericytes and vascular smooth muscle cells to regulate tumour angiogenesis and tumour-associated fibrosis (Tsai and Yang, 2013). PDGF activated perivascular cells also promote metastasis via tumour-associated macrophages and act on tumour cells that have undergone epithelial-mesenchymal transition (EMT) to promote migration through the extracellular matrix (Tan et al., 2015, Y. Yang et al., 2016). PDGF induced cell migration has been widely studied through small GTPases

Rac1, ERK MAPK and JNK kinases (Doanes et al., 1998, Ridley et al., 1992, Zhan et al., 2003). Collapsin response mediator protein-2 (CRMP2), also known as Dihydropyrimidinase-related protein 2; DPYSL2, is a phospho-protein that highly expressed in nervous system and mediate cellular processes upon external stimuli such as ephrins, neurotrophins and semaphorins (Uchida et al., 2005, Yamashita et al., 2007). CRMP2 has been mainly studied in nervous system and found to regulate a fundamental function such as cytoskeletal remodeling, neurotransmission and axon guidance through interaction with microtubules (Arimura et al., 2004, Inagaki et al., 2001, B. P. Liu and Strittmatter, 2001). Higher phosphorylated form of CRMP2 has been found in Alzheimer's disease suggested the contribution of CRMP2 in the development of this disease (Cole et al., 2007, Soutar et al., 2009). It has been previously demonstrated that glycogen synthase kinase-3 beta (GSK3β) can phosphorylates CRMP2 at Thr^{509}, Thr^{514} and Ser^{518} thereby inhibit CRMP2 activity leading to microtubule depolymerisation and destabilization (Gu et al., 2000, Wakatsuki et al., 2011). Despite the biological significance of these activities relatively little is known about the downstream intracellular pathways that mediate these responses beyond canonical RTK signalling pathways such as PLC, AKT and MAPK/ERK (Heldin and Lennartsson, 2013). To gain further insight into how PDGFs mediate their biological effects we analysed a differential phosphoproteomics dataset of PDGF-stimulated mouse embryo fibroblasts (MEFs). This shows that, in MEFs, PDGFβR activation by PDGF-BB elicits a broad range of phosphorylation events mediated by downstream Ser/Thr kinases which impact on mediators of cytoskeletal function and cell motility. In particular, we show CRMP2 is dephosphorylated in response to PDGFβR activation and is required for PDGF-induced cell migration.

These results demonstrate an intersection between PDGF and Ephrin signalling with broad implications for tissue repair and tumor development.

5.2. Results

5.2.1. Global quantitative phosphoproteomics of the PDGF signalling in MEFs

To identify novel PDGF downstream effectors, we determined the phosphoproteome of mouse embryonic fibroblasts (MEFs) in response to PDGF stimulation. Two populations of MEFs cells were SILAC-labeled by culturing them in light R0K0 or medium R6K4 SILAC media (**Figure 5.1**). Cells were left unstimulated or stimulated with PDGF-BB for 7 min. In total, 989 phosphosites from 611 proteins were detected with high localization scores (localization probability >0.75; score difference >5) in one or more experimental replicates.

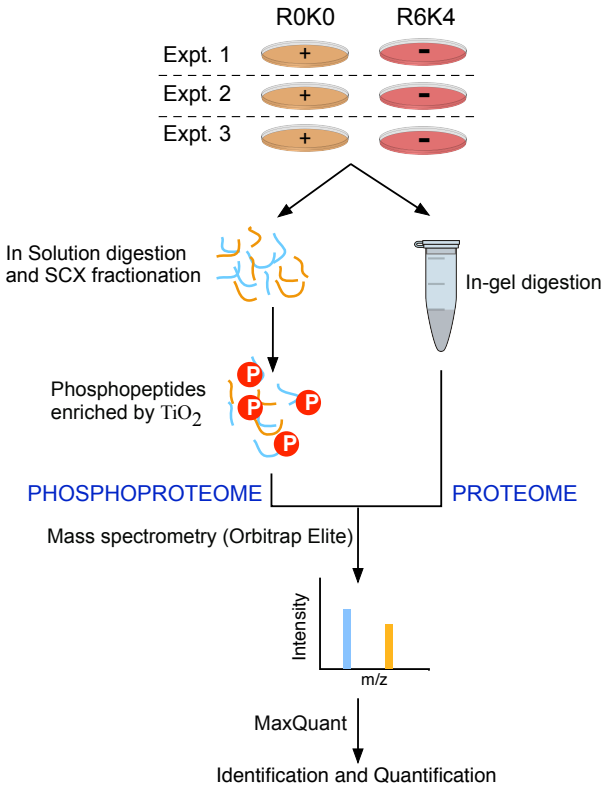

Figure 5. 1. Schematic overview of experimental design. Plus (+) stimulated with PDGF for 7 min, minus (-) nonstimulated.

Correlation coefficients for the peptide ratios measured across the biological and technical replicates, ranged from 0.77 to 0.93 (**Figure 5.2A**), indicating good biological reproducibility. Within the phosphoproteome data set, the phosphosites were composed of 896 (90.6%) serine, 76 (7.6%) threonine and 17 (1.7%) tyrosine phosphorylation sites (**Figure 5.2B**).

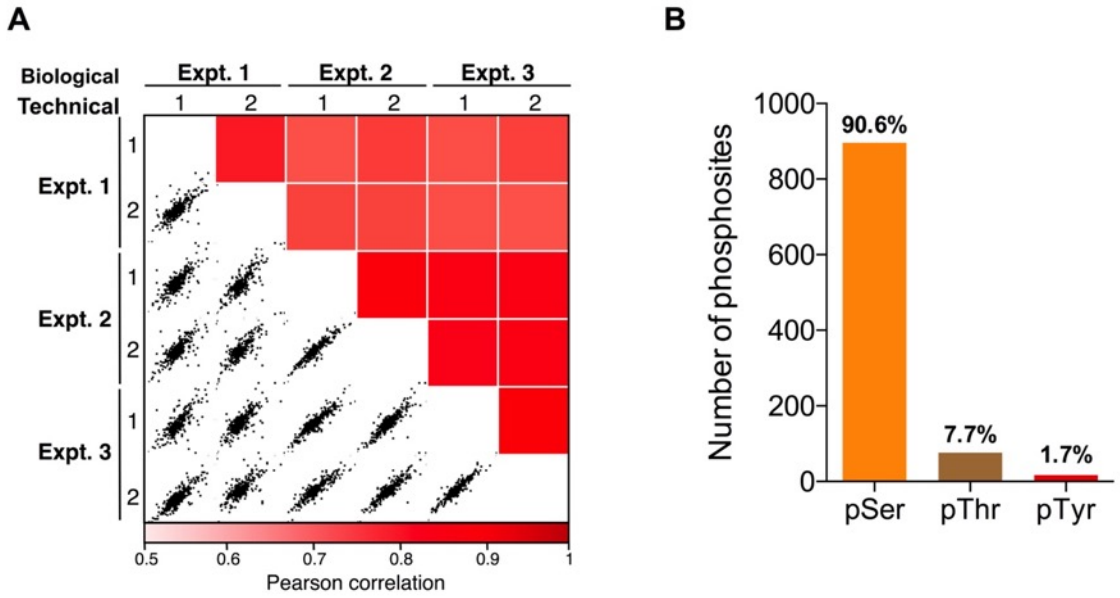

Figure 5. 2. The biological reproducibility and classification of phosphorylated peptides within phosphoproteome data set. A, Heat map of Pearson correlation coefficients and multi scatter plots demonstrating reproducibility between phosphoproteome of biological and technical replicates. **B**, Numbers of serine, threonine and tyrosine phosphorylation sites identified within our PDGF-regulated phosphoproteome data set are presented.

Out of these, 161 phosphorylation sites showed a significant change in abundance ($p < 0.05$; >1.5-fold change). A total of 116 phosphosites were upregulated and 45 downregulated upon PDGF treatment (**Figure 5.3B**). Within the proteomic data set, a total of 703 proteins were identified in two or more biological replicates. Not surprisingly, there is no significantly differences in protein abundance upon 7min PDGF stimulation (**Figure 5.3A**).

We have observed proteins that have been shown to be involved in PDGF signalling. For instance, ERK1/2 are extensively studied and to be hyperphosphorylated in response to growth factors such as PDGF and mediated diverse physiological functions such as cell growth, survival, cell adhesion, migration and differentiation (Nishimoto and Nishida, 2006, Qi and Elion, 2005, Z. Yao and Seger, 2009). In line with many other studies, we found the phosphorylation of ERK1 is highly increased on Tyr^{205} (ratio; 15.10) and Thr^{203} (ratio; 14.13). ERK2 is also hyperphosphorylated on Tyr^{185} (ratio; 16.77). The phosphorylation of these sites on ERK1/2 is essential for the kinase activity. We have also identified increase in the phosphorylation of Bcl2-associated agonist of cell death (BAD) at Ser^{136} upon PDGF stimulation. This site is a target for Akt and the phosphorylation of Ser^{136} on BAD leads to enhance cell survival (Datta et al., 1997).

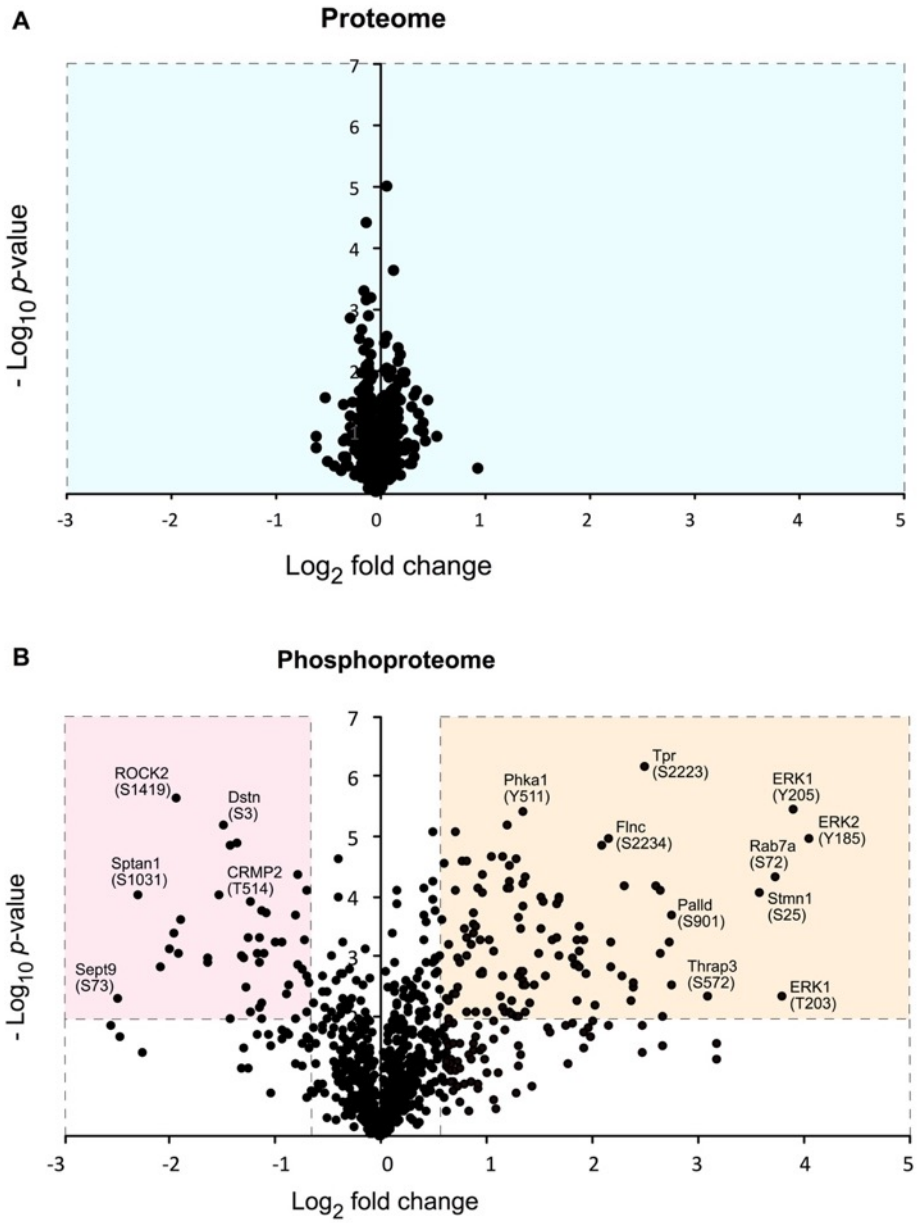

Figure 5. 3. Global phosphoproteomics and proteomics to identify novel PDGF signalling components. Volcano plots showing the ratio (log2 fold change) and significance (–log10 Benjamini-Hochberg adjusted p-value) of the proteome (**A**) and phosphoproteome (**B**) in response to PDGF stimulation. Significantly upregulated phosphopeptides are marked in light brown box and the downregulated phosphopeptides are marked in pink box (adjusted p value <0.05; >1.5 fold-change).

5.2.2. Novel regulatory phosphosites regulated by PDGF

From 161 phosphosites, we identified 110 (67%) that have not been reported as a PDGF regulatory sites (PhosphoSitePlus) (Hornbeck et al., 2012) and are considered novel (**Figure 5.4**). Known and novel PDGF regulatory proteins were cluster according to their cellular compartments (**Figure 5.4**). Cellular compartments analysis show that phosphoproteins localized in almost every part in the cell indicating the diversity of PDGF signalling. Within the phosphoproteomics data set, we found insulin receptor substrate 2 (IRS2), adaptor proteins that bind to the insulin receptor and regulate insulin signalling pathways. IRS2 can binds directly to insulin receptor (IR) leading to recruits p85-p110 heterodimer to their substrate, PI-4,5P2, at the cell membrane which in turn initiate PI3K/Akt signalling pathways (Hemmings and Restuccia, 2012, Lizcano and Alessi, 2002). We observed Ser^{303} on IRS2 and was largely upregulated upon PDGF stimulation (**Figure 5.4**). Interestingly, it has been shown that the activation of PKC isoforms, β and δ by phorbol esters (PMA) or the induction of angiotensin II significantly induce the phosphorylation of Ser^{303} on IRS2 in endothelial cells leading to inhibit insulin signalling (Park et al., 2013). Taken together, it has been reported that PDGF activates PKC via PLCγ (Weiqun Li et al., 1994b, Moriya et al., 1996) suggesting a novel role for PDGF as a negative regulator for the insulin signalling.

The most significant upregulated response after ERK1/2 was seen for Ras-related protein Rab7a (Rab7a) (**Figure 5.3B**). Rab7a is a small GTPase protein involved in the regulation of endo-lysosomal trafficking (T. Wang et al., 2011). The activity of Rab7a is regulated by GEF (guanine-nucleotide exchange factor) and GAP (GTPase activation protein). It has been shown that HOPs (homotypic fusion and protein sorting

complex) acts as GEF for Rab7 (Wurmser et al., 2000), whereas TBC1D15 serve as GAP (X. H. Zhang et al., 2005). Recent study revealed that the phosphorylation of Rab7a on Ser^{72} inactivate the protein and impaired the recruitment to endosomal membranes and delays the transport of EGFR from early to late endosomes (Shinde and Maddika, 2016). It suggests that PDGFR activates an unkown kinase which in turn phosphorylates Ser^{72} on Rab7a leading to delay the EGFR trafficking indicating a crosstalk endocytosis signalling between two major RTKs, PDGFR and EGFR. In addition, we observed Slit-Robo GTPase-activating protein 3 (srGAP3), Rho GAP protein which involved in the development of the nervous system and has been reported as Robo receptor downstream protein (Y. Ma et al., 2013, Wong et al., 2001). We detected increase in phosphorylation of srGAP3 on Ser^{858}. This site has previously been reported to be phosphorylated by protein kinase A (PKA) which in turn promotes GAP activity of srGAP3 toward Rac1 leading to inhibit the cytoskeletal reorganisation in neurons (M. Li et al., 2015b).

Following stimulation, 45 novel PDGF-regulated phosphosites were observed to be significantly downregulated include Rho-associated protein kinase 2 (ROCK2; Ser^{1362}), Neurofibromin (NF1; Ser^{2517}), CLIP-associated protein 1 (CLASP1; Ser^{1193}), Parva (Ser^{14}), collapsin response mediator protein 2 (CRMP2; Thr^{514}) and collapsin response mediator protein 4 (CRMP4; Thr^{514}). These proteins are mainly involved in regulation of nervous system homeostasis including development, axon formation and growth cone guidance (Aizawa et al., 2001, Blaise et al., 2012, Charrier et al., 2003, M. Chen et al., 2013, Montani et al., 2009, Trovo-Marqui and Tajara, 2006), suggesting a common set of PDGF effectors exist in both fibroblasts and the nervous system and demonstrates that, in addition to the autophosphorylation and

phosphorylation of the PDGFR downstream proteins, PDGF can also induce the dephosphorylation of key downstream effectors.

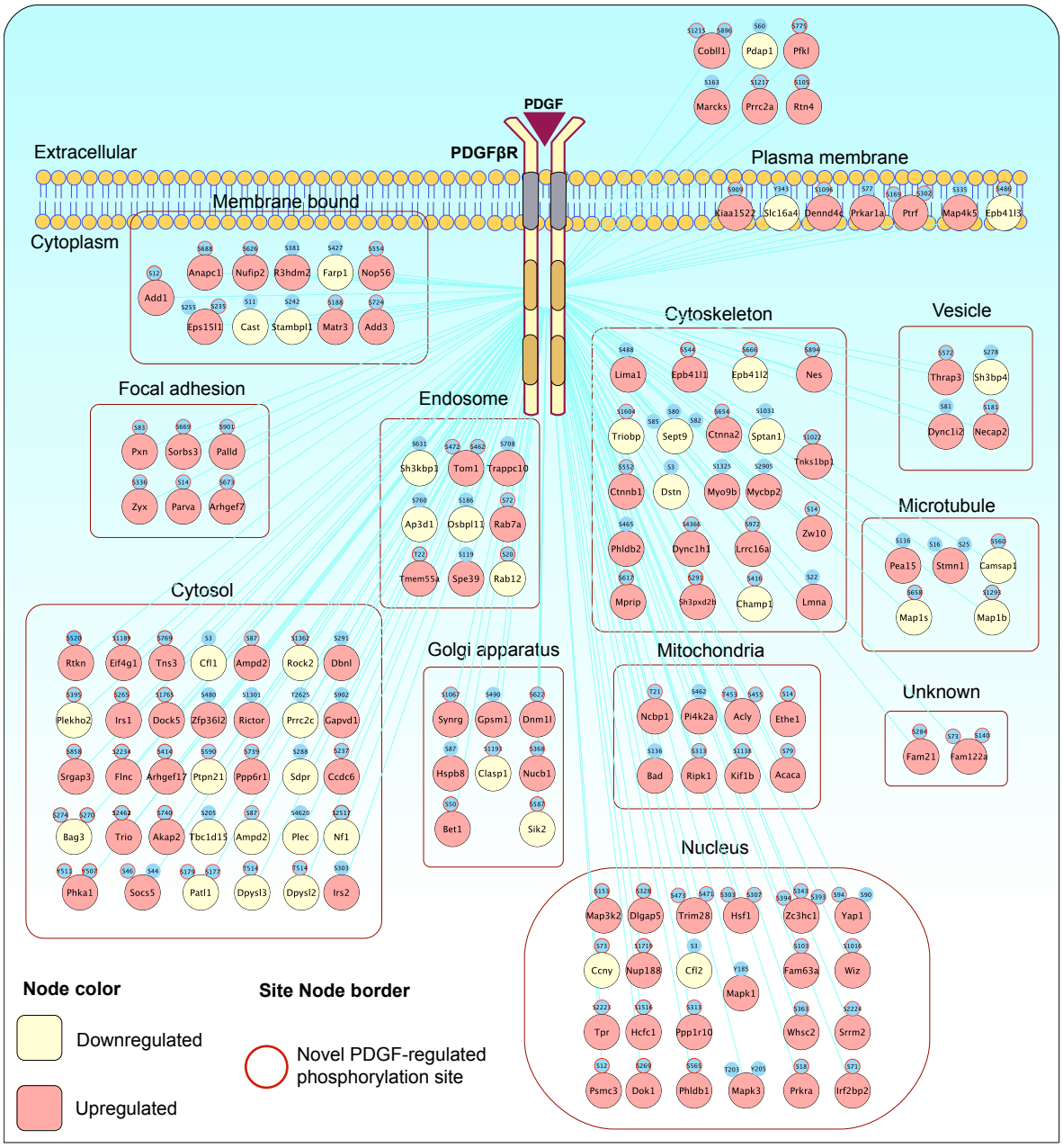

Figure 5. 4. Novel phosphosites are regulated by PDGF. All the identified Phosphopeptide and their sites were clustered according to their cellular component. Yellow node indicates downregulated phosphosites and red indicates upregulation. Sites with border are consider novel PDGF downstream effectors.

5.2.3. Functional analysis of the PDGF-regulated phosphoproteome

GO terms analysis revealed a number of actin cytoskeleton and microtubule proteins are enriched in our phosphoproteome data set including LIM domain and actin-binding protein 1 (Lima1), Rho guanine nucleotide exchange factor 17 (Arhgef17), Band 4.1-like protein 1 (Epb41l1) and Stathmin (Stmn1) (**Figure 5.5**). We observe increases in abundance of phosphopeptides for these proteins in response to PDGF stimulation. We also note the enrichment of biological processes associated with proteins involved in cell adhesion and migration including Reticulon-4 (Rtn4; also known as Nogo protein), a protein involved in the regulation of neuron development such as neurite growth and axon-axon adhesion and also has been shown to promotes the formation of endoplasmic reticulum tubules in neurons (Mathis et al., 2010, Petrinovic et al., 2010). We have found increases in phosphorylation of Nogo at Ser^{105} in PDGF-stimulated MEFs compared with non-stimulated.

Other prominent phosphopeptide linked by GO analysis to cell adhesion is paxillin (PXN). PXN is an adaptor protein involved in modulation of focal adhesions and mediated cell adhesion pathways (Huang et al., 2003, Schaller, 2001). PDGF is known to regulates PXN activity by promoting the phosphorylation of Tyr^{118} (Abedi and Zachary, 1995, Panetti, 2002). We have not identified this site on PXN in our phosphoproteomics data set, but instead we identified the phosphorylation of Ser^{83}. It has been reported that p38MAPK and ERK1/2 can phosphorylate PXN at Ser^{83} to regulate neurite extension and chemotaxis in neurons (Huang et al., 2004, S. H. Lee et al., 2012).

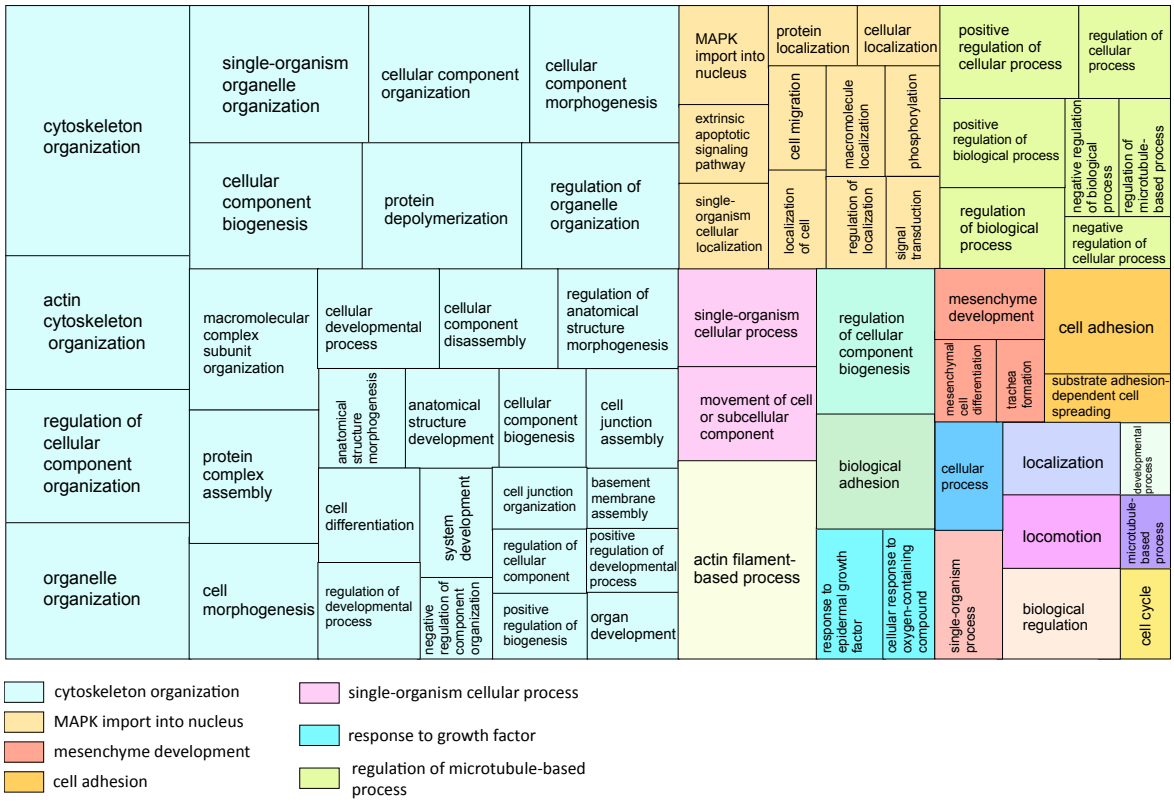

Figure 5. 5. GO terms analysis of the PDGF-regulated phosphoproteome. Proteins containing the 161 PDGF regulated phosphosites were analyzed in DAVID to identify enriched GO Terms. Biological processes are visualised using REVIGO as a Tree map and indicated by color key. The size of the box is indicated the number of genes in that category.

KEGG pathway analysis of PDGF-regulated proteins revealed enrichment of signalling pathways implicated in cell migration including focal adhesions, regulation of actin cytoskeleton, adherens junctions and transendothelial cell migration (**Figure 5.6A**). In addition, KEGG analysis of the dataset also identified neurotrophin signalling and axon guidance as PDGF-regulated processes (**Figure 5.6A**). To identify

which kinases are activated upon PDGF stimulation, we used the Kinase Enrichment Analysis (KEA) (Lachmann and Ma'ayan, 2009). KEA is a web-based software tool for the predication of kinase-substrate interactions and linking mammalian proteins with the upstream kinases that potentially phosphorylate them. KEA analysis revealed a significant enrichment of kinases for ribosomal protein S6 kinase (RSK2) (**Figure 5.6B**). It has been reported that RSK2 promotes cell motility and invasion by mediating the activity of integrin and actin cytoskeleton rearrangement (Gawecka et al., 2012, Kang et al., 2010, C. J. Lee et al., 2015, Sulzmaier and Ramos, 2013). RSK2 can also promote migration in epithelial cells through FGFR2-p38 kinase axis pathway (Czaplinska et al., 2014). KEA analysis also identified over-presented of other kinases include dual specificity mitogen-activated protein kinase kinase (MEK1) and c-Jun N-terminal kinase (JNK1) (**Figure 5.6B**). The activity of both kinases are known to be regulated by growth factors such as PDGF and they involved in regulation of many cellular functions including cell migration (Westerlund et al., 2011, Zhan et al., 2003).

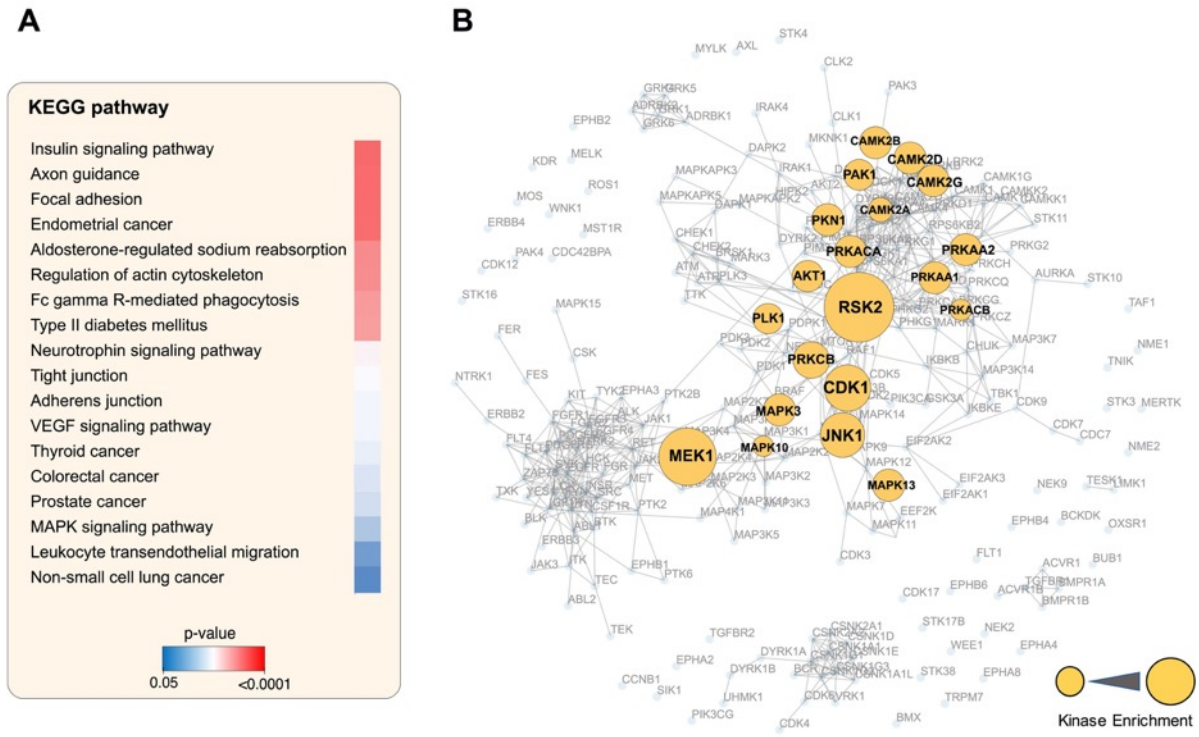

Figure 5. 6. PDGF regulated distinct signalling pathways. **A**, KEGG annotated pathways that were enriched in PDGF-regulated phosphoproteome. **B**, Kinase enrichment analysis of the PDGF regulated phosphopeptides. Network shows the enriched upstream kinases and node size corresponds to kinase enrichment.

5.2.4. CRMP2 is a novel PDGF-downstream protein

CRMP2 has been widely studied in the nervous system as a mediator of neuronal polarity, growth and axonal guidance (Arimura et al., 2004, J. P. Ip et al., 2014). We were able to detect the phosphorylation of CRMP2 at the highly-conserved site (Thr^{514}) in our screen and it was significantly downregulated upon PDGF stimulation (**Figure 5.7A** and **B**). To confirm that PDGF controls CRMP2 phosphorylation, we used phosphospecific antibody recognize Thr^{514}. Western blotting showed that phosphorylation of CRMP2 at Thr^{514} was downregulated following PDGF stimulation compared with non-stimulated cells (**Figure 5.7C**). These data clearly show that PDGF induces the activity of CRMP2 by promoting dephosphorylation at Thr^{514}.

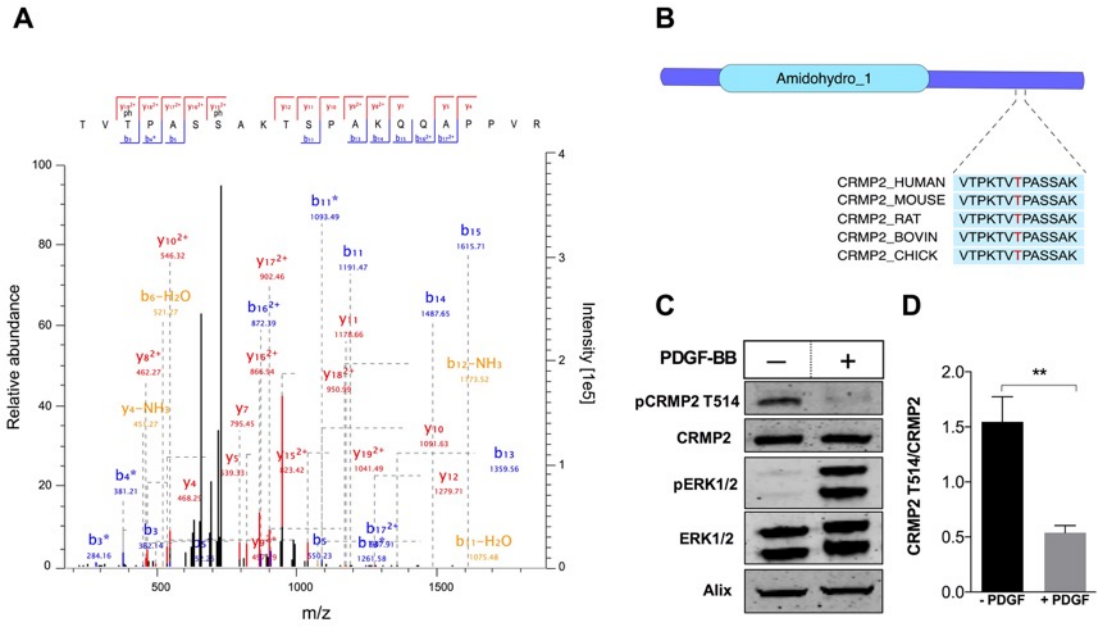

Figure 5. 7. CRMP2 is a novel PDGF effector. A, Tandem mass spectrum of CRMP2 phosphopeptide. **B**, schematic structure of CRMP2 shows the highly conserved motif around Thr^{514}. **C,** MEFs were stimulated with 20 ng/mL PDGF for 7 min. CRMP2, CRMP2 Thr514, ERK1/2, ERK 1/2 Thr202/Tyr204 and Alix in whole cell lysates were analyzed by Western blotting. **D**, Western blots were quantified and ratios of pCRMP to CRMP2 were plotted and statistical significance was calculated using Student's t-test (**, $p<0.01$).

Next we test if PDGF induces one of the Ser/Thr phosphatase in order to dephosphorylate CRMP2 for optimum activity. It has been previously shown that PP1 or PP2A can dephosphorylate CRMP2 at Ser^{518}, Thr ^{514} or Thr^{509} (Cole et al., 2008, Zhu et al., 2010). We tested the effect of okadaic acid on CRMP2 phosphorylation at Thr^{514} upon PDGF stimulation in MEFs. PP2A can be inhibited at low concentration (lower than 1μM), whereas the high concentration inhibits PP1 (Cohen et al., 1990,

Cohen et al., 1989). Western blot analysis showed no significant changes of okadaic acid at (0.01 μM or 1 μM) concentration compare to control (**Figure 5.8**). However, the higher concentration (1.5 μM) restored the phosphorylation of CRMP2 at Thr^{514} in response to PDGF stimulation (**Figure 5.8**). Together, these observations suggest that PDGF may promotes the PP1 to dephosphorylate CRMP2 at Thr^{514}.

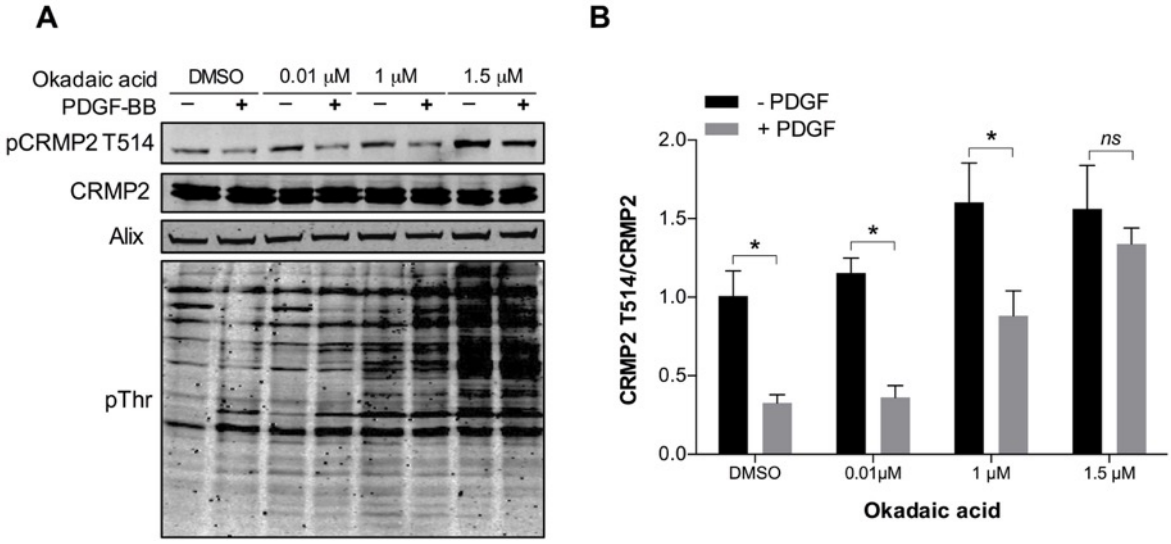

Figure 5. 8. PP1 dephosphorylates CRMP2 Thr514 in response to PDGF stimulation. A, MEFs were incubated with either DMSO or okadaic acid (0.01 μM, 1 μM or 1.5 μM) for 30 min prior stimulation with 20ng/mL PDGF for 7 min. CRMP2, CRMP2 Thr514, total phospho-threonine and Alix in whole cell lysates were analyzed by Western blotting. **B**, Western blots were quantified and ratios of pCRMP to CRMP2 were plotted and statistical significance was calculated using two-way ANOVA with Sidak's test (* $P<0.05$).

5.2.5. CRMP2 knockdown inhibits cell migration in MEFs

CRMP2 widely studied in nervous system and has been reported to regulate neuronal migration (J. P. Ip et al., 2014). For instance, α2-chimaerin mediates neuronal migration by upregulation of CRMP2 activity and the depletion of α2-chimaerin leads to disrupt migration in neurons (J. P. Ip et al., 2012). In addition, CRMP2 knockdown showed trapped neurons in intermediate cortical zone and resulted in morphological changes such as reduced in neuron processes (J. P. Ip et al., 2012). CRMP2 knockdown can also reduce migration of the T lymphocytes in virus-induced neuronal inflammation (Varrin-Doyer et al., 2012). However, the regulation of cell migration by CRMP2 outside nervous system has not been studied. Therefore, we asked whether CRMP2 regulates migration in fibroblast in response to PDGF stimulation. To test this, we used siRNA to knockdown CRMP2 and examined the consequences of CRMP2 depletion in a standard in vitro scratch wound healing assay. CRMP2 knockdown resulted in significant reduction in cell migration in response to PDGF stimulation (**Figure 5.9**). Here we have identified CRMP2 as a novel PDGF downstream effector and required for PDGF-induced cell migration in fibroblast.

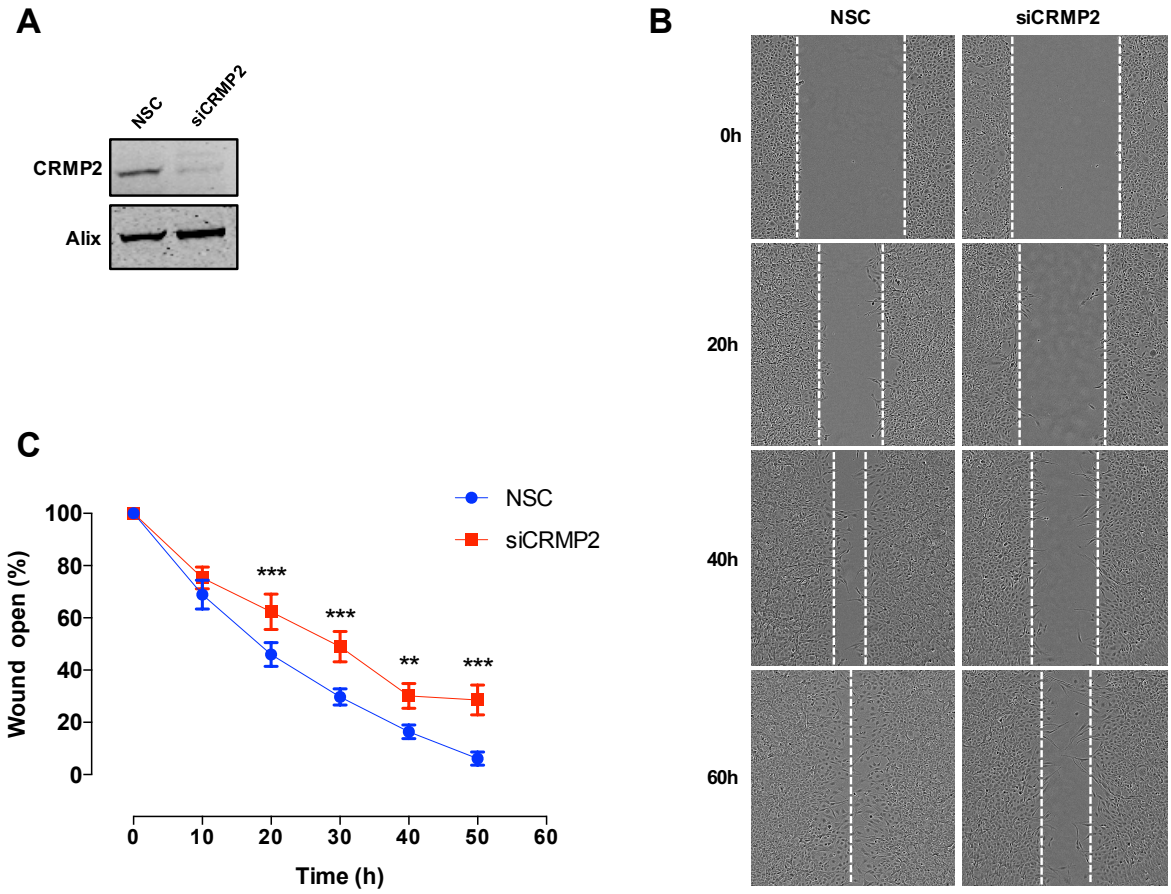

Figure 5. 9. PDGF-induced cell migration through CRMP2. **A**, MEFs were transiently transfected with CRMP2 siRNA (siCRMP2) or non-silencing control (NSC) prior wound healing assay. **B**, Cells were then wounded using an automated scratch maker and images of five separate wounds were taken at 0, 10, 20, 30, 40, 50, and 60 h post wounding using the IncuCyte ZOOM imaging system. **C**, Statistical significance was calculated using two-way ANOVA with Sidak's test (**P<0.01, ***P<0.001).

5.3. Discussion

PDGF ligands and their cognate receptors are expressed in a wide range of tissues during embryonic development (Hoch and Soriano, 2003). Extensive and detailed genetic studies of PDGF and PDGFR function in the mouse confirms that PDGF signalling from epithelial cells via PDGFRs is absolutely required for the expansion and migration of specific mesenchymal cell populations (Betsholtz, 2004). For example, in the kidney PDGF signalling is required both for the multiplication of mesangial cell precursors and their migration into the glomerular space (Lindahl et al., 1998). In the lung PDGF is required for the expansion of myofibroblasts and their migration along the alveolar epithelium during lung morphogenesis (Bostrom et al., 1996). In the central nervous system (CNS) oligodendrocyte precursor (O2A) cells require PDGF signalling both for population expansion and migration through the spinal cord (Calver et al., 1998).

PDGF signalling also has significant functions in adult tissue homeostasis and regeneration acting on cells of mesenchymal origin. PDGF is an established mitogen and chemoattractant for mesenchymal derived cells in vitro and has been studied for its ability to promote wound healing through promoting the migration, proliferation and extracellular matrix deposition by fibroblasts (Siegbahn et al., 1990, Werner and Grose, 2003). However, PDGFβR signalling networks initiated by PDGF other than PI3K, MAPK and PLC remain to be well established. Therefore, we have established the first global phosphoproteomics of the PDGF-dependent signalling network in fibroblasts. We have identified 989 phosphosites from 611 proteins in our screen. Out of these, 116 phosphopeptides were significantly upregulated. These data supported the notion that PDGF induction initiates signalling cascade by promoting

phosphorylation and autophosphorylation of the downstream molecules. Phosphoproteomics analysis also revealed novel 110 phosphosites that were regulated by PDGF. For example, Rab7, one of the key small GTPase protein which involved in the regulation of endocytosis and trafficking (T. Wang et al., 2011). Dephosphorylation of Ser^{72} on Rab7 by PTEN is essential for the protein activity and the transport of the protein target from early to late endosome for protein degradation (Shinde and Maddika, 2016). Our data showed that PDGF is highly induced the phosphorylation of Rab7 at Ser^{72} suggesting a role for PDGFβR in regulation of endocytosis. The explanation for this is that either PDGFβR directly phosphorylates Rab7 or indirectly by suppresses PTEN activity which in turn inducing the phosphorylation.

Interestingly, we were able to detected 45 phosphopeptides that were significantly downregulated. Therefore, our findings not only supported that PDGF promotes downstream proteins phosphorylation, but also showed that PDGF can induces dephosphorylation of crucial downstream proteins such as CRMP2 and CLASP1. CLASP1 is microtubule protein involved in stabilization of microtubules dynamics, cell motility and acts as a downstream for Abl protein tyrosine kinase mediating axon guidance (Akhmanova et al., 2001, H. Lee et al., 2004). Phosphoproteomic and western blotting analysis indicated that PDGF mediates dephosphorylation of CRMP2 at Thr^{514}. CRMP2 is highly expressed in neurons and has been reported to involved in regulation of many cellular functions in nervous system. The KEGG pathways analysis revealed enrichment of pathways that have not been linked with PDGF including insulin signalling, axon guidance, endometrial cancer and neurotrophin signalling pathway. Taken together, our phosphoproteomics screen revealed an evidence that

PDGF and PDGFβR are mediating neuronal signalling and showed the importance of PDGF signalling with broad implications for physiological homeostasis and development.

The downregulated phosphopeptides in our data set support the notion that PDGF can also regulate the activity of protein phosphatase. We have shown that inhibition of protein phosphatase 1 (PP1) by okadaic acid restored the phosphorylation of CRMP2 at Thr^{514}. Interestingly, CRMP2 cannot dephosphorylated in non-stimulated cells indicating that PDGF is essential for the activity of PP1. CRMP2 phosphorylation is controlled by GSK3β to deactivate CRMP2 in neurons which in turn leading to destabilizes microtubules. PDGF has been previously shown to induce cell migration via canonical pathways such as Rac, MAPK/ERK and JNK. Therefore, we examined whether PDGF regulates cell migration through novel downstream protein, CRMP2. We have shown that CRMP2 knockdown in fibroblasts significantly reduced cell migration in response to PDGF stimulation. This data clearly indicates CRMP2 induced cell migration outside nervous system in PDGF-dependent manner (**Figure 5.10**). It will be interesting to characterize whether the many other novel PDGF downstream proteins identified in this study play an important role in PDGFβR signalling pathways in health and disease.

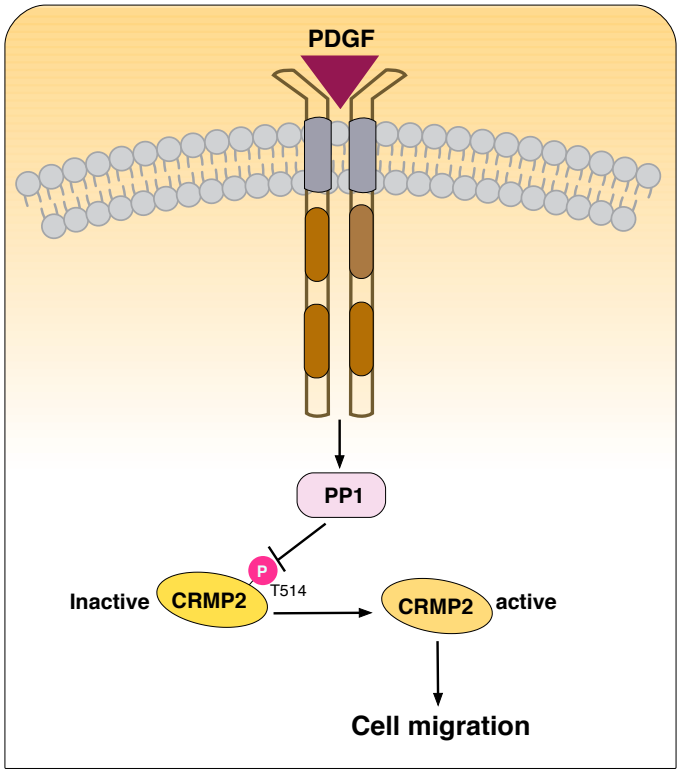

Figure 5. 10. Suggested model showing how PDGF regulates cell migration through CRMP2.

CHAPTER 6

Quantitative Proteomics Identifies a Role for LAR in Regulation of Mitochondrial Respiration

6. 1. Introduction

A key function of mitochondria is the production of energy (ATP) through a process called oxidative phosphorylation (OXPHOS) (Chandra and Singh, 2011). OXPHOS consists of ATP synthase proteins (complex V) and four respiratory chain complexes (I–IV) with all complexes located in the mitochondrial inner membrane (Poyton and McEwen, 1996). The OXPHOS system is a process involved in the transport of electrons generated by NADH (reduced nicotinamide adenine dinucleotide) through a number of carrier molecules called the electron transport chain or respiratory complexes (complexes I-V) (Smeitink et al., 2001). The passing of these electrons through the respiratory complexes leads to energy release, which can be used by ATP synthase proteins (complex V) to generate ATP from ADP (adenosine 5′-diphosphate) and phosphate (Poyton and McEwen, 1996, Smeitink et al., 2001, Zorov et al., 2014). Complex I (NADH-ubiquinone oxidoreductase) is the largest complex and contains more than 42 proteins (Brandt, 1997, Skehel et al., 1998). This complex is the main source for the production of reactive oxygen species (ROS) in the mitochondria (Smeitink et al., 2001). It has been reported that mutations in complex I subunits are responsible for neurological disorders such as Parkinson's disease (Gorman et al., 2016, Pagliarini et al., 2008, Perry et al., 1982, Spencer et al., 1998). Complex II

(succinate-ubiquinone oxidoreductase), also known as succinate dehydrogenase (SDH), are enzymes that oxidize succinate to fumarate during the Krebs cycle and electron respiratory chain (Yankovskaya et al., 2003). In complex III (ubiquinol-cytochrome c oxidoreductase), the electrons are passed from ubiquinol to cytochrome *c* by a mechanism called the Q-cycle, which in turn releases protons into the mitochondrial intermembrane space (F. L. Muller et al., 2004, Z. Zhang et al., 1998). Ultimately, enzymes in complex IV (cytochrome *c* oxidase) receive the electrons from complex III and convert them into water molecules leading to a membrane potential that can be used by complex V (ATP synthase) to produce ATP from ADP (Poyton and McEwen, 1996, Smeitink et al., 2001, Zorov et al., 2014).

In this chapter, the proteomic dataset was further analysed in order to identify protein expression regulated by LAR. Within the proteomic data set, a total of 2939 proteins were identified. Of these, 147 proteins showed a significant change in abundance in LARΔP MEFs compared with the WT MEFs, suggesting a role for LAR in regulation of protein turnover. GO term analysis revealed enrichment of metabolic processes including the oxidation-reduction process. In addition, higher mitochondrial respiration was observed in LARΔP MEFs compared to WT suggesting that LAR suppresses mitochondrial respiration by regulating protein turnover.

6.2. Results

6.2.1. Quantitative proteomics of LAR-regulated protein expression

For the proteomics analysis, 5 μg of light, medium, and heavy lysates from each experiment were mixed, run on SDS-PAGE gel and Coomassie stained (**Figure 6.1**). Each lane was cut into 10 bands and the protein digestion was carried out using In-gel digestion approach (Shevchenko et al., 2006). Within our proteome data set, a total of 1491 proteins were identified in at least one biological replicates and 1150 proteins were associated with quantitation data in two or more biological replicates. Of these, 147 proteins showed a significant change in abundance ($p<0.05$; >1.5-fold change) in the LARΔP cells compared with WT. Interestingly, 47 proteins were upregulated and 100 proteins were downregulated (**Figure 6.2**). This is a significant finding as 13% of the quantified proteome was changed because of the absence of LAR phosphatase activity suggesting that LAR may be involved in regulating protein turnover.

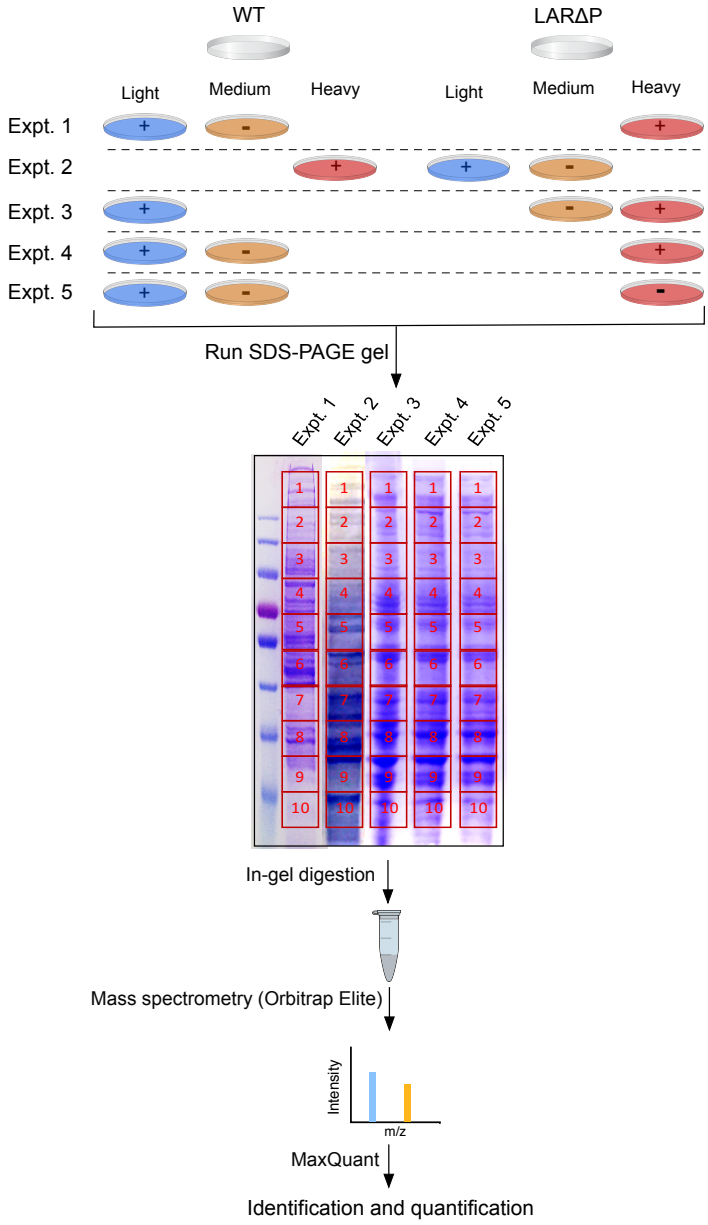

Figure 6. 1. Global proteomics to determine LAR-regulated protein expression. Schematic overview of experimental design. 5 µg of WT MEFs (SILAC light, medium, and heavy) or LARΔP MEFs (SILAC light, medium, and heavy) lysates from each biological replicate (including the reverse labelling experiment) were mixed, run on SDS-PAGE gel. Each lane was cut into 10 bands and the protein digestion was carried out using In-gel digestion approach for mass spectrometry based-proteomic analysis. The identification and quantification was carried out using MaxQuant software. (+) with PDGF-BB, (-) without PDGF-BB.

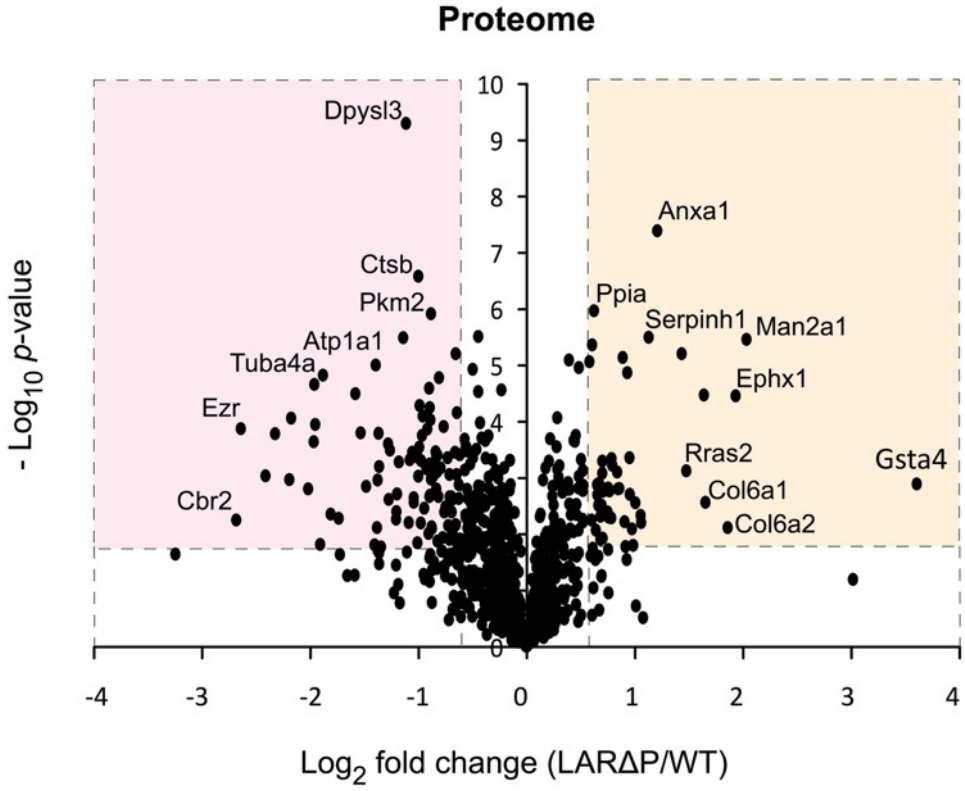

Figure 6. 2. Global proteomics to determine the contribution of LAR to protein expression. Volcano plots showing the ratio (log2 fold change) and significance (–log10 Benjamini-Hochberg adjusted p-value) of the proteome data set (adjusted p value <0.05; >1.5 fold-change). The upregulated and downregulated proteins are highlighted.

6.2.2. Functional analysis of the LAR-regulated proteome

KEGG pathway analysis of LAR-regulated protein levels revealed enrichment of pathways implicated in metabolic pathways including pyruvate metabolism, fatty acid metabolism and glutathione metabolism (**Figure 6.3**). These findings demonstrated that LAR may be involved in regulation of metabolic processes. In addition, KEGG analysis of the proteome dataset also identified focal adhesion and regulation of actin cytoskeleton as LAR-regulated processes.

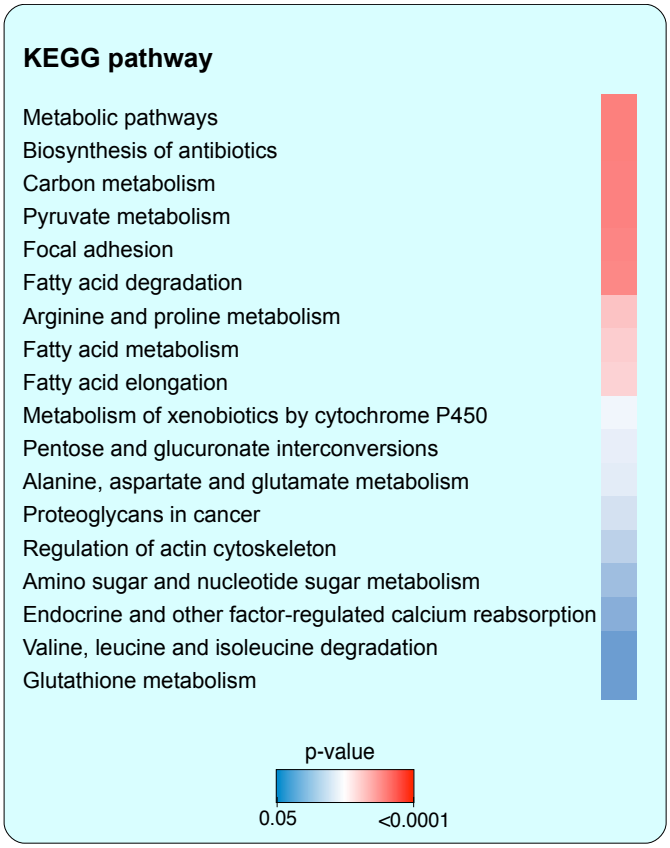

Figure 6. 3. Over-represented pathways of the LAR-regulated proteome. Significantly changed proteome data set were analysed in DAVID to identify enriched KEGG pathways.

Gene Ontology analysis of the proteome regulated by LAR revealed a number of enriched GO terms. All the significantly changed proteins are clustered according to their functional keywords as indicated in **Figure 6.4**. The dominant enriched functional group was associated with metabolic processes and cytoskeletal organisation. Metabolism proteins that were enriched in our proteome data set included: Alpha-mannosidase 2 (Man2a1), a protein involved in the protein glycosylation pathway (Moremen and Nairn, 2014); Adenine phosphoribosyl transferase (Aprt) as enzyme that regulate AMP synthesizes from adenine (Sassone-Corsi, 2012); Adenylate kinase 1 (Ak1), a kinase that play a key role in maintaining cellular energy homeostasis (Noma, 2005). We observed an increase in abundance of these proteins in LARΔP cells compared with WT.

We also noted the enrichment of biological process for proteins associated with oxidation-reduction process including Aldose reductase (Akr1b1), Succinate dehydrogenase flavoprotein, mitochondrial (Sdha) and Sodium/potassium-transporting ATPase subunit alpha-1 (Atp1a1) suggesting a role for LAR in regulation of mitochondrial respiration.

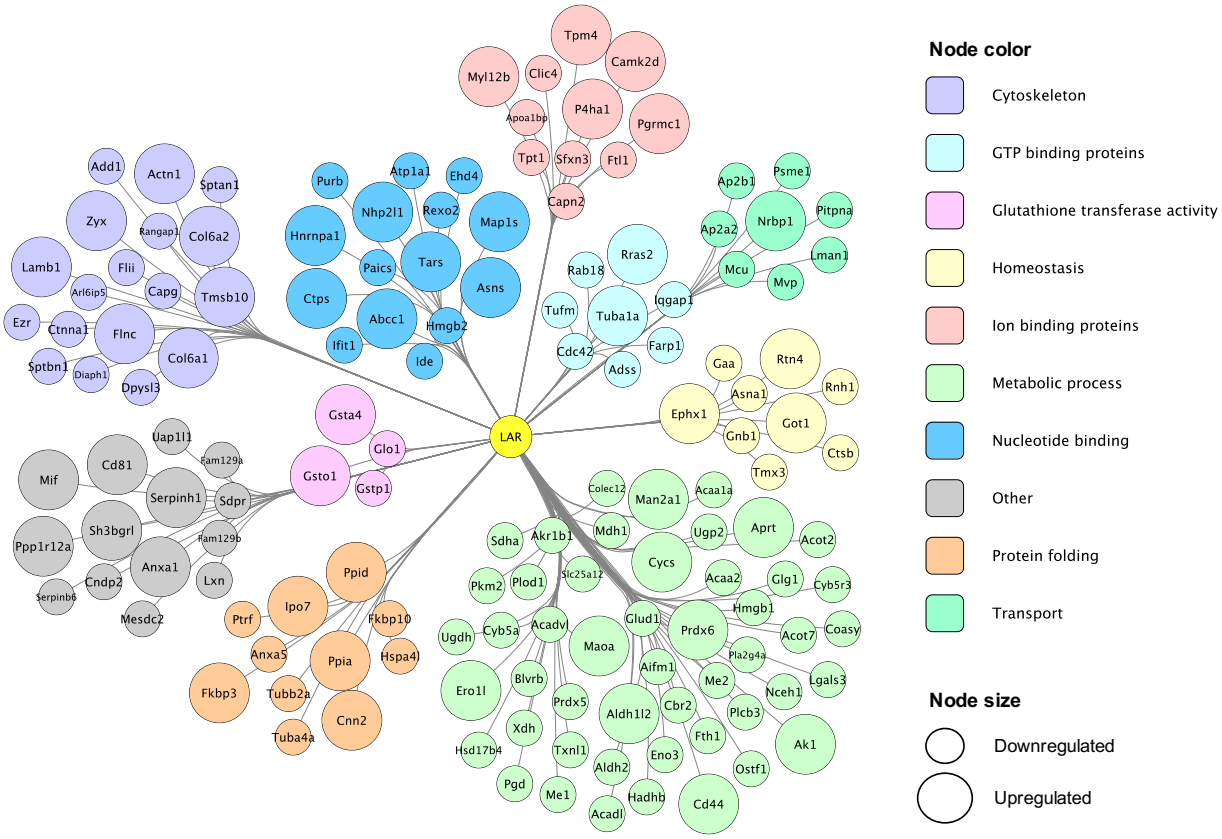

Figure 6. 4. LAR regulated distinct metabolic processes. Proteins were clustered according to their functional keywords. Large node size indicates that the protein was up-regulated in LARΔP cells and small node size indicates down-regulation.

6.2.3. Deletion of LAR phosphatase domains increase mitochondrial respiration

Having identified proteins that have a role in regulation of redox processes and mitochondrial respiration, we decided to study whether LAR was involved in the regulation of mitochondrial respiration. Mitochondrial respiration was analysed using a Seahorse XF Analyzer (Salabei et al., 2014). WT and LARΔP cells were either grown in complete or starvation media overnight and the basal respiration, ATP production, maximal respiration and non-mitochondrial respiration measured by serial injecting of oligomycin (Oligo), carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (FCCP) and rotenone/ antimycin A (Rot/AA). Oligo inhibits complex V, whereas FCCP enhance the production of ATP. Rotenone and antimycin A can be used to inhibit complex I and III (Salabei et al., 2014).

In complete medium, both basal and maximal respiration rates were significantly higher in LARΔP cells compared with WT (**Figure 6.5**).

In starvation media, mitochondrial respiration was significantly reduced in WT cells compared with LARΔP cells. Interestingly, mitochondrial respiration was higher in LARΔP cells compared to WT cells and even higher when compared with LARΔP cells that grown in complete media (**Figure 6.6**).

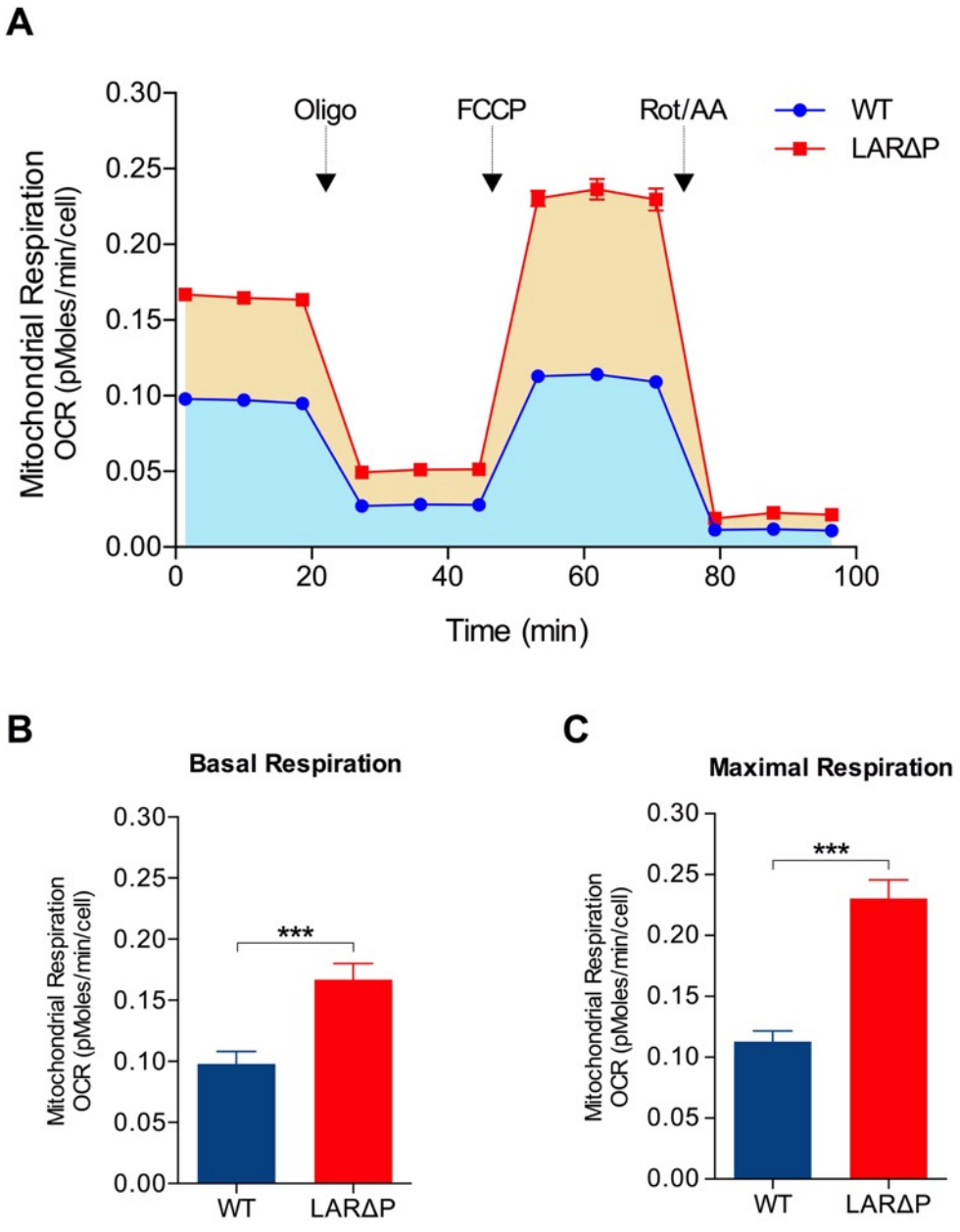

Figure 6. 5. Role of LAR on mitochondrial respiration. A, the profile mitochondrial respiration test in WT and LARΔP cells for the measurement of basal respiration (**B**) and maximal respiration (**C**). WT and LARΔP cells were seeded to 40,000 cells/ well in XF24 cell culture plates and incubate in complete media for 24 h prior to the assays. Oxygen consumption rate (OCR) was measured under basal state followed by the sequential injection of oligo (1 µM), FCCP (1 µM), and rotenone/ antimycin A (0.5 µM) to determine the ATP production and maximal respiration. Statistical significance was calculated using Student's t-test (***, $p<0.001$).

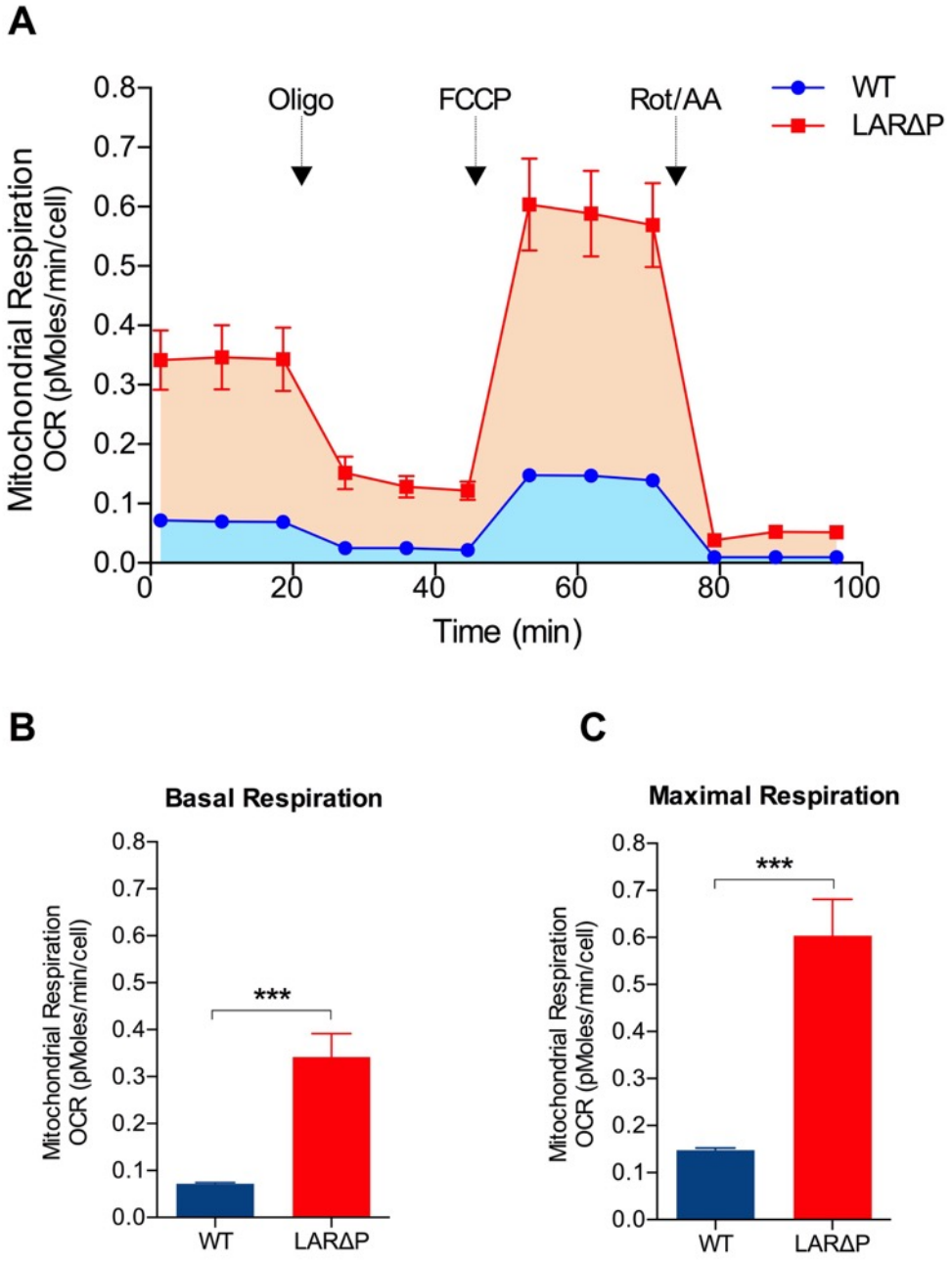

Figure 6. 6. Role of LAR on mitochondrial respiration. A, the profile mitochondrial respiration test in WT and LARΔP cells for the measurement of basal respiration (**B**) and maximal respiration (**C**). WT and LARΔP cells were seeded to 40,000 cells/well in XF24 cell culture plates and incubate in starvation media for 24 h prior to the assays. Oxygen consumption rate (OCR) was measured under basal state followed by the sequential injection of oligo (1 µM), FCCP (1 µM), and rotenone/ antimycin A (0.5 µM) to determine the ATP production and maximal respiration. Statistical significance was calculated using Student's t-test (***, $p<0.001$).

6.2.4. Increased expression of succinate dehydrogenase protein in LARΔP cells

Since the XF analysis revealed higher mitochondrial respiration in LARΔP cells compared with WT in either complete or starvation media (**Figure 6.5** and **6.6**), the expression of proteins involved in the regulation of the OXPHOS system was further investigated in WT and LARΔP MEFs. Immunoblotting analysis was carried out using an antibody that recognizes one protein from each of the OXPHOS complexes including NADH dehydrogenase alpha subcomplex subunit 8 (Ndufa8) from complex I, succinate dehydrogenase [ubiquinone] iron-sulfur subunit b (Sdhb) from complex II, cytochrome b-c1 complex subunit 2 (Uqcrc2) from complex III, cytochrome c oxidase subunit 1 (Mtco1) from complex IV and ATP synthase subunit alpha (ATP5A1) from complex V. All the subunits showed no significant difference in expression, except succinate Sdhb from complex II which significantly increased in expression in LARΔP compared with WT cells (**Figure 6.7**).

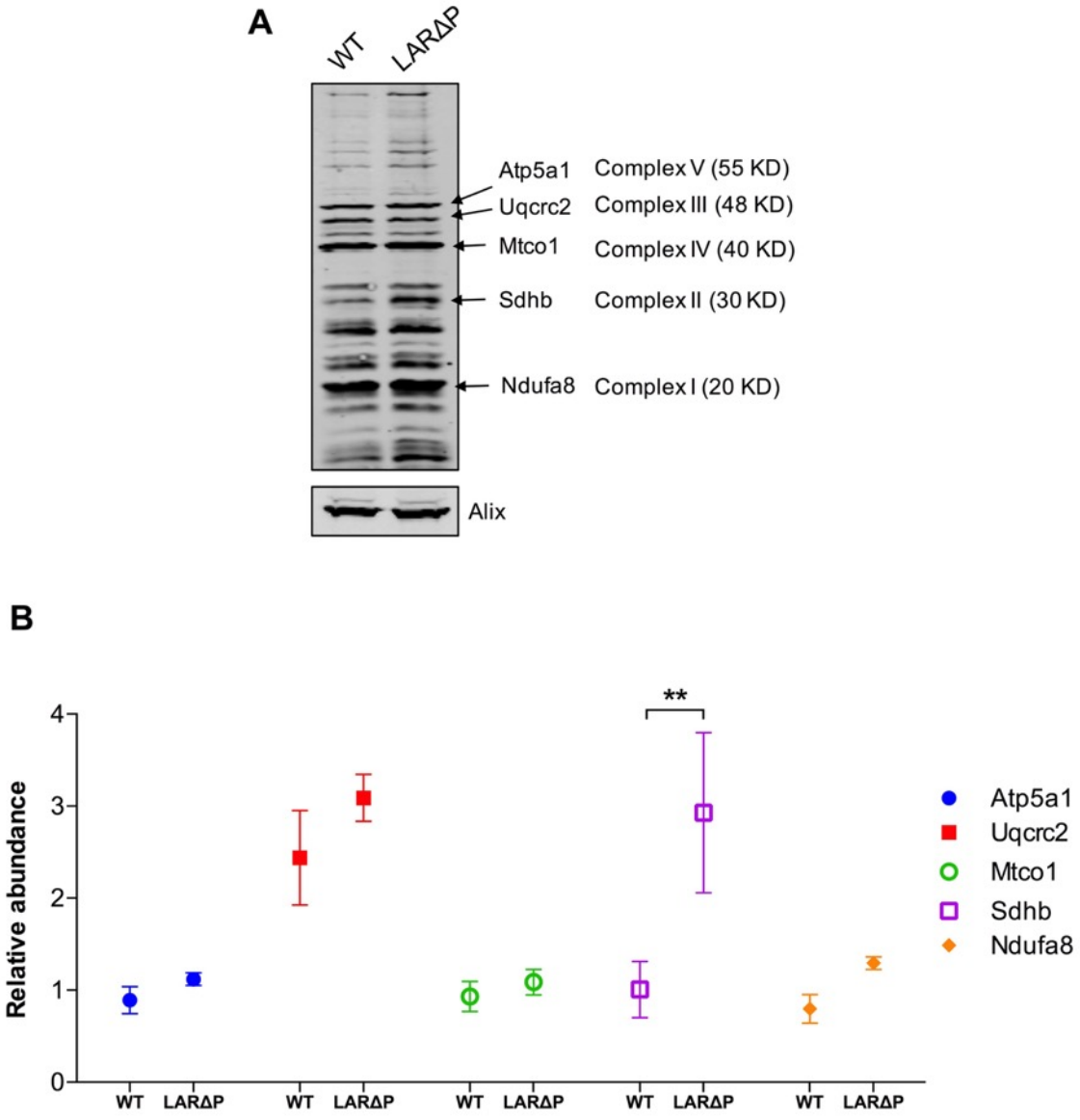

Figure 6. 7. Up-regulation of the mitochondrial SDHB expression in LARΔP MEFs. A, Expression of the proteins involved in oxidative phosphorylation was analysed using an OXPHOS cocktail antibody and Alix was used to control for loading. **B**, Results from western blotting analysis (n=3) were quantified and ratios of OXPHOS to Alix plotted. Statistical significance was calculated using two-way ANOVA with Sidak's test (** $p<0.01$).

6.3. Discussion

Mitochondria are involved in regulation of multiple cellular functions, signal transduction and metabolic pathways (Chandra and Singh, 2011, Saraste, 1999). Mitochondria is mainly involved in the production of energy through oxidative phosphorylation (OXPHOS) system (Smeitink et al., 2001). In this chapter, the proteomics datasets were further analysed to identify and characterize protein expression regulated by LAR. A total of 1491 proteins were identified in at least one biological replicate and 1150 proteins were associated with quantitation data in two or more biological replicates. Of these, 147 proteins showed a significant change in abundance. Interestingly, 47 proteins were significantly upregulated and 100 proteins were downregulated suggesting that LAR may be involved in regulation of protein turnover since 13% of the quantified proteome data was changed due to the absence of LAR phosphatase activity.

KEGG pathway analysis of the significantly changed proteins revealed over-representation of several metabolic pathways, demonstrating that LAR may be involved in regulation of metabolic processes. In addition, GO term analysis identified many proteins involved in regulation of metabolic processes such as Adenylate kinase 1 (Ak1), a kinase that has a key role in maintaining cellular energy homeostasis by catalyzes adenine nucleotides (Noma, 2005). It has been shown that Ak1 knockout mice have significantly reduced ATP levels in myocardial cells leading to myocardial dysfunction (Dzeja et al., 2007) suggesting a key role for Ak1 in controlling the myocardial energy state and homeostasis. Upregulation of Ak1 was observed in LARΔP cells compared with WT. This is suggesting that LAR could suppresses the

expression of this protein at either protein or mRNA level and thus modulate energetic homeostasis.

Since the bioinformatics analysis of the proteome data set identified proteins that have a role in regulation of redox processes and mitochondrial respiration, mitochondrial respiration was investigated to determine whether LAR has a role in this process. Seahorse XF analysis clearly showed a significant increase in the basal and maximal mitochondrial respiration in LARΔP MEFs compared to WT. Interestingly, serum starvation causes suppression of respiration in WT cells. However, starvation did not affect the respiration of LARΔP cells. Instead, the basal and maximal respiration was even higher when compared with LARΔP cells grown in complete media. This demonstrates that LAR phosphatase activity has a negative effect on mitochondrial activity and this is could be via suppression of mitochondrial proteins expression.

Mitochondria are mainly involved in the production of ATP and this function is regulated by a process called OXPHOS (Chandra and Singh, 2011, Murphy, 2009). OXPHOS is consisted of ATP synthase proteins (complex V) and four respiratory chain complexes (I–IV) (Poyton and McEwen, 1996). In order to identify if LAR controls the protein levels in these complexes, western blotting analysis was carried out using an antibody that recognized one protein from each of the OXPHOS complexes. Upregulation in one of the main proteins in complex II (Sdhb) was observed in LARΔP cells compared with WT. Sdhb is involved in the oxidization of succinate to fumarate during the Krebs cycle (Yankovskaya et al., 2003). In addition, Sdhb was identified as a tumor suppressor (Bayley et al., 2006, Benn et al., 2003, Gimenez-Roqueplo et al., 2002). Sdhb overexpression has been linked to inhibition of cell proliferation, invasion and induced apoptosis in human ovarian cancer through the

suppression of AMPK-HIF-1α pathway (L. Chen et al., 2014). Taken together, these data suggest that LAR could decrease mitochondrial respiration through suppression of Sdhb.

CHAPTER 7

LAR Protein Tyrosine Phosphatase Enhances PDGFβR Signalling by Suppression of GRK2 Activity

7.1. Introduction

Many activities that occur in cells such as growth, survival, motility, proliferation and differentiation, are regulated by growth factors such a PDGF. PDGF is a stimulator of cellular processes in many cells including fibroblasts, smooth muscle cells, neurons and endothelial cells (Ataliotis and Mercola, 1997). Several protein tyrosine phosphatases (PTPs), including Shp-1, Shp-2 and TC-PTP, are able to dephosphorylate tyrosine residues on PDGFβR, which in turn deactivates the receptor kinase activity. It has been previously discovered that, unlike the other protein tyrosine phosphatases, leukocyte common antigen related (LAR) can control PDGFβR activity by enhancing PDGFβR phosphorylation (Zheng et al., 2011).

LAR is widely expressed in human tissues such as brain, heart and bladder (Johnson et al., 2006). Several studies have found that LAR plays an important role in regulation of cell signalling and regulates cellular functions such as neuronal growth and neuronal regeneration (Sethi et al., 2010). Several phenotypic disorders have been recognized in LAR-deficient mice including defects in the development of mammary glands, neuron degeneration, axon guidance defects, reduction in the rate of hepatic glucose production, diabetes and cancer (Andersen et al., 2001, Kanakaraj et al., 1991, Sethi et al., 2010, Van Lieshout et al., 2001).

G protein-coupled receptor kinase 2 (GRK2), also called β-adrenergic receptor kinase-1, is a serine/ threonine kinase which play an important role in the regulation of cellular signalling (Black et al., 2016). GRK2 phosphorylates PDGFβR leading to a reduction in receptor autophosphorylation and desensitization (J. H. Wu et al., 2005). Protein kinase C (PKC) phosphorylates and activates GRK2 promoting translocation of GRK2 to the cell membrane (Krasel et al., 2001, Winstel et al., 1996). Furthermore, Src-mediated tyrosine phosphorylation of GRK2 promotes kinase activity (Penela et al., 2001, Sarnago et al., 1999). ERK1 can phosphorylate GRK2 at Ser^{670} inhibiting its kinase activity (Elorza et al., 2000, Pitcher et al., 1999). GRK2 overexpression has been linked to many pathological conditions such as heart failure and hypertension (Elorza et al., 2003). GRK2 overexpression has also been shown to decrease proliferation of smooth muscle cells (Aragay et al., 1998). More recently, investigators have revealed that overexpression of GRK2 increases reactive oxygen species (ROS) production in cardiac myocytes by promoting the expression of NADPH oxidase 4 (Nox4) leading to heart failure (Theccanat et al., 2016).

Na^+/H^+ exchanger regulatory factors NHERF1 (also called EBP50) and NHERF2 (also known as E3KARP) are adaptor proteins which can bind with cytoskeletal proteins ezrin, radixin, moesin, and merlin comprised through their PDZ domains (Dunn and Ferguson, 2015, Karthikeyan et al., 2001, Voltz et al., 2001). NHERF1 is expressed in restricted areas such as kidney, intestine, prostate and mammary tissue, while NHERF2 is widely expressed in many cells (Weinman et al., 1995). NHERF1 and NHERF2 form homodimers and heterodimers with each other via their PDZ domains and with other PDZ-containing proteins creating a membrane scaffold that can bind transmembrane or intracellular proteins such as transporters, ion channels

and signal transduction molecules (Fouassier et al., 2000, Shenolikar et al., 2001, Weinman et al., 2000). NHERF1 has been shown to bind to PDGFβR via its PDZ1 domain and to potentiate receptor autophosphorylation and downstream signalling activity (Maudsley et al., 2000). Another study reported that NHERF2 also binds to the c-terminal of PDGFβR enhancing ERK and Akt signalling (Jung Kang et al., 2004). In contrast, depletion of NHERF1 or NHERF2 in MEFs enhances PDGFR signalling suggesting a different role for NHERFs whereby they promote the recruitment of the phosphoinositide phosphatase (PTEN) to the PDGFR, which in turn attenuates receptor signalling (Takahashi et al., 2006). In 2004, Hildreth and co-workers published a paper in which they identified that GRK2 can phosphorylate PDGFβR on Ser^{1104}, preventing the association of Na^+/H^+ exchanger regulatory factor 1 (NHERF1) with the receptor, and hence decreased receptor signalling (Hildreth et al., 2004).

Here, it was determined that LAR is required for PDGFβR activation. We have also identified GRK2 as a part of a negative feedback loop regulating PDGFβR signalling. Therefore, this suggests a signalling pathway connecting LAR and GRK2 to the regulation of PDGF signalling.

7.2. Results

7.2.1. LAR positively regulates PDGFβR tyrosine phosphorylation

Many protein tyrosine phosphatases (PTPs) are known to modulate PDGFβR by dephosphorylating tyrosine residues on the receptor. For example, PTP1B dephosphorylates Tyr^{579} residue on PDGFβR preventing Src and Shc binding, thereby terminating the signalling cascade (Ekman et al., 2002, Persson et al., 2004). However, PTPs, such as SHP-2, can also promote PDGFβR signalling (Ekman et al., 2002, Lechleider et al., 1993). LAR dephosphorylates receptor tyrosine kinases such as the insulin receptor and decreases their activity (Tsujikawa et al., 2001).

To examine whether LAR is involved in the regulation of PDGFβR activity, total tyrosine phosphorylation of PDGFβR was analysed in WT and LARΔP MEFs. We found a significant reduction in tyrosine phosphorylation in LARΔP cells compared with WT cells **Figure 7.1**). These data indicating that LAR phosphatase domains are required for PDGFβR tyrosine phosphorylation.

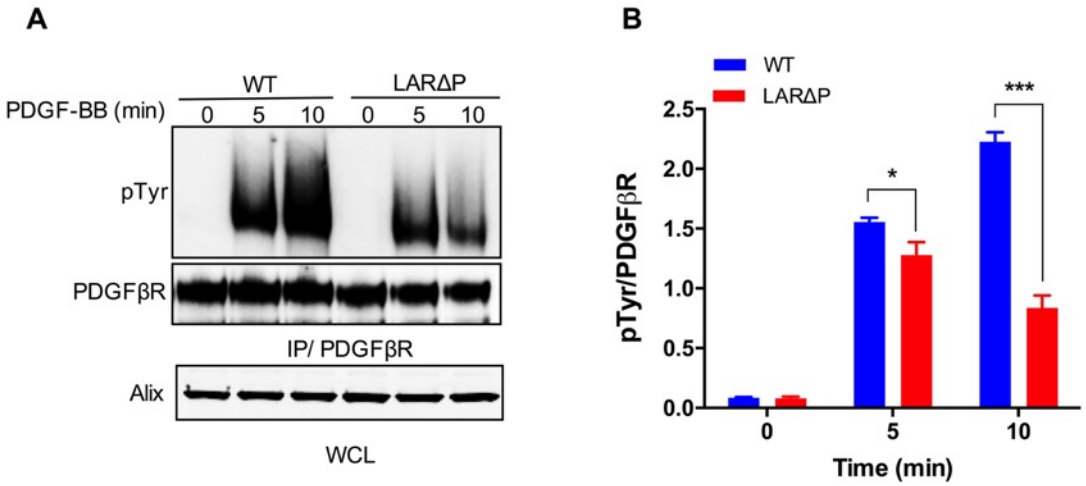

Figure 7. 1. Deletion of LAR phosphatase domains reduces PDGFβR tyrosine phosphorylation. **A,** WT and LARΔP cells were stimulated with 20 ng/mL PDGF-BB for indicated times. PDGFβR was immunoprecipitated using a PDGFβR-specific antibody and immunoprecipitates were immunoblotted with either PDGFβR or total phosphotyrosine (PY99) antibodies. To control for expression levels, whole cell lysates (WCL) were immunoblotted with Alix antibody. **B**, Western blots (n=3) were quantified and ratios of pPDGFβR to PDGFβR were plotted over time. Statistical significance was calculated using two-way ANOVA with Sidak's test (*** $p<0.001$; * $p<0.05$).

7.2.2. GRK2 phosphorylation is regulated by LAR

Having identified that LAR potentiates PDGFβR tyrosine phosphorylation, we further investigated the underling mechanism on how LAR regulates PDGFβR. It has been previously shown that serine phosphorylation of the receptor can lead to reduced receptor tyrosine autophosphorylation and kinase activity. GRK2 regulates PDGFβR by mediating serine phosphorylation leading to receptor desensitization and reduces receptor autophosphorylation (J. H. Wu et al., 2005). To examine whether LAR regulates GRK2 activity, the tyrosine phosphorylation of GRK2 was investigated. We observed higher GRK2 phosphorylation in LARΔP cells compared with WT (**Figure 7.2**).

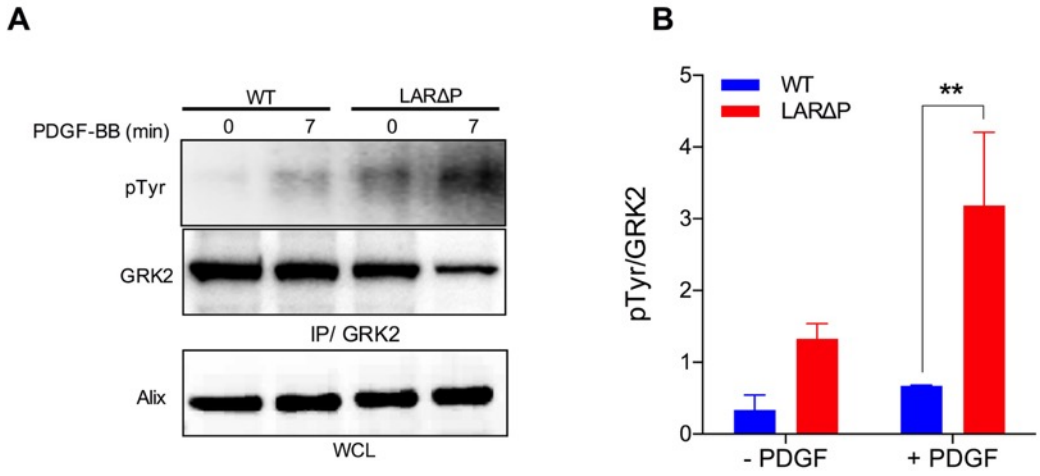

Figure 7. 2. Higher GRK2 tyrosine phosphorylation in LARΔP cells. **A**, WT and LARΔP cells were stimulated with 20 ng/mL PDGF-BB for 7 min. GRK2 was immunoprecipitated and immunoblotted with either GRK2 or total phosphotyrosine (PY99) antibodies. To control for expression levels, whole cell lysates (WCL) were immunoblotted with Alix antibody. **B**, Western blots (n=3) were quantified and ratios of PY99 to GRK2 were plotted. Statistical significance was calculated using two-way ANOVA with Sidak's test (** $p<0.01$).

In collaboration with a Masters student (Assel Bektassova) in our lab, I carried out a quantitative SILAC proteomics study to characterise phosphorylation of GRK2 as well as identify interacting proteins following PDGF stimulation (**Figure 7.3A**). WT and LARΔP MEFs were grown in either 'Heavy' R10K8 or 'Light' R0K0 SILAC media, respectively. Cells were stimulated with PDGF for 15 min prior to immunoprecipitation of GRK2 and subsequent mass spectrometric analysis. Within our quantitative proteomics data, we identified a 2-fold increase in the phosphorylation of Ser^{670} on GRK2 in WT cells compared to LARΔP cells (**Figure 7.3B**). This suggests that LAR is involved in the regulation of GRK2 activity, since the phosphorylation of this residue leads to inhibition of kinase activity (Elorza et al., 2000, Pitcher et al., 1999).

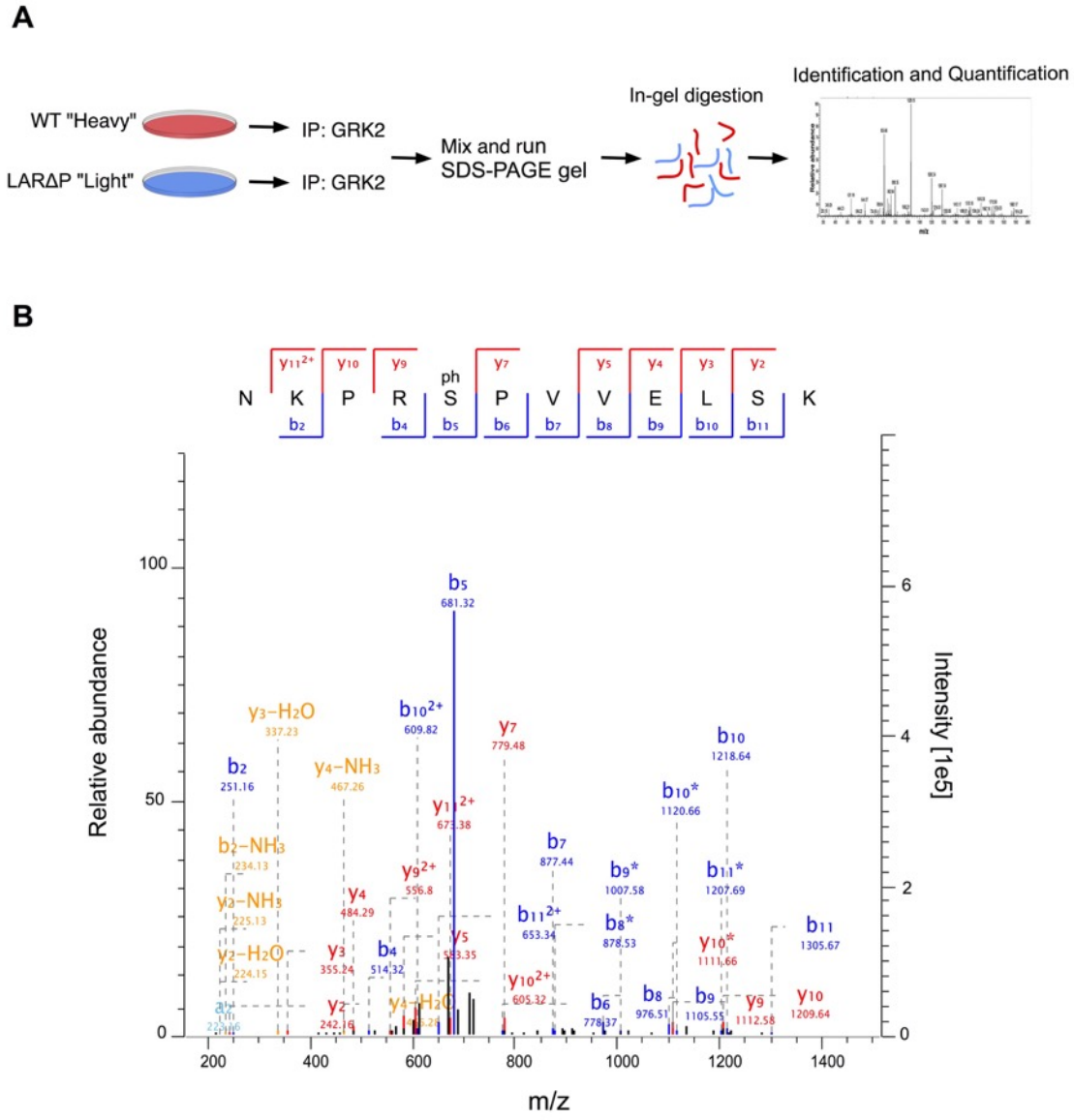

Figure 7. 3. GRK2 is regulated by LAR. A, Schematic overview of experimental design**. B,** Tandem mass spectrum of GRK2 phosphopeptide around Ser670.

7.2.3. GRK2 depletion rescued the activity of PDGFβR downstream signalling in LARΔP MEFs

Next we examined whether GRK2 is involved in the regulation of PDGFβR signalling by LAR. Consistent with previous data, the activity of ERK1/2 and Akt were much lower in LARΔP than WT cells demonstrating that LAR activity is required for the PDGF induced downstream signalling pathways (**Figure 7.4**). To examine if the higher activity of GRK2 in LARΔP cells is responsible for the reduced PDGFβR signalling, RNA interference was used to knockdown GRK2 in LARΔP MEFs and the activity of PDGFβR downstream proteins were analysed by western blotting. GRK2 depletion in LARΔP MEFs partially restored the phosphorylation of ERK1/2 and Akt suggesting that LAR could positively regulate PDGFβR activity via GRK2 (**Figure 7.4**).

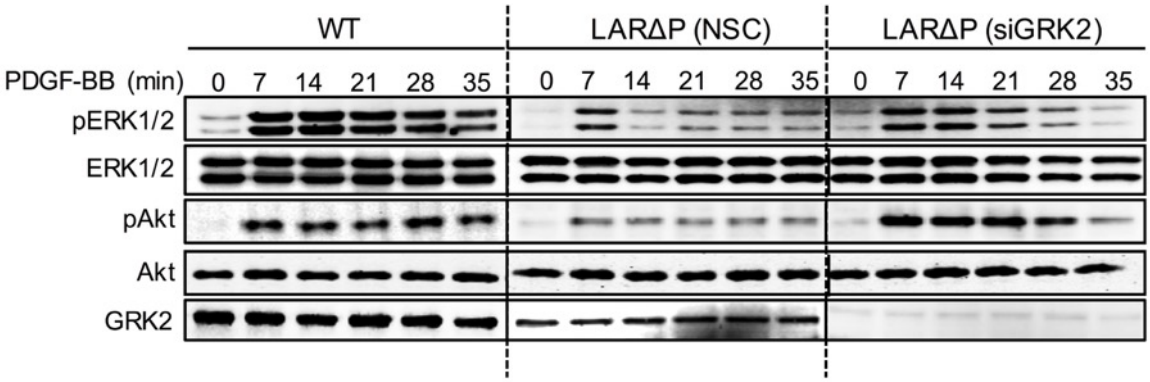

Figure 7. 4. Knockdown GRK2 restored the activity of PDGFβR signalling. LARΔP MEFs were transiently transfected with siRNA oligonucleotides targeted against GRK2 or with control non-silencing oligonucleotides (NSC). At 48 h after transfection, cells were stimulated with 20ng/mL PDGF-BB for indicated time periods. ERK1/2, ERK 1/2 Thr202/Tyr204, Akt, Akt Thr308 and Alix in whole cell lysates were analyzed by Western blotting (n=3).

7.2.4. Increased interaction of NHERF2 and PDGFβR in WT MEFs

NHERF2 is known to interact with, and regulate the activity of, PDGFβR. Therefore, to test if LAR controls the interaction of NHERF2 with PDGFβR, WT and LARΔP cells were transfected with both NHERF2 and PDGFβR plasmids. We found more NHERF2 associated with PDGFβR in WT cells compared with LARΔP MEFs following PDGF stimulation (**Figure 7.5**). These data suggest a novel role for LAR in regulation of PDGF signalling via GRK2.

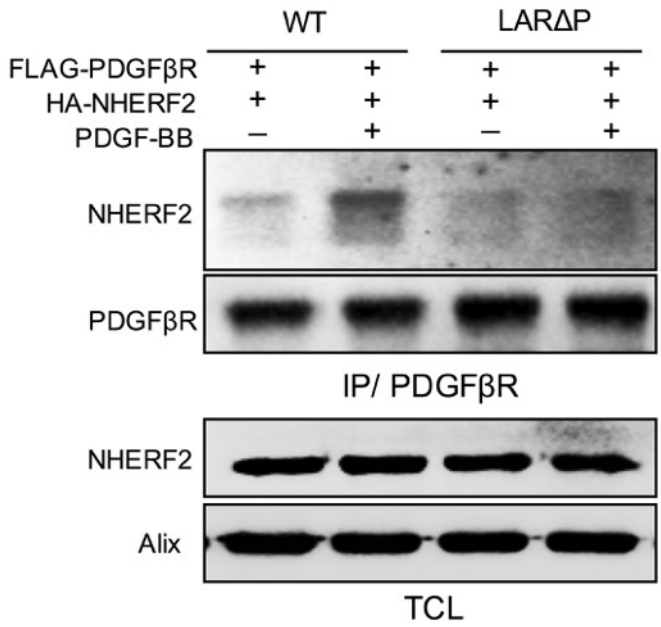

Figure 7. 5. LAR is required for the interaction of NHERF2 with PDGFβR. FLAG-tagged PDGFβR along with NHERF2 were co-expressed in either WT or LARΔP cells. PDGFβR was immunoprecipitated using anti-FLAG beads and immunoprecipitates were immunoblotted with either PDGFβR or NHERF2 antibodies. To control for expression levels, whole cell lysates (WCL) were immunoblotted with Alix antibody (n=1).

7.3. Discussion

PDGF is involved in regulation of many cellular function including growth, survival, motility, proliferation and differentiation (Heldin and Lennartsson, 2013). In this chapter, we identified that LAR positively regulates PDGFβR tyrosine phosphorylation and the activation of downstream proteins (**Figure 7.1** and **7.4**). Having identified LAR potentiates PDGFβR tyrosine autophosphorylation and positivity regulates canonical PDGF signalling pathways such as mTOR and JNK (**Chapter 3**), we further investigated the underling mechanism. We have shown that LAR is directly regulates c-Abl activity by mediating the dephosphorylation of tyrosine residues on c-Abl (**Chapter 4**). This suggests that, in LARΔP cells, the higher c-Abl activity leads to either a direct modulation of PDGFβR activity or an indirect mechanism involving regulation of activity of downstream targets involved in attenuating PDGF signalling. One of the possibilities is the phosphorylation of serine residues on PDGFβR which in turn leads to reduced tyrosine phosphorylation and kinase activity. For example, GRK2 has been shown to regulate PDGFβR by mediating serine phosphorylation (Hildreth et al., 2004).

It has shown that the activation of MAP kinase promotes GRK2 degradation (Elorza et al., 2003). In addition, ERK1 has reported to deactivate GRK2 by the phosphorylation of Ser^{670} (Elorza et al., 2000, Pitcher et al., 1999). Quantitative proteomics revealed a significant increase in the phosphorylation of GRK2 at Ser^{670} in WT cells following PDGF stimulation (**Figure 7.3**). GRK2 was highly tyrosine phosphorylated in LARΔP MEFs, further indicating that LAR controls the activity of GRK2 (**Figure 7.2**). Furthermore, the finding that knockdown of GRK2 restored the activity of PDGFβR downstream kinases, Akt and ERK1/2 (**Figure 7.4**) suggests that

LAR keeps c-Abl and GRK2 inactive leading to enhanced and prolonged PDGFβR autophosphorylation and activation. This would be consistent with the observation that loss of LAR activity was accompanied by decreased ERK1/2 phosphorylation, which would remove the inhibitory effect of ERK1/2 on GRK2, leading to activation of GRK2 and inactivation of PDGFβR. This is supported by the observation that in WT cells, ERK1/2 was highly activated, with less GRK2 activity, indicating that LAR enhances PDGFβR activity through suppression of GRK2 activity.

We also detected the interaction of NHERF2 with PDGFβR in WT cells following PDGF stimulation (**Figure 7.5**). Taken together, these data suggest that LAR regulates PDGFβR by inhibition of GRK2 activity which affects association of NHERF2 with PDGFβR, leading to stabilized the receptor dimerization, enhancing the tyrosine autophosphorylation and downstream signalling molecules. In summary, we propose a novel mechanism whereby LAR enhances PDGFβR signalling through suppression of GRK2.

CHAPTER 8

Discussion and Future Directions

Platelet-derived growth factors are known to stimulate of multiple cellular functions in cells of mesenchymal origin. PDGFs and their receptors are expressed in most tissues and play important roles during embryonic development leading to cell proliferation and differentiation (Heldin and Westermark, 1999). In addition, PDGFs also play a crucial post-natal role regulating wound healing, constriction of blood vessels, angiogenesis, cell migration, chemotaxis and tissue remodeling (Fredriksson et al., 2004, Hansen et al., 1996, Hellberg et al., 2010).

PDGF receptor overactivation has been linked to several diseases including breast cancer, glioblastoma, sarcoma, atherosclerosis and renal fibrosis (Aguilar, 2016, Alehagen et al., 2016, Appiah-Kubi et al., 2016, Lewandowski et al., 2016a, Lewandowski et al., 2016b, Pietras et al., 2003). Furthermore, overexpression of PDGFβR has also reported in lung cancer, demonstrating a crucial role for PDGF in the development of lung cancer (Tsao et al., 2011). Taken together, these findings demonstrate that PDGF receptor overactivation contributes to the development of cancer and invasiveness.

PDGFβR signalling is highly controlled through several mechanisms that modulate the receptor activity. Dephosphorylation of the phosphorylated tyrosine residues by protein tyrosine phosphatases (PTPs) is one of the key mechanisms for controlling the receptor activity and downstream signalling pathways (Nicholas K Tonks, 2006b). It

has been reported that many PTPs directly dephosphorylate selected tyrosine residues on PDGFβR to terminate the receptor downstream signalling molecules. For instance, low molecular weight protein tyrosine phosphatase (LMW-PTP) has been found to dephosphorylate Tyr^{716} and Tyr^{857} on PDGFβR to inhibit the receptor kinase activity and prevent the interaction of Grb2 with receptor (Chiarugi et al., 2002). T-cell protein tyrosine phosphatase (TC-PTP) can dephosphorylate Tyr^{1021} on PDGFβR, which is a binding site of phospholipase C gamma1 (PLCγ1), leading to decreased cell migration in response to PDGF stimulation (Persson et al., 2004). Tyrosine-protein phosphatase non-receptor type 11 (SHP-2) can dephosphorylate the binding site of Ras-GAP Tyr^{771} leading to enhanced activity of the Ras signalling pathway by preventing Ras-GAP interacting with PDGFβR (Ekman et al., 2002). Moreover, PTP1B dephosphorylates Tyr^{579} residue of PDGFβR where Src and Shc can bind, to terminate the signalling initiation by these proteins (Ekman et al., 2002, Persson et al., 2004). Finally histidine domain-containing protein tyrosine phosphatase (HD-PTP) is recruited to PDGFβR in response to PDGF-BB and is required for PDGFβR degradation (H. Ma et al., 2015a). In this thesis, global SILAC-based phosphoproteomics approach was applied to gain insight into the signal transduction pathways regulated by PDGF. In addition, this technique was used in combination with bioinformatics and computational biology to study the role for the one of receptor-type protein tyrosine phosphatase (LAR) on PDGFβR downstream signalling pathways and PDGF-induced cellular functions including cell adhesion, migration and proliferation. This type of comprehensive analysis of the LAR regulated phosphoproteome in response to PDGF stimulation has not been done before.

A total of 29,734 phosphopeptides were identified across the four biological replicates including redundant and non-redundant peptides. In LARΔP cells, a total of 270 phosphopeptides from 205 proteins showed a significant change in abundance ($p<0.05$; >1.5-fold change) upon PDGF stimulation. Of these, 255 (95%) are serine phosphorylation sites, 9 (3%) threonine, and 6 (2%) were tyrosine phosphorylation sites indicating that LAR could contribute to the regulation of these phosphorylation sites via modulation of the activity of specific kinases, or via direct dephosphorylation of the tyrosine sites.

Most of the previous studies on LAR were focused on its role in the regulation of the nervous system including neuronal development, growth, neuronal regeneration and axon guidance (Mélanie J Chagnon et al., 2004a, Sethi et al., 2010, Fang Wang et al., 2012, Julie S Zhang and Longo, 1995). However, GO term analysis revealed an enrichment of LAR-mediated phosphorylation events on proteins involved in many signal transduction pathways affecting the actin cytoskeleton, cell adhesion, endocytosis and metabolism. Kinase prediction tools have identified a key role for LAR in regulating ERK, mTOR and JNK signalling pathways in response to PDGF stimulation. These data have been confirmed by immunoblotting. Importantly, re-expression of WT LAR in LARΔP MEFs rescued the activity of these signalling pathways, providing further support that PDGF-induced mTOR, ERK and JNK pathways is LAR-dependent phenotype. Collectively, these results significantly expand the range of proteins implicated downstream of LAR and provide an extensive publicly available dataset for further scrutiny.

The activity of GRK2 was higher in LARΔP MEFs. GRK2 is a serine/threonine kinase that has previously been shown to regulate PDGFβR by mediating serine

phosphorylation (Hildreth et al., 2004). ERK has been reported to downregulate GRK2 activity through phosphorylation of Ser^{670} (Elorza et al., 2000, Pitcher et al., 1999). Quantitative proteomics revealed a significant increase in the phosphorylation of GRK2 at Ser^{670} in WT cells following PDGF stimulation and this supports the notion that GRK2 is overactivated in LARΔP MEFs. A possible explanation for this might be that LAR is regulating PDGFβR signalling through the inhibition of GRK2 activity since the knockdown of this protein partially restored the activity of ERK1/2 and Akt in LARΔP cells. Another possible explanation for this is that loss of LAR activity in LARΔP MEFs was accompanied by decreased ERK1/2 activity, which would remove the inhibitory effect of ERK1/2 on GRK2, leading to GRK2 activation, which in turn phosphorylates serine residues on the PDGFβR. This is also supported by the observation that in WT cells, ERK1/2 was highly activated, with less GRK2 activity, indicating that LAR enhances PDGFβR downstream signalling through the suppression of GRK2 activity. Further work is required to establish the regulation of GRK2 by LAR. Is it direct tyrosine dephosphorylation or indirectly via mediator proteins? To answer these questions, quantitative phosphoproteomics and mutation approaches could be used to map the phosphorylation of tyrosine residues on GRK2 and identify which site is regulated by LAR. Further research should be also undertaken to investigate the biological functions of these sites. In addition, CRISPR-Cas9 approach could be used to knockout LAR in mammalian cells to better understand the role of LAR in regulation of signal transduction pathways.

Actin cytoskeleton and cell adhesion were the most predominant GO terms identified in the phosphoproteome data set. Therefore, we decided to investigate if LAR regulates cell adhesion. Decreased adhesion complex formation and cell adhesion to

fibronectin were observed in LARΔP MEFs, identifying a novel role for LAR as a regulator of cell adhesion in mammalian cells. It was further investigated the underling mechanism on how LAR regulates cell adhesion. Kinase motifs analysis of phosphoproteome data set identified CDK1 as a predominant node and predicated to phosphorylate the majority of phosphosites. In addition, CDK1 activity was essential for the formation of focal adhesion complexes. By using LAR substrate-tapping mutants, this phenotype is mainly regulated through c-Abl-Akt pathway. Since the inhibition of c-Abl can restored the activity of Akt and CDK1 in LARΔP cells. Akt1 has previously been shown to be required for phosphorylation of CDK1 at Thr^{161} (Nogueira et al., 2012). It is possible, therefore, that Akt acts a downstream of LAR and c-Abl leading to activation of CDK1 and promoting the formation of focal adhesions. Taken together, these findings reveal a novel pathway downstream of LAR that regulates cell adhesion in c-Abl, Akt and CDK1 dependent manner.

In future work, it would be interesting to study if LAR regulates cell adhesion to other extracellular proteins such as collagen or laminin. Total Internal Reflection Fluorescence (TIRF) microscopy technique can be used to study if LAR colocalize with cell adhesion proteins such as paxillin, talin or vinculin in presence and absent of PDGF. We identified a connection between LAR and integrin expression in the phosphoproteomics data set. It would be interesting to assess the effects of LAR on integrin expression and whether LAR enhances cell adhesion or other function via integrin interaction. This may allow whether LAR involved in metastasis since integrins regulate many cellular functions that are crucial to the initiation and progression of cancer. If LAR regulates integrin expression, then LAR protein can be used as a cancer biomarker.

Part of the work presented in this thesis provides the first systematic study of PDGF signalling using a quantitative phosphoproteomics approach. In total, 989 phosphosites from 611 proteins were detected in response to PDGF stimulation. Within the phosphoproteome dataset, the phosphosites comprised 896 (90.6%) serine, 76 (7.6%) threonine and 17 (1.7%) tyrosine phosphorylation sites. Of these, 161 phosphorylation sites showed a significant change in abundance ($p < 0.05$; >1.5-fold change). Interestingly, from 161 phosphosites, 110 (67%) have not been reported as PDGF-regulated sites and are considered novel. These results significantly expand the range of proteins implicated in PDGF signalling pathways.

PDGF-induced cell migration has been widely studied and involves the small GTPase Rac1, ERK MAPK and JNK kinases (Doanes et al., 1998, Ridley et al., 1992, Zhan et al., 2003). The identification of CRMP2 as a PDGF-stimulated regulator of cell migration adds another effector to this list. CRMP2 is dephosphorylated in response to PDGFβR activation and is required for PDGF-induced cell migration. It is possible that PDGFβR modulates the activity of CRMP2 via an unknown adaptor protein. Another possible explanation for these findings is that PDGFβR activates serine/ threonine phosphatases that then dephosphorylate CRMP2 at Thr^{514} leading to activation. This explanation is supported by the fact that inhibition of protein phosphatase 1 (PP1) can restored the phosphorylation of Thr^{514} on CRMP2 upon PDGF stimulation. Further work is needed to identify how PDGFβR is involved in regulation of PP1 activity.

The proteome datasets were analysed in order to identify protein expression regulated by LAR. Within the proteomic data set, a total of 2939 proteins were identified. Of these, 147 proteins showed a significant change in abundance in LARΔP MEFs

compared with the WT. GO terms analysis of these proteins revealed enrichment of metabolic processes. Therefore, we examined the role of LAR in mitochondrial respiration. Higher mitochondrial respiration was observed in LARΔP MEFs compared with WT suggesting that LAR could suppresses mitochondrial respiration by regulating protein turnover. Immunoblotting analysis identified overexpression of succinate dehydrogenase subunit b (Sdhb) in LARΔP cells when compared with WT cells. The evidence from this study suggests that LAR could decrease mitochondrial respiration through regulation of Sdhb expression. However, further studies are required to confirm and validate these findings. For instance, it would be interesting to study if LAR localizes to mitochondria, as there is mass spectrometry evidence showing that LAR can be found in mitochondria (Smith and Robinson, 2016, Stauch et al., 2014). In addition, different LAR constructs could be expressed to identify which domains are responsible for suppressing mitochondrial activity. Further research is also required to determine whether LAR can regulate protein turnover or mRNA level to modulate energetic homeostasis.

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Appendix I

Published work

Sarhan, A.R., Patel, T.R., Creese, A.J., Tomlinson, M. G., Hellberg, C., Heath, J. K., Hotchin, N. A., and Cunningham, D. L. (2016) Regulation of Platelet Derived Growth Factor Signalling by Leukocyte Common Antigen-related (LAR) Protein Tyrosine Phosphatase: A Quantitative Phosphoproteomics Study. **Molecular & Cellular Proteomics**, 15: (6): 1823-1836.

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