Characterisation of the SH2 domain of the melanoma associated adaptor protein, ShcD/RaLP

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Statement

The thesis entitled “Characterisation of the SH2 domain of the melanoma associated adaptor protein, ShcD/RaLP” is submitted for the degree of Doctor of Philosophy. Unless otherwise acknowledged by references or in the text, the thesis based on the experimental work has been fulfilled by the author in the Department of Molecular and Cell biology at the University of Leicester during November 2016 and September 2020. This Thesis has not been submitted for any other degree at this or any other university.

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Characterisation of the SH2 domain of the melanoma associated adaptor protein, ShcD/RaLP Abstract

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ShcD/RaLP is the least characterised member of the Shc adaptor family of proteins. Shc adaptor proteins share the modular structural domains known as phosphotyrosine binding (PTB), Src homology 2 (SH2), and one or two collagen homology (CH1/CH2) domains. These domains are involved in binding activated receptor tyrosine kinases and co-ordinating the activation of various signalling pathways. ShcD is highly expressed in melanomas where high expression levels correlate with increasing vertical growth phase and invasive properties. Since ShcD is a signalling adaptor, its role in migration and invasion is likely to depend on the proteins with which it forms complexes. Previous studies in this lab have shown that the signalling scaffolding protein Gab1 interacts with the SH2 domain of ShcD. Interestingly a possible phosphorylation site has been identified by mass spectrometry within the SH2 domain of ShcD which could regulate binding to Gab1. I have made a S551D phosphomimic mutation in the SH2 domain of ShcD and shown that this inhibits interaction with Gab1 and other tyrosine phosphorylated proteins by GST pull-downs. This suggests that phosphorylation of the SH2 domain by a Ser/Thr kinases may negatively regulate ShcD-Gab1 induced signalling. Future studies will examine the effect of the mutation on ShcD mediated migration and identifying the biological function of kinases that phosphorylate the SH2 domain of ShcD.

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Finally, I would like to thank my family members for encouragement and support. I am sure without my family I would not reach this point. I am really grateful to them.

Research Impact Statement form

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Supervisor	Sally Prigent
Thesis title	Characterisation of the SH2 domain of the melanoma associated adaptor protein, ShcD/RaLP

Research Impact Statement:

The Covid-19 pandemic had an impact on research.

- Lab closures and loss of research materials
- Inability to collect data due to restricted access to laboratory and/ microscopy facility.
- Long term (sample-based experiments impacted)
- The destruction of cells due to restricted lab and travel restriction
- Cancellation of research plans due to lab closures

As a result of the pandemic, planned wound healing assays and thorough microscopy analysis could not be completed.

Student's signature *[by typing your name, you are providing your electronic signature]*	YoungHwan Song
Supervisor signature *[by typing your name, you are providing your electronic signature]*	Sally A Prigent

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Abbreviation

APS: adapter protein with a PH and SH2 domain

BDNF: brain-derived neurotrophic factor

bp: base pair

BSA: bovine serum albumin

cAMP: cyclic AMP

cDNA: complementary DNA

DMEM: Dulbecco’s modified Eagle’s medium

DMSO: dimethyl sulphoxide

DNA: deoxyribonucleic acid

DTT: dithiothreitol

ECL: enhanced chemiluminescence

ECM: extracellular matrix

EDTA: Ethylene Diamino ethane Tetra Acetic acid

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

ERK: extracellular signal regulated kinase

FBS: foetal bovine serum

GAB 1/2: Grb2 associated binding 1/2

GPCR: G-protein coupled receptor

Grb2: growth factor receptor bound protein 2

GST: glutathione S transferase

h: hour

HCl: hydrochloric Acid

HGF: hepatocyte growth factor

H_2O_2: hydrogen peroxide

IL: interleukin

IGF: insulin like growth factor

IPTG: Isopropyl β-D-1-thiogalactopyranoside

IR: insulin receptor

IRS: insulin receptor substrate

Kb: kilo base pairs

kDa: kilodalton

MAPK: Mitogen activated protein kinase

mRNA: messenger RNA

mTOR: mammalian target of rapamycin

MuSK: muscle specific kinase

MW: molecular weight

NaF: sodium fluoride

Na_3VO_4: sodium orthovanadate

OD: optical density

PAK4: Serine/threonine-protein kinase 4

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PDGF: platelet-derived growth factor

PEG: polyethylene glycol

PI3K: phosphatidylinositide 3 kinase

PIP2/PI(4,5)P2: Phosphatidylinositol 4,5 bisphosphate

PIP3/PI(3,4,5)P3: Phosphatidylinositol 3, 4,5 bisphosphate

PKA: protein kinase A

PKC: protein kinase C

PMSF: phenylmethanesulphonylfluoride

PTEN: phosphatase and tensin homolog

RNA: ribonucleic acid

ROS: reactive oxygen species

rpm: revolution per minute

RTK: receptor tyrosine kinase

SDS: sodium dodecyl sulfate

Shc: Src Homology and Collagen

SHIP2: The SH2 domain containing inositol 5-phosphatase 2

SH-PTP2/SHP2: Src homology region 2 domain-containing phosphatase-2

siRNA: small interfering RNA

SOS: son of sevenless

TAE: tris-acetate-EDTA buffer

TBS: tris-buffered-saline

VEGF: vascular endothelial growth factor receptor

WT: wild type

YPD: yeast extract peptone dextrose

2x YT Yeast Extract Tryptone broth

Chapter 1

Introduction

1.1 The Src Homology and Collagen (Shc) adaptor protein

Cell signaling is a complex multistep process involving various types of protein-protein interactions which can be initiated by the binding of proteins that have SH2 (Src homology 2) or PTB (Phosphotyrosine binding) domains to a phosphotyroine motif on an activated receptor tyrosine kinase (RTKs). The Shc (Src homology and collagen) protein family are known as adapter proteins that play a crucial role in signal transduction and have both SH2 and PTB domains among all Shc family members (Ahmed and Prigent., 2017). As the members of the Shc protein family have both SH2 and PTB domains it allows the proteins to interact with several receptor tyrosine kinases (RTKs) and signaling transducer proteins like Grb2 (growth factor receptor bound protein 2) in cells, which lead to regulating various cell responses such as migration, apoptosis or cell proliferation via cell signaling pathways (Wills *et al*., 2014). Shc proteins in mammalian cells comprise 4 major members and 7 isoforms known as ShcA/Shc (p46, p52 and p66), ShcB/Sli (p68), ShcC/Rai (p52 and p64) and ShcD/RaLP (p69) (Ahmed and Prigent., 2017) as shown Figure 1.1.

The four members of the Shc protein and their isoforms are encoded by four different genes with alternative initiation codon usage and splicing patterns in the production of Shc proteins (Hawley *et al*., 2011). ShcD protein is the most recently discovered member of the Shc adaptor protein family, identified by TBLASTn search on each ShcA, ShcB and ShcC sequences with both human and mouse genome (Fagiani *et al*., 2007). The analysis of Shc family proteins indicate that they share the common modular domain structure. There are three types of domains that form the structure of Shc proteins; an N-terminal Phosphotyrosine-binding (PTB) domain, a collagen homogy 1 (CH1) domain rich in both proline and glycine residues and lastly a C-terminal Src homology 2 (SH2) domain (Landry *et al.*, 2016). Certain Shc proteins such as p66ShcA, p68ShcB, P64ShcC and p69ShcD have an additional domain that forms the N-terminus known as a collagen homology 2 (CH2) domain (Wills and Jones., 2012).

Shc family proteins do share the modular domains however they have their unique expression patterns and function. ShcA proteins are widely expressed in adult tissues except in the nervous tissues with a main role of regulating cell proliferation and cell growth. ShcB and ShcC proteins are expressed only in the central nervous system tissues and in particular ShcC expression replaces that of ShcA during brain development suggesting a role for the survival of mature nervous cells (Pelicci *et al*., 2002). The ShcD protein is expressed mostly in the central nervous system but also present in skeletal/cardiac muscle, epithelia region of many organs and neural crest-derived tissues (Robeson *et al*., 2019).

The studies on ShcD protein have identified several biological roles such as ShcD's involvement in melanoma cell migration (Fagiani *et al*., 2007), its involvement in the brain-derived neurotropic factor (BDNF)-TrkB signaling pathway by interacting with TrkB via both SH2 and PTB domains (You *et al*., 2010). The BDNF binding with TrkB receptor induces many neuronal cells related biological functions via signaling cascades such as Shc linked Ras/ERK pathway leading to cell proliferation and differentiation while PI3K/AKT/mTOR pathway neuron cell survival/growth (Sasi *et al.,* 2017). In addition, BDNF-TrkB-tyrosine 816 acts as a binding site for PLC pathway which leads to release of calcium from intercellular store and regulatiessynaptic plasticity (Pradhan *et al*., 2019). ShcD binds Muscle-Specific Kinase (MuSK) through its PTB domain and possibly plays a role in MuSK receptor signaling pathway (Jones *et al*., 2007). MuSK is a receptor tyrosine kinase involved in formation of neuromuscular junctions via playing major role in signaling (Hubbard and Gnanasambandan., 2013). In addition, ShcD protein interact with the proto-oncogene tyrosine-protein kinase RET receptor upon Glial cell line-Derived Neurotrophic Factor (GDNF) stimulation and significantly decreased rate in cell migration and viability in neuroblastoma (Maburk *et al*., 2018). The GDNF is one of important and well-known growth factor that involves in survival and differentiation of neuron cells during neuronal development period via RET receptor signaling (Mulligan., 2019). Interestingly the interaction of ShcD with RET led to opposite biological responses to those observed in melanoma cells which indicates that the

biological responses vary depends on stimulation and cell types (Mabruk *et al*., 2018).

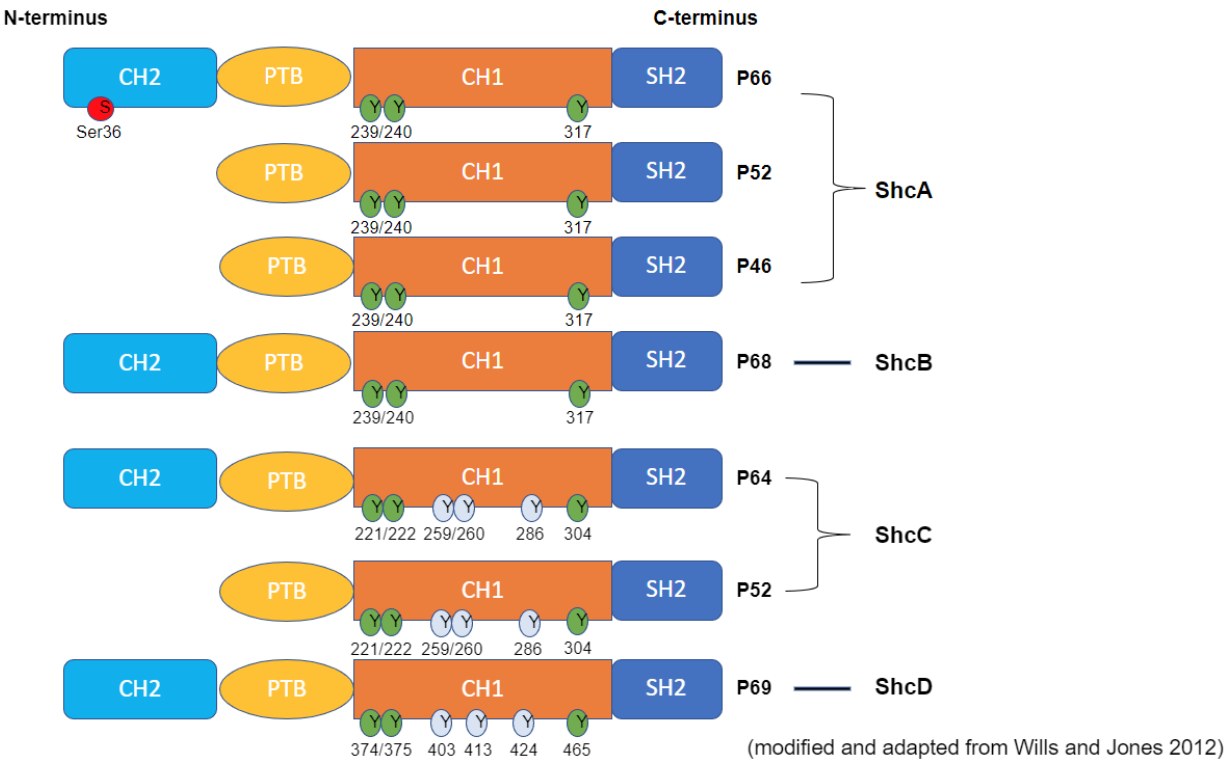

Figure 1.1: The modular domain structures of Shc protein family in mammalian cells. All Shc family members share the modular domain structure (CH2)-PTB-CH1-SH2. Three important tyrosine residues (green circle) in the CH1 domain are present in all Shc protein members. Those tyrosine residues are phosphorylated by RTKs and allow Shc protein members to recruit Grb2. The N-terminal CH2 domain is unique to certain Shc protein members like p66ShcA, p68ShcB, p64ShcC and p69ShcD. The CH2 domain of p66ShcA possesses a unique Serine residue [Ser36] (red circle) and that has a pro-apoptic function.

1.2. Structure of Shc Proteins

Shc family members share the modular structural domain sequences of (CH2)-PTB-CH1-SH2 and this unique modularity allows Shc proteins to form complexes with multiple proteins phosphorylated on tyrosine residues, and other SH2 or via PTB domain containing proteins

1.2.1. Src Homology 2 (SH2) domain

The SH2 domain forms the C-terminal domain of the Shc protein and although when Shc was first identified it was assumed that the SH2 domain would be the main domain for binding EGFR. This was before it was shown to have another phosphotyrosine binding domain the PTB domain. The PTB domain plays a more important role in interacting with receptors (Wills *et al*., 2014).

The SH2 domain of Shc has a positively charged binding pocket that recognizes the phosphorylated tyrosine (Wagner *et al*., 2013) and it is more likely to bind the phosphorylated peptides/receptors with a leucine or isoleucine residues positioned three residues C-terminal to the phosphotyrosine (+3 position) (Wollberg *et al.*, 2003). The structure of ShcA SH2 domain bound to a phosphopeptide from T-cell receptor has been solved by nuclear magnetic resonance (NMR) (Zhou *et al*., 1995) The position of the serine corresponding to ShcD S551 is shown in Figure 1.2

The SH2 domain binds to phosphorylated tyrosine residues on growth factor receptors such as EGFR, insulin receptor and platelet derived growth factor receptor which allows the Shc family member proteins to initiate the signal transduction cascades (Wagner *et al*., 2013; Huang *et al*., 2008).

The other adaptor proteins such as Grb2-SH2 recognize the pYXN motif where pY - phosphotryosine, X - any amino acid and N – asparagine and regulate the activation of Ras signaling pathways (Higo *et al*., 2013). The pYXN motif can be found in cytoplasmic tails of signaling receptors like EGFR and PDGFR (McDonald *et al*., 2010). In addition, a lot of other signaling proteins also have this motif such as Shc proteins. Furthermore, PI3K subunit P85 also have SH2 domain and this p85-SH2 domain recognize the pYXXM where pY - phosphotryosine, M - methionine, and X - any amino acid (Lee *et al*., 2011). Those two motifs are found in insulin receptor substrate IRS-1/IRS-2, Gab1 and other cellular proteins. The SH2 domain plays a critical role in RTK signaling pathways as SH2 domain is essential for signaling proteins to interacting with either docking complexes or phosphorylated RTK (Jadwin *et al*., 2016)

1.2.2. PhosphoTyrosine-Binding (PTB) domain

The N- terminal PTB domain is another one of the main structures that form the Shc protein and this domain recognizes the NPXY motif which allows the PTB domain to bind to integrin cytoplasmic tails and receptor tyrosine kinases like EGFR (McCleverty *et al*., 2007). In addition, The Shc-PTB domain recognizes and binds the NPXY motif on insulin receptors (Hanke and Mann. 2009).

The two specific binding regions of the PTB domain allow it to interact with both peptides and the headgroup of phosphatidylinositides as well to regulate the spatial orientation and membrane localization (Uhlik *et al*., 2005). In addition, the subfamily of PTB domains can also interact with peptide sequences containing unphosphorylated tyrosine (Sain *et al.*, 2016).

The analysis of PTB domain crystal structure revealed that all PTB domains appears to have same folding pattern, the ligand binding pocket consists of a β5 strand [two virtually orthogonal β-sheets forming β- sandwich] with a carboxy-terminal α-helix located at ligand binding region (Wagner *et al*., 2013). Interestingly, PTB domains have a similar folding structure to pleckstrin homology (PH) domains, however PH domains do not have conserved residues for recognizing specific phosphotyrosines. (Eck *et al*., 1996).

The research using the yeast two hybrid assay showed that the bait SH2 and PTB domains of ShcD can interact with TrkB and further studies showed that brain derived neurotrophic factor (BDNF) stimulation leads to the interaction of the ShcD/RaLP PTB domain with the NPQY motif on TrkB resulting in an increase of the MAPK activation (You *et al*., 2010). In addition, recent studies indicate that ShcD protein is expressed in the central nervous system along with ShcB and ShcC, and only ShcD is expressed in the Olfactory bulb (Robeson *et al*., 2019) Interestingly ShcD has been shown to be involved in inhibiting the Erk activation downstream of TrkB which suggests that ShcD possible have a role in neural cell growth (Wills *et al*., 2017).

1.2.3. The Collagen homology 1 (CH1) domain

Shc protein's CH1 domain is located in between the PTB and SH2 domains. The CH1 domain is rich in glycine/proline and has several PXXP motifs which act as binding sites for other proteins containing SH3 domains (Ravichandran., 2001). ShcA proteins have three important tyrosine residues Y239, Y240 and Y317 on the CH1 domain and those tyrosine residues are present within a YXN motif which upon phosphorylation in response to growth factor stimulation and receptor binding, act as binding sites for the SH2 domain of Grb2 (Hardy *et al*., 2007). In addition, Y239/Y240 tyrosine residues induce the c-Myc activation while Y317 was shown to activate the MAPK signaling pathway (Ravichandran., 2001 and Geer *et al*., 1996). However, any mutation on either one or both tyrosine Y239/Y240 or Y317 shown reduction of interaction with Grb2 and mutation on tyrosine Y239/Y240 lead to

decreased c-Myc transcription (Patrussi *et al*., 2005). This suggests that tyrosine Y239/Y240 and Y317 are all important for signaling.

The phosphorylation of tyrosine Y317 of ShcA is recognized by Grb2-SH2 in complex with other signaling proteins like SOS and mediates the mitogenic signaling via Ras/MAPK signaling pathway (Siegel and Muller., 2008) while Y239/240 mediate the c-Myc dependent cell survival

ShcC isoforms and ShcD protein have additional conserved tyrosine residues 259/260/286 and 403/413/424 respectively as shown Figure 1.1. When ShcC conserved tyrosine residues phosphorylated, tyrosine Y286 interacts with other proteins like Crk while tyrosine Y259/Y260 appears to have a role for providing binding sites for other signaling proteins with either SH2 or PTB domains that recognize the phosphorylated tyrosine residues (Nakamura *et al*., 2002). The studies of ShcD protein identified that ShcD-CH1 domain conserved tyrosine residues Y374/375, Y465 and Y424 providing two additional binding sites for Grb2 and act as regulator of Erk repression but tyrosine residues Y403/Y413 appear to be not involved in phospho-Erk responses (Wills *et al*.,2017).

1.2.4. The Collagen homology 2 (CH2) domain

The CH2 domain is located at the N-terminus and is only found on certain Shc proteins such as p66ShcA, p68ShcB, p64ShcC and ShcD/RaLP proteins as shown in Figure 1.1. ShcA protein's CH2 domain differs from the CH1 domain in that the CH2 domain does not get phosphorylated on tyrosine residues but is phosphorylated on Serine (Ser36) which has pro-apoptotic function (Bhat *et al*., 2015). In the CH2 domain of p68ShcB and p64ShcC Ser36 is not conserved, but proline rich motifs act as recognition sites for SH3 domain containing proteins (Jones *et al*., 2007).

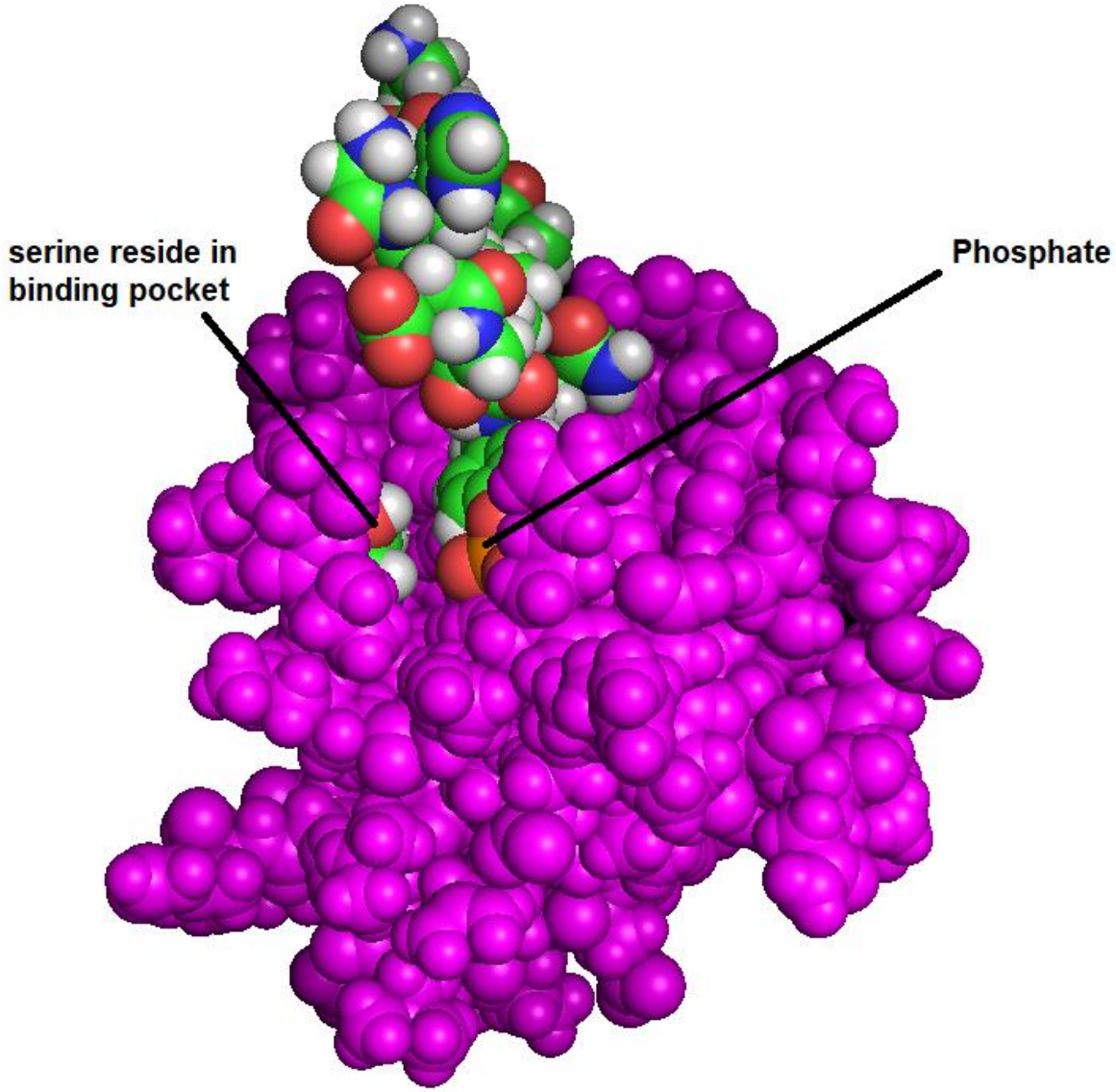

Figure 1.2: NMR structure of ShcA SH2 domain bound to a phosphopeptide from T-Cell receptor. The 3D structure of ShcA SH2 domain used as model for ShcD-SH2. The position of the serine corresponding to S551 in ShcD is indicated along with the phosphate group of the phosphotyrosine residue in the bound phosphopeptide (Zhou *et al.*, 1995).

1.3. Biological function of Shc proteins

Although Shc proteins share a similar structure they have different functions. p52ShcA is involved predominantly in cell proliferation, p66ShcA has a role in apoptosis [CH2-Ser36], p64ShcC and p68ShcB have been shown to contribute to neuron cell survival and ShcD/RaLP has been shown to contribute to melanoma cell migration (Fagiani *et al*., 2007 and Jones *et al*., 2007). All Shc proteins recognize activated RTKs and induce the signaling pathway to regulate cellular functions such as the Ras/MAPK for cell proliferation, PI3K for neuronal cell survival and stress induced apoptosis (Wills and Jones 2012). The most recent identified Shc member ShcD/RaLP also induces the Ras/MAPK signaling pathway in some cells, but unlike p52ShcA, ShcD function induces a Ras-independent pathway that stimulates melanoma cell migration (Fagiani *et al*., 2007).

1.4. Signaling transduction pathway involved in melanoma progression

1.4.1. Mitogen activated protein kinase (MAPK) signal transduction

MAPK signal transduction is one of the well characterized pathways and a major target for anti-cancer research. The MAPK signaling pathway mediates the various cellular responses including cancer cell proliferation, apoptosis, migration and differentiation (Kim and Choi., 2010). In most of melanomas the MAPK signaling pathway is activated, and they frequently have activation mutations in either NRas (15-20% cutaneous melanoma) or BRAF (40-50%) (Sullivan and Flaherty., 2013).

In the MAPK signaling pathway, Ras GTPase is the most critical mediator that is activated downstream of RTKs like EGFR, growth promoting integrin and G-protein coupled receptors (Dhillon *et al*., 2007).

The ShcA involvement in MAPK signaling pathway via interacting with activated RTKs is well characterized as shown Figure 1.3. When growth factors, like EGF or PDGF, bind to their receptors leading to activation, Shc protein recognizes specific phosphorylation sites on the receptor and interacts via the SH2 domain or PTB domain. This interaction leads to phosphorylation of tyrosine residues in the CH1 domain of ShcA. The Grb-SH2 binds to phosphorylated tyrosine of CH1 domain and recruit the SOS which results in activation of the Ras/MAPK signaling pathway and leads to cellular responses like cell proliferation, migration and survival (Lee *et al*., 2007)

1.4.2 Shc and phosphatidylinositol-3-kinase (PI3K) pathway

The PI3K signaling pathway has been identified to be very commonly activated in cancers is a very promising therapeutic target. The PI3K signaling pathway starts with activation of RTKs. The stimulation of PI3K leads to phosphorylation of phosphatidylinositol-4,5-biphosphate [PI(4,5)P2] to make phosphatidylinositol-3,4,5-triphosphate [PI(3,4,5)P3], which results in the activation of AKT. In addition, phosphatase and tensin homolog (PTEN) negatively regulates the PI3K/AKT pathway by dephosphorylation of PI(3,4,5)P3 (Chalhoub and Baker., 2009) as shown in Figure 1.4.

The studies of PI3K signaling pathway reported that it can be stimulated with various types of growth factors and interact with several proteins which lead to cell proliferation, migration, survival and differentiation (Yang *et al*., 2019).

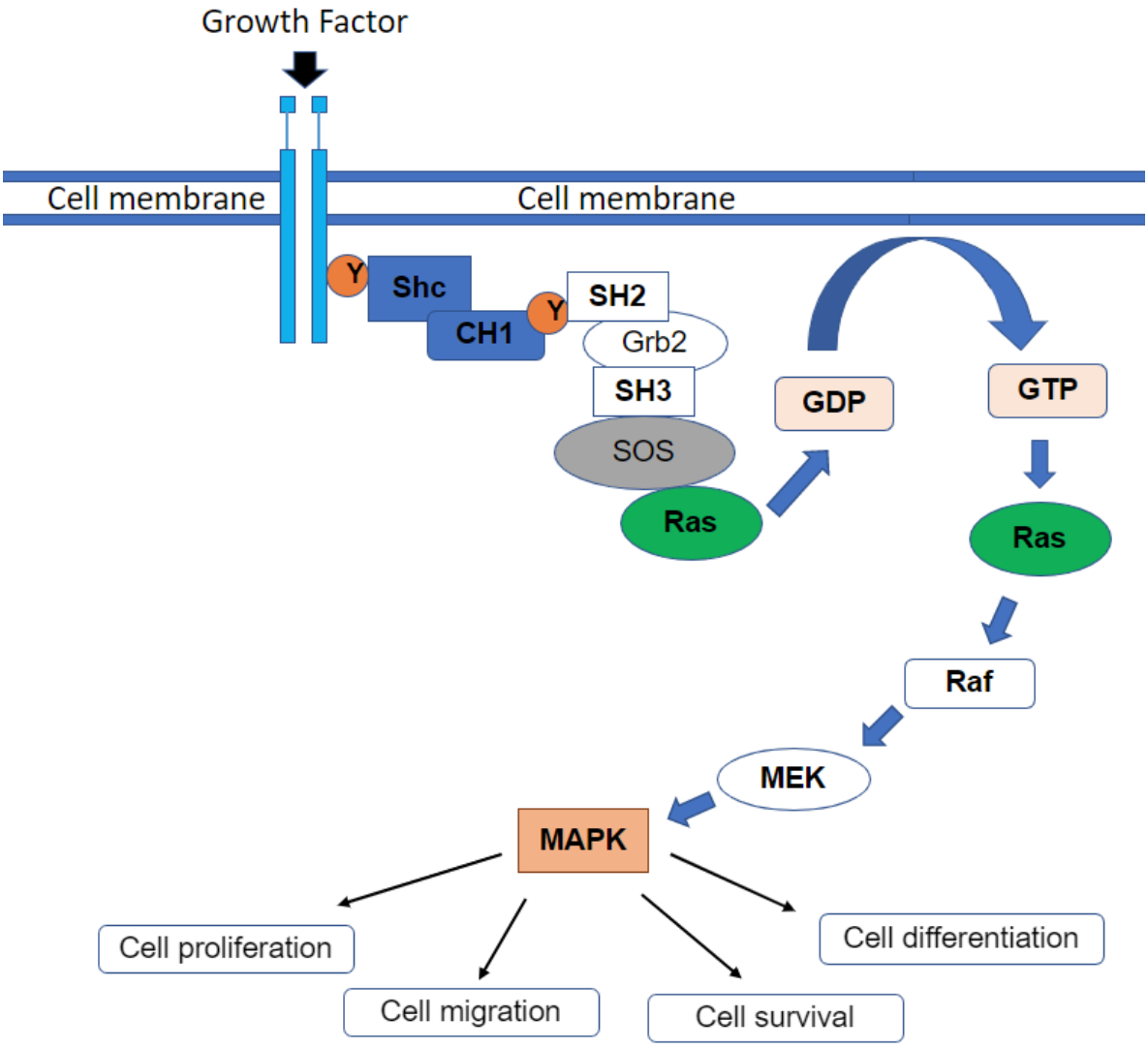

Figure 1.3: ShcA-Grb2/SOS/Ras mediated MAPK signaling pathways. Upon growth factor stimulation, Shc protein interacts with activated RTKs via either SH2 or/and PTB domains. Tyrosines in the CH1 domain of Shc are phosphorylated and Grb2-SH2 domain interacts with specific phosphotyrosine residues. The interaction leads to recruitment of SOS, and activation of Ras. The Grb2-SOS complex localizes to cell membrane and stimulates Ras/MAPK signaling transduction resulting in cellular responses such as cell proliferation, migration, survival and differentiation.

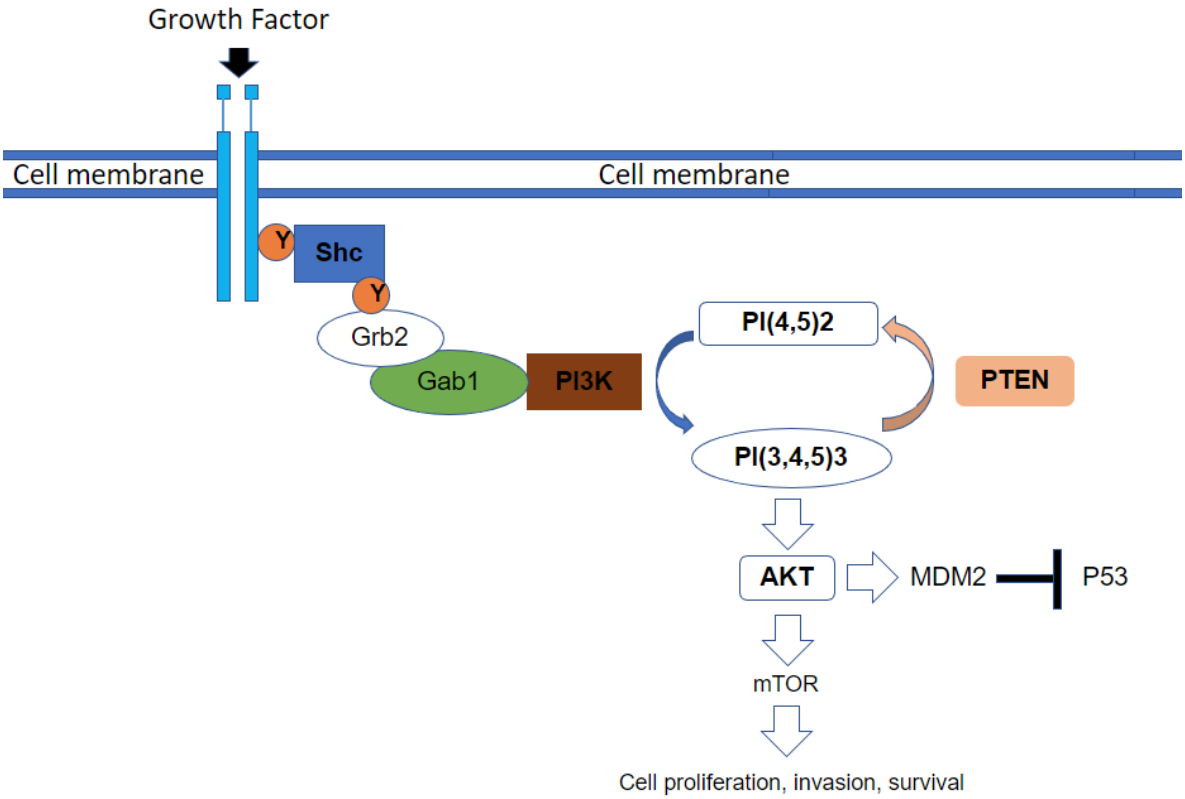

Figure 1.4: diagram of possible Shc protein involvement in PI3K signal transduction. Shc protein interacts with phosphorylated tyrosine residues at RTK. This interaction leads to recruitment of Grb2 and Gab1 to stimulate the PI3K/AKT pathway. PTEN act as switch that negatively regulates PI3K signaling and MDM2 also negatively regulates the P53 that preventing the transcriptional initiation.

As shown in Figure 1.4, the Shc protein plays a role in PI3K signaling pathway by linking activated RTKs and Grb2-Gab1 complexes which leads to activation of AKT (Xie *et al*., 2018). The activated AKT can phosphorylate mammalian target of rapamycin (mTOR) resulting cell proliferation, invasion and survival. In addition, activated AKT can also phosphorylate MDM2 (Zou et al., 2020). As shown in Figure 1.4

1.5 ShcD/RaLP

ShcD is the most recently identified Shc adaptor protein Shc family member and as a member of Shc family, it has the same modular domain structure as other Shc proteins as shown in Figure 1.1. ShcD was discovered by running TBLASTn analysis on human and mouse genetic sequences with ShcA, ShcB and ShcC sequences (Jones *et al.*, 2007). The result of TBLASTn analysis identified that ShcD also has the same modular structure domain CH2-PTB-CH1-SH2 as other Shc protein. However, ShcD appears to have most conserved sequence with ShcA compared to other members. The ShcD protein conserved PTB region has (69%-76%) while for the SH2 region share 55-60%; the CH1 region have 18-24% and the CH2 region share 17-25% identity compare to overall protein identity level for ShcA, ShcB and ShcC being 45%, 41% and 37% respectively (Fagiani *et al*., 2007). Although ShcD-CH2 domain identity percentage is around 17-25%, the CH1 domain still contains conserved tyrosine residues (374/375/465) and has additional tyrosine phosphorylation sites (403/413/424) as shown in Figure 1.1.

The ShcD protein mainly localizes to the cytoplasm and around 5% of ShcD localized in the cell membrane without any stimulation (Fagiani *et al*., 2007). The ShcD can also bind to EGFR RTKs, like other Shc protein members ShcA and ShcB but interestingly, ShcC protein cannot interact with either EGFR or Grb2 and go through phosphorylation like other Shc protein members (Wills *et al*., 2014).

The overexpression of ShcD was detected in metastatic melanoma cancers (Fagiani *et al*., 2007). In normal circumstances, melanoma progression begins with normal melanocytes then progress to a benign nevus. The benign nevus can lead to radical growth phase melanoma and once melanoma has expanded into the epidermis during radial growth phase it acquires the possibly to develop into vertical growth phase melanoma (Elder., 2006). After those stages, melanoma eventually develops into metastatic cancer. The initial studies on ShcD reported that it interacts with EGFR and insulin like growth factor receptor in metastatic melanoma, and expression of endogenous ShcD leads to increased MAPK signaling (Fagiani *et al*., 2007).

Interestingly, when ShcD expression was knocked-down using siRNA in metastatic melanoma cell lines, this resulted in a decrease in the rate of cell migration and invasion (Fagiani *et al*., 2007). In addition, the MAPK signaling pathway was not affected by ShcD knockdown in melanoma cells indicating that ShcD can stimulate cell migration via either Ras dependent or independent pathways (Fagiani *et al.*, 2007). Those results clearly suggested that ShcD plays a critical role in late stages of melanoma and migration.

The ShcD was identified to be expressed in skeletal muscle and binds to muscle specific kinases (Musk) (Jones *et al*., 2007). The ShcD protein was also identified in the central nervous system, and in the CNS, expression overlaps with other Shc proteins (Hawley *et al*., 2011). The ShcD protein expression in CNS suggests that ShcD has a possible role in the CNS such as cell survival.

The analysis of ShcD via affinity purification-mass spectrometry using HEK 293T cells identified that Shp2 phosphatase co-immunoprecipitated with ShcD in the presence or absence of TrkB indicating that it can bind to the ShcD leading to a decrease of ShcD phosphorylation (Wills *et al.*, 2017). In addition, You and colleagues (2010) demonstrated ShcD binds TrkB through the PTB and SH2 domain by yeast two hybrid and co-immunoprecipitation experiments. Firstly, ShcD-PTB interacts with Y518 of TrkB in the juxta-membrane region which is contained within an NPQY motif as identified by yeast two hybrid assay and Co-IP. Additionally, ShcD protein regulates the BDNF mediated MAPK signaling pathway and the western blotting analysis indicated a slight increased MAPK phosphorylation using anti-pErk antibody (You *et al*., 2010). This suggests that ShcD can be phosphorylated by TrkB and any BDNF stimulation leads to increased ShcD interaction with TrkB.

1.5.1 Biological function of ShcD

The ShcD biological function and involvement in melanoma progression has not been fully understood. The analysis of ShcD suggests that ShcD can directly associate with several different cell surface receptors such as EGF, HGF and IGF (Mabruk *et al*., 2018).

Analysis of the CH2 domain of ShcD has revealed the presence of a potential nuclear export sequence "^{83}LCTLIPRM^{90}" and oxidative stress induces modest nuclear accumulation of ShcD suggesting a role for ShcD in the nucleus (Ahmed and Prigent., 2014).

1.5.2 ShcD and melanoma

The ShcD protein is overexpressed in 50% of late-stage melanomas, and the expression level of the protein increases as melanoma progresses from radial growth to vertical growth (Fagiani *et al.*, 2007). The high level of ShcD expression in metastatic melanoma was confirmed by western blotting. In addition, overexpression of ShcD was shown to maintain the amoeboid movement by restricting Dedicator of cytokinesis 4 (DOCK4) in the cytoplasm via inhibition of the Ras-related C3 botulinum toxin substrate 1 (Rac1) signaling pathway (Aladowicz *et al*., 2020). The DOCK4 is part of Guanine nucleotide exchange factors (GEFs) group which play a role in cell signaling process via activating Rho GTPase Rac1 such as cell migration and proliferation (Yu et al.,2015).

EGF stimulation of melanoma cell lines resulted in increased ShcD phosphorylation and MAP activation. However, the PI3K signaling pathway was not shown to be activated by EGF or IGF-1 stimulation as there was no effect on the phosphorylation level of AKT (Fagiani *et al*., 2007).

The siRNA experiment to knock-down ShcD in melanoma dramatically reduced the cell migration and cancer development rate (Fagiani *et al*., 2007). Furthermore, silencing the ShcD expression in metastatic melanoma also resulted in a decrease of the rate of migration and metastasis of melanomas without affecting MAPK signaling pathway (Pasini and Lanfrancone., 2009). Those studies suggest that ShcD plays a critical role in migration and metastasis of melanomas.

1.6. Grb2 associated binding 1 (GAB1) protein

The Gab1 protein was identified as a binding partner for ShcD and by screening a yeast two-hybrid library with the ShcD-SH2 domain as a bait protein (Chung, 2013). Two possible tyrosine phosphorylation sites Y162 and Y183 were identified in the fragment of Gab1 isolated (Prigent, unpublished). Site directed mutagenesis confirmed Y183 to be the binding site. Grb2 associated binding (GAB) adapter proteins play a crucial role in signaling and there are 3 GAB proteins found in the mammalian cells known as Gab1, Gab2 and Gab3. All Gab proteins have an amino-terminal pleckstrin homology (PH) domain and proline-rich sequences (PXXP) which are potential binding sites for SH3 domain containing proteins (Nishida and Hirano, 2003).

Various growth factors can induce phosphorylation of Gab1 and this phosphorylation allows Gab1 proteins to interact with various other signaling proteins like PLCγ, Crk, and CrkL (Gu and Neel., 2003). Gab1 contains multiple tyrosine phosphorylation sites which can interact with SH2 or PTB domain-containing proteins as shown in Figure 1.5. The interaction of Gab1 with other proteins leads to several signaling pathways and Gab1 is the best characterized protein among Gab proteins. Gab1 is a critical regulator that is involved in many signaling pathways initiated by cell surface receptors which results in cellular responses like migration and proliferation.

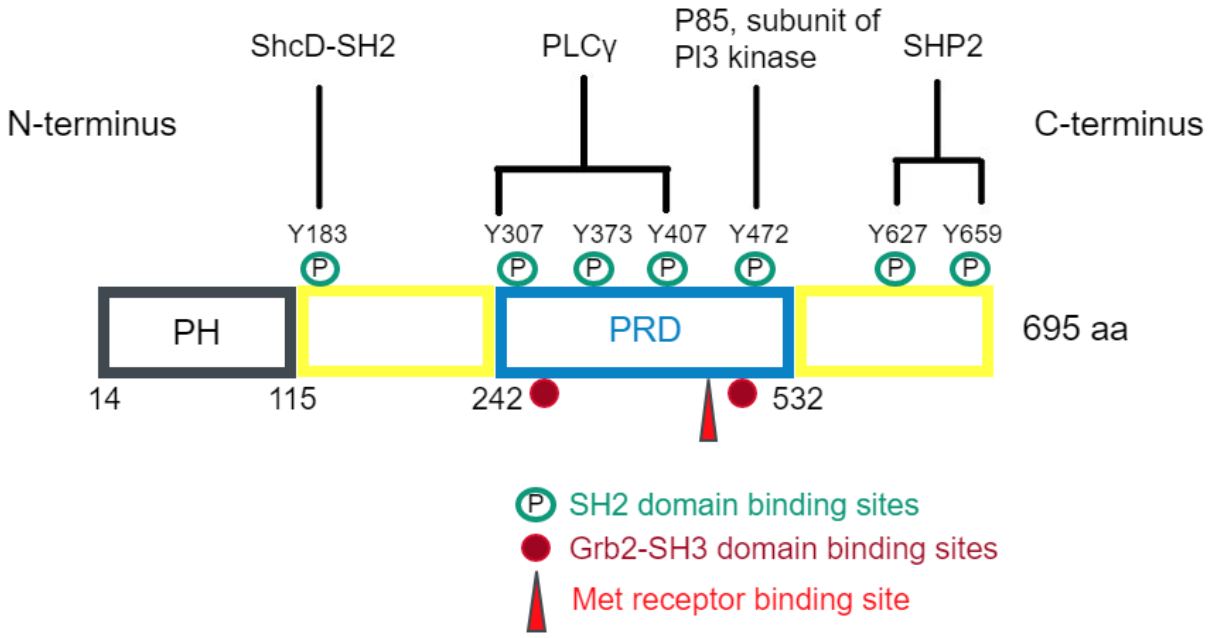

Figure 1.5: Domain structure of mammalian Gab1 protein illustration. Gab1 protein structure is made up of an N-terminal PH domain, a proline-rich (PRD) domain where tyrosines Y307, Y373, and Y407 can bind PLC, Y472 binds p85 subunit of PI3 kinase, Y627 and Y659 bind SHP2. The binding sites for SH2 domain as (green), Met receptor is indicated in (red) and the Grb2-SH3 domain binding site are shown in brown (adapted from Nishida and Hirano., 2005; Chung, 2013).

1.6.1. The Pleckstrin Homology (PH) domain of Gab1 protein

The N-terminal PH domain is the most conserved domain in Gab protein. The PH domain allows Gab1 protein to interact with cell membrane lipids, specifically phosphoinositides phosphorylated on the 3'-OH group (Gu and Neel., 2003). The PH domain plays as important role in recognizing the membrane and localizing Gab1 upon RTK stimulation such as Met activation (Maroun *et al*., 1999).

The Gab1 protein is recruited to PI(3,4,5)P3 or EGFR via Grb2 depending on the EGF stimulation signal strength (Chen *et al*., 2015). The strong EGF stimulation leads Gab1 to interact with receptor directly through Grb2 while weak EGF stimulation resulting Gab1 to binds PI(3,4,5)P3.

1.6.2. Proline rich domain (PRD) of Gab1 protein

The PRD domain in Gab proteins has multiple PXXP motif residues which bind to SH3 domain and act as possible binding sites for other proteins (Wöhrle *et al*., 2009). The analysis of the Gab protein PRD sequence revealed two motifs (PXXPXR and PX_3RX_2KP) which allow Grb2 adaptor proteins to dock in complex with other signaling transduction proteins like SOS and SOS also interacts with Grb2 via its SH3 domain so you wouldn't expect Grb2 to bind SOS and Gab1 at the same time. Grb2 SH2 domain is free to bring other proteins to the complex such as Shc (Liu and Rohrschneider., 2002).

The PRD domain of Gab1 has various tyrosine residues that can interact with other proteins, and one particular/unique site allows Gab1 protein to bind the activated Met receptor as shown in Figure 1.5 and 1.6 A. The PRD region of the Gab1 protein allows interaction with Met directly or indirectly via Grb2 (Furge *et al*., 2000). In addition, PRD domain of Gab1 is essential for EGF stimulated signaling. An experiment mutating PRD tyrosine Y307, Y373 and Y407 to phenylalanine in Gab1 showed no interaction between Gab1 and PCLŸ upon EGF induced Akt activation (Mattoon *et al.*, 2004).

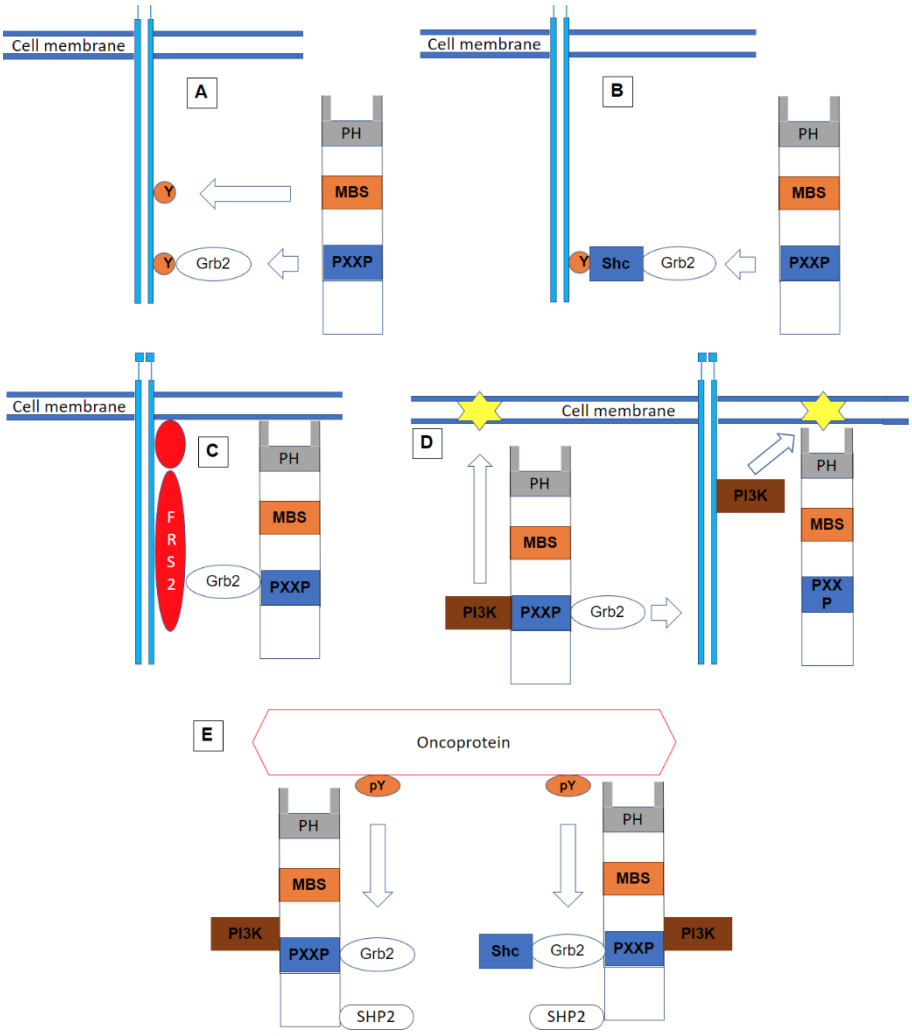

Figure 1.6: Gab1 recruitment and stimulation of signaling pathways. (A) Gab1 protein recruitment either directly via Met-Binding Sequence (MBS) or indirectly via Grb2 to the HGF receptor (c-Met) to stimulate signaling. (B) Gab protein can interact with other adapter protein Shc and Grb2 to IL3/IL-5 receptors. (C) Recruitment of Gab1 protein via scaffolding protein. Where Gab1 is recruited to the Grb2 complex and binds to the scaffolding protein FRS2-Fibroblast growth factor receptor. (D) Gab family protein members are recruited via PH domain. Some signaling pathway require initial recruitment before binds to receptors like (B-Cell Receptor) or EGFR for sustained signaling. (E) Gab proteins recruitment via oncoprotein. Gab proteins involved in several oncogenic stimuli via recruitment of Shc-Grb2-Gab1 complex or Gab2 to Y177 of Bcr-Abl as part of Grb2-Gab2 complex (Adapted from Gu and Neel., 2003).

1.7. Gab1 protein induced signaling pathway via RTKs in melanoma

1.7.1. c-Met receptor signaling pathway

The analysis of Gab1 and c-Met receptor showed that c-Met receptor has two tyrosines Y1349, that directly bind Gab1 and stimulate downstream signaling (Schaeper *et al.*, 2000). The interaction of Hepatocyte Growth Factor (HGF) with c-Met receptor led to the activation of numerous intercellular signaling pathways which regulate embryogenesis and wound healing in normal cells but in cancer cell, c-Met gene mutations, amplification or overexpression can lead to development and rapid growth of cancer via stimulating various signaling pathways such as PI3K/AKT, Ras/MAPK, SRC and JAK/STAT (Zhang *et al*., 2018).

The HGF binding to c-Met receptor triggers dimerization and activation of autophosphorylation, this leads to recruitment of various signaling proteins such as Gab1, Grb2, SOS, SRC, Shc, PI3K, PLCŸ, SHIP2 and STAT3 to stimulate signaling pathways (Organ and Taso., 2011). The c-Met signaling stimulated by HGF induces cellular responses such as angiogenesis, cell proliferation, migration, mobility and invasion via activation of MAPK, Erk, PI3K/AKT and STAT (*Wang et al*.,2020).

The overexpression and abnormal activation of c-Met was found in melanoma cell lines which correlated with melanoma cell growth and metastasis (Demkova and Kucerova., 2018). In addition, high expression level of HGF/c-Met has been shown to be associated with promoting cell survival of melanoma cells, and protection from apoptosis via stimulation of MAPK and PI3K pathways (Czyz., 2018).

1.7.2. EGF receptor signaling pathway

EGFR is an important RTK in cancer and induces various cell signaling pathways such as cell proliferation, cell migration, and differentiation (Fraguas *et al*., 2011). When growth factors like EGF bind to RTKs [EGFR] this leads to dimerization and phosphorylation causing the tyrosine kinase to become activated. Upon activation of EGFR tyrosine kinase, it phosphorylates and recruits other adaptor proteins like Shc or Grb2 as shown in Figure 1.7.

In this EGFR signaling Shc or Grb2 proteins play a critical role as directly or indirectly recruiting other adaptor proteins (Hashimoto *et al*., 1999). The process of signaling cascade is regulated by Grb2, because Grb2 can interact with either Gab1/PI3K to induce survival or Shc-Grb2-SOS to activate Ras/MAPK signaling to induce the cell proliferation (McCubrey *et al*., 2012).

Gab1 mediated PI3K/AKT activation also appears to play a role in cancer angiogenesis (Wang *et al*., 2016).

The EGFR expression level increases significantly as benign nevi progress to melanoma indicating EGFR is crucial for development of early melanoma (Akslen *et al*., 2008).

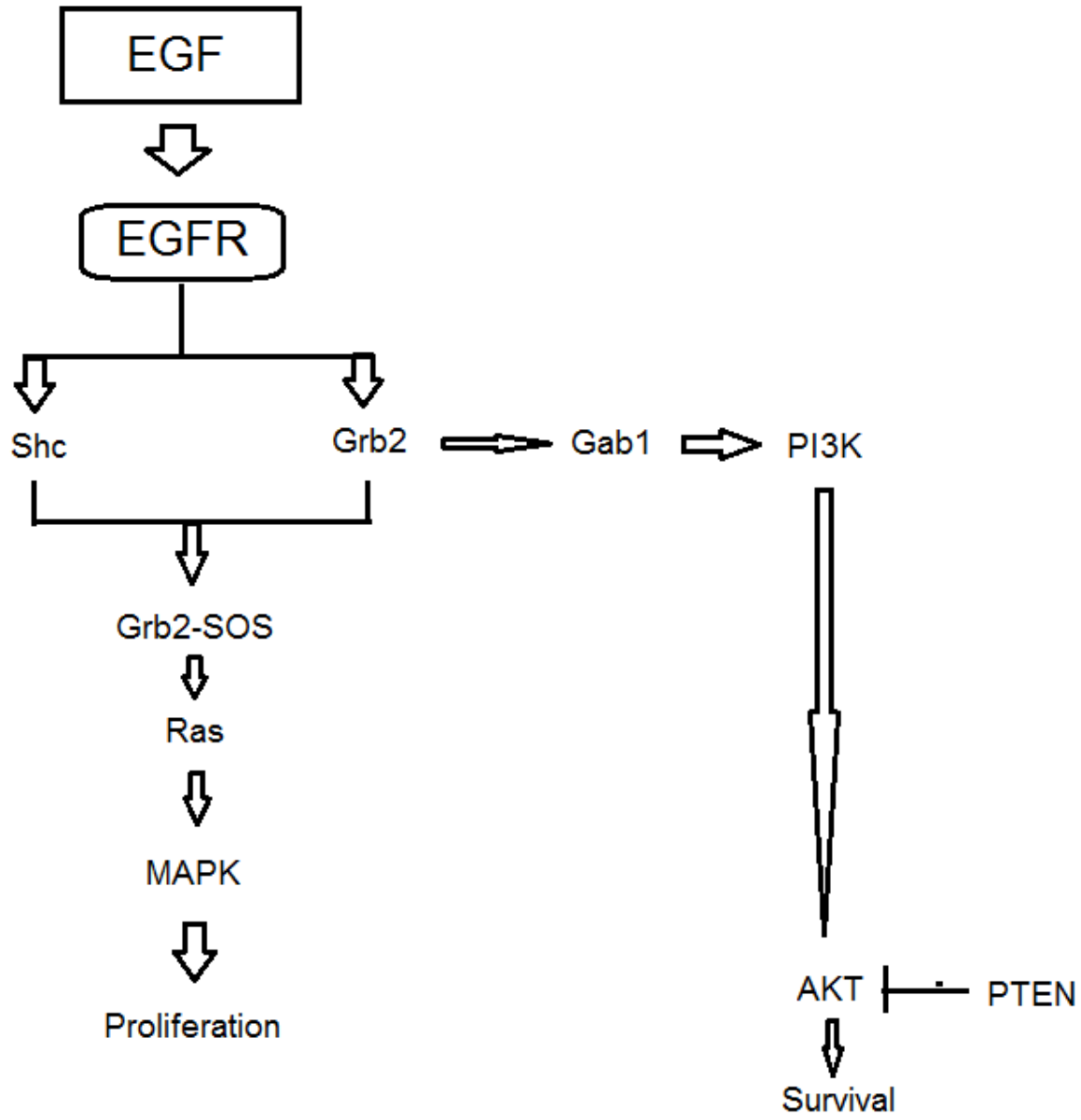

Figure 1.7: Shc and Gab1 protein involvement in EGFR signaling pathway. The binding of EGF to receptor lead to EGFR to dimerization and tyrosine phosphorylation. The phosphorylation of tyrosines recruit with the adaptor proteins Shc or Gab1 via Grb2 and form the Shc-Grb2-SOS to induce the Ras/MAPK signaling for proliferation or Grb2-Gab1 to stimulate the survival via PI3K/AKT signaling pathway (Adapted from Chung., 2013).

1.8 Aim and objective of this project

The ShcD has an apparent unique function among the Shc family in melanoma cells inducing migration. Since the function of ShcD is to act as scaffolding protein, its role in migration depends on complex formation with the other proteins. It has been shown in this lab previously that SH2 domain of ShcD interacts with Gab1 through tyrosine phosphorylation site Y183. This ShcD-SH2 interaction with Gab1 might be important as both adaptor proteins play a critical role in melanoma cells. Further studies in this lab have identified a number of phosphorylation sites on ShcD by mass spectrometry.

In this project I am particularly interested in Ser 551 which is inside the phosphotyrosine binding pocket of ShcD-SH2 domain. I hypothesize that phosphorylation of this site would inhibit interaction with Gab1 and may negatively affect cell migration.

Preliminary work in this lab revealed that introducing an aspartic acid at Ser551 inhibited binding to Gab1 in a yeast two hybrid assay. I therefore wished to extend these studies to determine the effects of mutation of S551 to S551D in mammalian cells and the effect on cell migration. In addition, I sought to identify the kinases that phosphorylate ShcD-SH2 domain and possible signaling pathways involved, as these could influence the ability of ShcD to induce melanoma cell migration.

Chapter 2

Material and Methods

2.1. Materials and methods

Chemicals and reagents

Common chemicals were purchased from Sigma, Fisher or VWR Chemicals

2.2. Standard solutions

2.2.1. Western blotting

10x Tris-buffered Saline (TBS) - 0.1 M Tris (pH 7.4) and 1.5M NaCl in 1000ml of distilled water

1x Tris-buffered-Saline-Tween-20 (TST) - 1x Tris-buffered Saline,0.1%v/v Tween-20

10x PBS – 80g NaCl, 2g KCL, 14.4g Na_2HPO_4, 2.4g KH_2PO_4 dissolved in 700ml dH_2O pH adjusted to 7.4 and made up to 1L with dH_2O. (Prior to use require autoclave for cell culture)

3x Sample Buffer – 6%v/v of SDS, 18.8ml of 1M Tris (pH 6.8), 15mM DTT, 30ml of Glycerol and tip of bromophenol blue

10x SDS-PAGE running buffer – 30.3g Tris powder, 144 Glycine, 10g SDS dissolved in 1000ml of dH2O

5% Blocking Buffer – Dried-skimmed milk powder dissolved in 1x Tris-buffered-Saline-Tween-20 (TST)

Blot Stripping buffer – 100ml of 10%v/v SDS, 31.25ml of 1M Tris (pH 6.8) and 369ml of dH_2O.

Enhanced Chemiluminescent (ECL) detection solution – 2ml of 1M Tris (pH 8.5), 18 ml dH_2O 44 µl of solution A, 100µl of solution B and lastly 6µl of H_2O_2 added to light tight container

- Solution A: 0.15g of p-Coumaric acid in 10ml DMSO
- Solution B: 0.44g of Luminol in 10ml DMSO

Transfer buffer – 3.6g Glycine, 0.75g Tris powder, 0.25g SDS and 50ml MeOH were dissolved and made up to final volume of 250ml with dH_2O

2.2.2. Bacterial culture and transformation

2x YT medium – 16g of bacterial tryptone, 5g of NaCl and 10g of yeast extract were dissolved in 1000ml of dH_2O then autoclaved. The solution was autoclaved on the day it prepared.

Luria Bertani (LB) broth – 10g NaCl, 10g bacterial tryptone, 5g yeast extract were dissolved in 1000ml of dH_2O. The solution was autoclaved on the day it prepared.

Luria Bertani Agar– 5g NaCl, 5g bacterial tryptone, 2.5g yeast extract, 7.5g bacterial purpose agar were dissolved in 500ml of dH_2O. The solution was autoclaved on the day it prepared.

NZY$^+$ broth – 2.5g NaCl, 2.5g yeast extract and 5g NZ amine were dissolved in dH_2O then adjust to pH 7.5 and made final volume of 500ml with dH_2O. The solution was autoclaved on the day it prepared then sterilized 5ml 2M glucose, 6.25ml 1M $MgCl_2$ and 6.25ml $MgSO_4$ were added to solution.

1x Tris-EDTA (TE) solution – 10mM Tris (pH 7.5) and 1mM EDTA [autoclaved]

$Ca^{2+}Mn^{2+}$ solution (2x stock) – 70mM $MaCl_2 4dH_2O$ (pH 5.5), 40mM $NaOAc 3H_2O$ and 100mM $CaCl_2 2H_2O$ were filtered for sterilize. To make 15% v/v glycerol: 3ml autoclaved 50%v/v glycerol and 2ml dH_2O into 5ml 2x stock solution in sterile environment.

2.2.3. Agarose Gel Electrophoresis

10x Tris-Acetate-EDTA (TAE) solution – 48.4g Tris Base powder and 7.44g EDTA were dissolved in 800ml of dH_2O then 11.4ml of Glacial acetic acid added to solution and made up to 1000ml with dH_2O.

6X Orange G loading Dye – 10mM Tris-HCl (pH 7.6), 60mM EDTA, 0.15g of Orange G and 60%v/v glycerol.

2.2.4. Immunoprecipitation

Triton lysis buffer – 0.05M Tris-HCl (pH 7.5), 0.005M EGTA, 0.15M NaCl, 0.025M Benzamidine, 1%v/v TritonX-100, 0.05M NaF, 0.001M Na_3VO_4 and protease inhibitor cocktail (Sigma) (1/100) were added prior to use.

Triton Wash buffer – 0.1%v/v TrionX-100, 0.15M NaCl and 0.05M Tris-HCl (pH 7.4)

2.2.5. GST-pulldown assay buffers

Extraction buffer - 0.05M Tris-HCl (pH 7.5), 0.005M DTT, 0.15M NaCl, 0.001M PMSF, 0.002M EDTA, 0.005M Benzamidine, final concentration of 1%v/v TritonX-100 and 10%v/v Glycerol. Prior to use protease inhibitor cocktail 10 µg/ml was added

Lysis buffer - 0.05M Tris-HCl (pH 7.5), 0.01M DTT, 0.15M NaCl, 0.001M PMSF, 0.002M EDTA, 0.01M Benzamidine, 0.05M NaF, 0.001M Na_3VO_4. final concentration of 1% TritonX-100 and 10% Glycerol. Prior to use protease inhibitor cocktail 10 µg/ml were added

Wash buffer - 0.05M Tris-HCl (pH 7.5), 0.001M DTT, 0.15M NaCl and final concentration of 1% TritonX-100 and 10% Glycerol.

Elution buffer + Glutathione – 10mg of reduced Glutathione (Sigma) dissolved in every 1.5ml 50 mM Tris (pH 8.0) (10mM) made freshly prior to every experiment.

2.2.6. SDS-PAGE Gel Staining solutions

Coomasie Blue Staining solution – 0.625g Brilliant blue R-250 was dissolved in mixed solution consist of 250ml dH_2O, 25ml of Glacial acetic acid and 112.5ml of Methanol. The solution was filtered using Whatman paper.

Destaining solution – 35ml Glacial acetic acid, 125ml Methanol and 340ml of dH_2O

2.2.7. Yeast two hybrid buffers

YPD broth – 10g bacterial peptone, 5g Yeast Nitrogen base without amino acids were dissolved, pH was adjusted to between 5.8-6.0 and solution made up to 500ml. The solution was autoclaved on the day it was made to prevent contamination. 25 ml sterile filtered 40% glucose was added to 500 ml YPD broth before use.

YPD agar plate – 9g bacterial agar powder was added per 500ml YPD broth + 25 ml 40% glucose then autoclaved.

Selective drop out plate (-Trp-Ura-Lys) - 6.7g Yeast Nitrogen base without amino acids, 0.55g drop out amino acid mix (CMS-HIS-TRP-LEU-URA-Lys), 50mg His, 100mg Leu, were dissolved in 800ml dH_2O then adjust pH to between 5.8-6.0. The solution was made up to 1000ml with dH_2O then divided into 2 x 500ml. 9g bacterial agar powder was added to the one of 500ml solution then autoclaved. 25ml 40% glucose was added to prior to use.

Selective drop out plate (-Trp-Leu) - as above but 50 mg histidine, 100 mg Lysine and 100mg Uracil were added to supplement the drop-out amino acid mix.

Selective drop out plate (-Trp-Leu-Ura-Lys) - as above but 50mg histidine only was added to supplement the drop-out mix.

Z buffer (pH 7.0) - 8.05g Na_2HPO_4-$7H_2O$, 0.375g KCl, 2.75g Na_2HPO_4-H_2O, 0.125g $MgSO_4$- $7H_2O$ dissolved in 300ml dH_2O then made up to 500ml with dH_2O.

Z buffer/X-gal substrate solution - 25 ml Z buffer, 0.0675 ml β-mercaptoethanol and 0.4175 ml X-gal stock solution

X-gal stock solution – 20 mg 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal] dissolved in 1 ml of dimethylformamide and stored in aluminum foil covered tube at –20 ^{O}C

2.2.8. Plasmid DNA Mini/Midi preparations

Qiagen spin miniprep kit cat no:/ID: 27104 was used to perform the plasmid DNA miniprep

ZymoResearch ZymoPURE II Plasmid Midiprep kit was used to perform the plasmid DNA midiprep

2.2.9. Radioactive Kinase assay solutions

Kinase buffer – 50mM Tris-HCl (pH 7.5), 20mM EGTA, 10mM $MgCl_2$, 1mM DTT, 1 mM beta-glycerol phosphate [made 1 ml aliquots and stored at –20]

ATP (10mM) - 60mg of ATP dissolved in 0.8 ml of Tris-HCl (pH 7.5) then made up to 1 ml. The ATP was aliquoted in 10 µl aliquots and stored at –80 ^{o}C.

Gamma ^{32}P-ATP – 10 mCi/ml (10 µCi/µl)

2.2.10. SDS-PAGE gel preparation

Resolving gel					**Stacking gel**
Reagent (ml)	**6%**	**8%**	**10%**	**15%**	**3%**
Protogel	**4**	**5.33**	**6.67**	**10**	**1**
Tris-HCL	**7.5 (pH 8.8)**	**7.5 (pH 8.8)**	**7.5 (pH 8.8)**	**7.5 (pH 8.8)**	**1.2 (pH 6.8)**
10% SDS	**0.1**	**0.1**	**0.1**	**0.1**	**0.1**
dH_2O	**8.2**	**6.9**	**5.6**	**2.2**	**7.6**
10% APS	**0.15**	**0.15**	**0.15**	**0.15**	**0.075**
TEMED	**0.02**	**0.02**	**0.02**	**0.02**	**0.012**

Table 2.1: SDS-PAGE gel composition

2.2.11. Primary and Secondary Antibodies

Western blotting

Company	Name of antibody	Antibody type	Dilution ratio
Sigma-Aldrich	**Anti-FLAG M2**	**Mouse Monoclonal**	**1:1000**
Cell signaling	**Anti-Gab1**	**Rabbit Polyclonal**	**1:1000**
Cell signaling	**Anti-Gab2**	**Rabbit Polyclonal**	**1:1000**
Made in Leicester university by Dr. Sally Prigent	**Anti-GST**	**Rabbit Polyclonal**	**1:5000**
Santa Cruz	**Anti-HA**	**Rabbit Polyclonal**	**1:1000**
Santa Cruz	**Anti-PY99**	**Mouse Monoclonal**	**1:1000**
Cell signaling	**Anti-SHIP2**	**Rabbit Polyclonal**	**1:1000**
Santa Cruz	**Anti-SH-PTP2**	**Mouse Monoclonal**	**1:1000**
Cell signaling	**Anti-phospho-Cofilin**	**Rabbit Polyclonal**	**1:1000**
Cell signaling	**Anti-phospho-Cerb1**	**Rabbit Polyclonal**	**1:1000**
Santa Cruz	**Anti-phospho-Erk**	**Mouse Monoclonal**	**1:1000**
Cell signaling	**Anti-phospho-SAPK/JNK**	**Mouse Monoclonal**	**1:1000**

Table 2.2: information of primary antibodies used for western blotting

2.2.11.1 Polyclonal antibody generation to S551 phosphopeptide

Rabbit polyclonal antibodies were generated by Pepceuticals using the following protocol. Two rabbits (37 and 86) were immunised on day 1 using 250 micrograms of phophopeptide (VKDGDFLVRE[pS]ATSPGQYVLC) conjugated to KLH via the C-terminal Cysteine. The second injection of 250 micrograms of antigen was performed on day 19-21, a third injection of 300-600 micrograms of antigen on day 49-51 and final injection of 300-600 micrograms of antigen on day 79-81. Rabbit serum was collected on day 86. Antisera were purified by affinity chromatography using either the phosphopeptide, or the non-phosphorylated peptide, covalently coupled to a solid matrix. Purified antibody from rabbit 37 was used in these studies

Secondary antibodies

Company	**Name of antibody**	**Dilution ratio**
Jackson ImmunoResearch	**Anti-mouse HRP**	**1:5000**
Jackson ImmunoResearch	**Anti-rabbit-HRP**	**1:1000**

Table 2.3: information of secondary antibodies

Immunofluorescence microscopy antibodies and reagents

Company	**Name of antibody**	**Dilution ratio**
Sigma-Aldrich	**Anti-FLAG M2 (mouse)**	**1:500**
Life Technologies	**Texas red-phalloidin**	**1:100**
Thermo Fisher Science	**Hoechst 33342 20mM in Dimethyl sulphoxide**	**1:1000**
Molecular probes	**AlexaFluor 488 conjugated goat anti-mouse**	**1:200**

Table 2.4: information of antibodies and reagents used for immunofluorescence microscopy

2.3. Methods

2.3.1. Molecular biology

2.3.1.1. Competent E. coli DH5α and BL21 cell preparation

A single competent DH5α or BL21 bacteria colony was picked and applied into 10ml of 2x YT medium then incubated at 37°C for overnight with shaking. The following day 2ml of DH5α bacterial culture was applied to pre-warmed 200ml of 2x YT medium and incubated at 37°C in incubator with shaking. The culture was incubated until OD_{600} measurement reach 0.2 [approximately 2 hours] then 1M $MgCl_2$ was applied to culture to make a final concentration of 20mM. The bacterial cell culture was incubated for approximately 50 mins until OD_{600} measurement reached 0.45-0.55 and then the culture was divided into 4x 50ml of Falcon tubes. The tubes were incubated on ice for 2 hours (set centrifuge machine's temperature to 4°C) and centrifuged at 3000 rpm for 5 minutes at 4°C. 25ml of ice cold $Ca^{2+}Mn^{2+}$ solution was applied to pellet for resuspension and incubated on ice for 45 minutes. The bacterial suspension was centrifuged at 3000 rpm for 5 minutes and resuspended using 5ml of $Ca^{2+}Mn^{2+}$ solution with 15% v/v glycerol. The resuspended DH5α cells were aliquoted into pre-chilled 1.5ml Eppendorf tubes (100-200 µl) then quickly frozen using a dry ice/isopropanol bath. The frozen competent cells were stored at -80°C.

2.3.1.2. Bacterial transformation

2.3.1.2.1. Standard DH5α and BL21 transformation method

The competent cells DH5α or BL21 were thawed gently on the ice. 50µl of competent cells were transferred into pre-chilled 1.5 ml of Eppendorf tube. 50ng of desired DNA was applied to the competent cells and mixed by gently flicking. The transformation mixture was incubated on ice for 30 mins then heat shocked at 42°C water bath for 45-50 seconds and then incubated on ice for 2 minutes. 500 µl of autoclaved LB broth was added to mixture and grown at 37°C for 1.5 hours. The Eppendorf tube was gently inverted every 10-15 minutes throughout 1.5 hours incubation period. 250 µl of grown culture was plated on LB agar plate containing antibiotics and incubated at 37°C overnight.

2.3.1.2.2. XL10 Gold Ultracompetent Cells – Quickchange II – for transformation of product of mutagenesis reaction

XL10 Gold Ultracompetent Cells were gently thawed on ice. 45µl of cells were transferred into pre-chilled 14 ml BD Falcon polypropylene tube. 2µl of β-Me (provide from Kit) was applied to cells and incubated on ice for 10 minutes with swirling every 2 minutes during incubation period. 50ng of DNA (or Dpn1 digested DNA) was applied to cells then mixed by gently swirling and incubated on ice for 30 minutes (pre-heat the NZY+ broth at 42°C water bath). The transformation mixture was heat shocked at 42°C water bath for 30 seconds then incubated on ice for 2 minutes. 500µl of pre-heated NZY+ broth was added to tube and grown at 37°C with shaking 225-250 rpm for 1 hour. 250µl of transformation reaction was plated on agar plate containing antibiotics and incubated at 37°C overnight.

2.3.1.3. Plasmid DNA purification

2.3.1.3.1 Miniprep plasmid DNA purification - QIAGEN spin columns

A single transformed bacteria colony was picked and transferred into 5ml of LB containing appropriate antibiotic. The cells were incubated at 37°C with shacking at 225-250 rpm for overnight. The bacteria cell culture was transferred to 1.5ml Eppendorf tube and centrifuged at 8000 rpm for 1 minute. The supernatant was discarded, and pellet was resuspended using 250µl of P1 buffer (contain RNase) until pellet was resuspended completely. 250µl of P2 buffer was applied and mixed by inverting the tube 6 times and then 350µl of N3 buffer was added and mixed immediately by inverting the tube 6 times. The mixture was centrifuged for 10 minutes at 13000rpm and then supernatant was transferred into QIAprep spin column in a collection tube. The tube was centrifuged for 1 minute at 13000 rpm, the supernatant was discarded and then spin column was washed with 500µl of PB buffer and then centrifuged for 1 minute at 13000 rpm, the supernatant was discarded. The spin column was washed by applying 750µl of PE buffer (containing ethanol) to column and centrifuged for 1 minute, the supernatant was discarded, and an additional centrifugation was done for remove any possible residue of PE buffer. The spin column was placed in a fresh 1.5ml Eppendorf tube and 50 µl of Elution Buffer (EB) was applied to column to elute the DNA. The sample was incubated at room temperature for 1 minute and centrifuged for 1 min at 13000 rpm. The DNA was stored at -20°C

2.3.1.3.2. ZymoPURE™ II Plasmid Midiprep (centrifuge) method

The Plasmid Midiprep was performed following the manufacturer protocol.

2.3.1.4. Measuring Plasmid DNA concentration

2µl of purified plasmid DNA was diluted in 1ml of dH_2O and absorbance was measured using spectrophotometer (setting 260nm). The measured concentration was used to calculate the DNA concentration assuming an OD_{260} of 1.0 is equivalent to 50 µg/ml for double stranded DNA.

2.3.1.5. Agarose gel electrophoresis DNA analysis

In order to confirm the quality of the purified DNA, 1% agarose gel was prepared to separate DNA. The agarose powder was mixed in 1x TAE and dissolved by using microwave for 1-2 minutes. The liquid form of agarose gel was cooled down on the bench (about 50°C) and 2µl of Ethidium bromide was added. The liquid gel solution was poured into the agarose gel casting tray and allowed to set before transferring to a gel tank and submerging in 1 x TAE buffer. 6x loading Orange G dye was applied to DNA and loaded into agarose gel along with 1Kb plus DNA ladder (Invitrogen). The DNA was run at 100 Volts until dye front reached 70% of the length of the gel and DNA was analyzed using a UV light transilluminator (Syngene G:box system).

2.3.2. Mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit)

The site-directed mutagenesis reaction was prepared using 5µl of 10x reaction buffer, 10ng of dsDNA template, 125ng of both Forward and Reverse oligonucleotide primers, 1µl of dNTP mix, 3µl of QuikSolution and ddH_2O to a final volume of 50µl. 1µl of PfuUltra HF DNA polymerase (2.5 U/µl) was added to reaction.

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	1 minute /kb of plasmid length
3	1	68°C	7 minutes

Table 2.5: Mutagenesis PCR reaction setting for PCR machine

Gene	Primer sequence (5' -> 3')
Full-length ShcD-SH2-S551D Forward	GGACTTTTTGGTTCGAGAGGATGCAACATCCCCTGGC
Full-length ShcD-SH2-S551D Reverse	GCCAGGGGATGTTGCATCCTCTCGAACCAAAAAGTCC
ShcD-SH2-S551A Forward	GGACTTTTTGGTTCGAGAGGCTGCAACATCCCCTGGC
ShcD-SH2-S551A Reverse	GCCAGGGGATGTTGCAGCCTCTCGAACCAAAAAGTCC
HA-PAK4-S445N Forward	GACATCAAGAGCGACAATATCCTGCTGACCCATG
HA-PAK4-S445N Reverse	CATGGGTCAGCAGGATATTGTCGCTCTTGATGTC

Table 2.6: Forward and Reverse primers for the mutagenesis reaction.

Once the PCR reaction finished, 1µl of Dpn I restriction enzyme (10 U/µl) was applied to directly to reaction sample. The reaction samples were mixed gently and properly by pipetting samples. The PCR reaction was transferred into 1.5ml Eppendorf tube and centrifuged for 1 minute. The reaction was incubated at 37°C for 1.5 hours for digesting methylated template DNA. The digested mutagenesis reaction sample was transformed using **XL10 Gold Ultracompetent Cells – Quickchange II** method.

2.4. Cell lines

Human embryo kidney, HEK293 were originally from ATCC

F4 – HEK293 cells expressing Flag-ShcD were generated in previously in this lab.

DAUV melanoma cell line was a gift from Dr Mike Browning, University of Leicester.

WM226-4 melanoma cell line gift from Dr Eugene Tulchinsky, University of Leicester.

U2OS Human Bone Osteosarcoma Epithelia cell line from Professor Andrew Fry

2.4.1. Maintain Cell lines for experiment

All cell culture reagents were purchased from Gibco. The cell lines were maintained using DMEM medium supplemented with 1% Penicillin/Streptomycin, 10% FBS. The cell lines were grown at 37°C in humidified 5% CO_2 incubator and when cell reached around 80% - 90% confluence, the cells were split using 1xTrypsin/EDTA solution at sterile fume hood.

2.4.2. Thawing cell line

The frozen cells were recovered from the liquid nitrogen tank and thawed quickly at 37°C in a water bath. The cells were transferred into a 12 ml Falcon tube containing 10 ml complete medium, centrifuged at 500x g for 5 minutes and supernatant was discarded. Cells were resuspended in complete medium and used to seed cell culture dishes or flasks as required.

2.4.3. Preparing frozen cell line stock for long term storage

The cell lines were grown until the confluence around 80% - 90% and washed with 1x PBS (autoclaved) 1 time. 2ml of 1x Trypsin/EDTA was applied to washed cells and swirled gently to let Trypsin/EDTA cover the entire cell surface then discarded. The cells were incubated at 37°C in cell culture incubator with 5% CO_2 for 2 – 3 minutes and then resuspended using 2ml of FBS. The cell culture was transferred into a sterile universal tube and an equal amount of filtered Freezing medium added in drop wise to cell culture (consist of 20ml complete medium and 5ml DMSO). The 1ml of cell mixture was aliquoted into 2ml cryovials and cryovials were transferred to Mr. Frosty™ Freezing Container (Thermo Fisher Scientific) for slow freezing for a few days before storing in liquid nitrogen storage.

2.4.4. Cell transfection via Turbofect reagent

Transfection was carried out when cell confluence reached 70% - 80% confluence. The Turbofect reagent (Themo Scientific] was used for transfection following the manufacturer's protocol. Typically, for a well of a six well plate, 4 μg DNA and 6 μl of Turbofect was used.

2.5. Yeast two hybrid assay

2.5.1. L40 Yeast preparation

The L40 Yeast was thawed gently on ice and streaked on YPD agar plate containing glucose for growing overnight at 30°C. The following day, a single colony was picked and streaked onto selective drop out plate (-Trp-Ura-Lys) for preventing contamination. The streaked plate was incubated at 30°C for overnight and then it stored at 4°C. A single colony from selective drop out plate was picked and transferred into 5ml of YPD (+glucose) and grown at 30°C for overnight. 100 – 200µl of L40 yeast cell culture was transferred to 50ml of YPD(+glucose) and incubated approximately 12 hours at 30°C for overnight until OD_{600} measurement reach range between 1-2. The yeast cell culture was transferred to 50ml falcon tube and centrifuged at 3000 x g for 5 minutes. 50ml of 0.1M lithium acetate in TE was applied to the yeast pellet for washing and centrifuged again. 1ml of 0.1M lithium acetate in TE was applied to yeast pellet and resuspended. The resuspended yeast cells were incubated at 30°C with shaking for 1 hour and then aliquoted into Eppendorf tube.

2.5.2. L40 yeast transformation

The L40 yeast from preparation step was used to carry out the yeast transformation. 100µl of L40 yeast was aliquoted into Eppendorf tube and 1µg of ‘bait’ and ‘prey’ DNAs of interest (table 5) were applied to aliquoted tube. 400µl of 50% PEG3350 was added and mixed by inverting several times. The yeast transformation mixture was incubated in shaking incubator at 30°C for 30 minutes then 42°C for 20 minutes. The tubes were centrifuged for a few seconds to gather yeast cell pellet and supernatant was removed. 100µl of sterile 1x PBS was added to yeast cell pellet and resuspended. The yeast transformation mixture was spread onto selective drop out plate (-Trp-Leu-Ura-Lys) and incubated at 30°C until yeast colonies formed.

Bait vectors used	Prey vectors used
ShcD-SH2-pBTM116-PDGFR	ShcD-SH2-pLEICS82 (pVP16)
TShcD-CH1-pBTM116-PDGFR	Grb2-SH2-pVP16
ShcD-CH2-pBTM116-PDGFR	Gab1-pVP16
ShcD-PTB-pBTM116-PDGFR	ShcD-FL-pLEICS82 (pVP16)
Ret-PTC - pBTM116	

Table 2.7: L40 Yeast transformation plasmids

All Bait plasmids had been constructed previously in the lab except Ret-PTC which was a gift from Prof Susan Taylor (Durick et al., 1996). Grb2-SH2-pVP16 and Gab1-pVP16 constructs had been isolated previously in this lab by screening a mouse embryo yeast two hybrid library. The ShcD-SH2-pLEICS82 and ShcD-FL-pLEICS82 constructs were made by PROTEX, University of Leicester, using pLEICS82, a derivative of pVP16 modified to improve ease of cloning.

2.5.3. B-Galactosidase Assay (X-gal)

The Circular Whatman grade 50 filter paper were placed on top of selective drop out plates (-Trp-Leu) and transformed yeast were streaked onto the filters using sterile toothpicks. The streaked yeast were grown at 30°C overnight.). Whatman 3MM blotting paper circles were placed into petri dishes and soaked with X-gal substrate. The Yeast streaked Whatman filter was placed into liquid nitrogen for 10 seconds to permeabilize the yeast cells and then transferred on top of X-gal substrate solution soaked Whatman paper, with yeast facing upwards. The plates were incubated at 30°C for around 15 minutes - 4 hours until reaction change yeast color from pink to blue.

2.6. SDS-Polyacrylamide (PAGE) gel electrophoresis

The prepared samples were boiled at 100°C heating block for 5 – 10 minutes and loaded into pre-made SDS-PAGE gel along with Molecular weight marker. The % of SDS-PAGE gel was determined by molecular weight of the protein of interest and 6%, 8%, 10%, 15% gels were used. The gels were run at 60 – 80 volts until marker and samples reach resolving gel then voltage was increased to 120 – 150. The Page Ruler Pre-Stained Protein Ladder (Themo Scientific) was used for molecular weight marker.

2.6.1. Western blotting/Immunoblotting

After electrophoresis, the gel was soaked in transfer buffer for 10 minutes at room temperature. The transfer sandwich was prepared using 3 Whatman papers cut to the same size as the gel soaked in transfer buffer (at the anode), followed by nitrocellulose membrane, also soaked in transfer buffer, then the SDS-PAGE gel, followed by another 3 Whatman papers soaked in transfer buffer (cathode).

The TE77 ECL Semi dry transfer unit [Amersham Cytiva / GE Healthcare Life Sciences] was used for performing the transfer. The transfer was carried out for 1 hour at 0.8 mA/cm^2 gel surface area. Nitrocellulose membrane was gently washed with dH_2O and the nitrocellulose membrane was incubated in blocking buffer either 1 hour (room temperature) or overnight (4°C) to prevent nonspecific binding. After the blocking period, the primary antibody diluted in blocking buffer was apply to nitrocellulose membrane and incubated either 1 hour at room temperature or overnight at 4 °C.

Following incubation, the nitrocellulose membrane was washed 4 times with 1x TST for 10 minutes each at room temperature and then incubated with secondary antibody for 1 hour at room temperature. After secondary antibody incubation, the membrane was washed 4 times with 1x TST for 10 minutes each. The ECL solution was applied to membrane for 1 minute in the dark room for detecting bounded secondary antibodies. The protein of interest was visualized on X-ray films and developed using the Compact X4 developer (Xograph Imaging Systems).

2.6.2. Stripping membrane

The membrane was rinsed a few times with dH_2O and transferred into container with lid. 50ml of blot stripping buffer and 350ul of β-mercaptoethanol were added to container. The container with membrane was incubated at 55°C in a water bath for 1 hour. The membrane was rinsed with dH_2O and washed with 1x TST 4 times for 10 minutes each on a rocking platform. After the washing, the membrane was blocked using blocking buffer for 1 hour (room temperature) or overnight (4°C) before applying primary antibody.

2.6.3. Dot blotting

The nitrocellulose membrane was cut into 2 x 6 cm strips. Defined quantities (0.01 - 100ng) of synthetic peptide, either phosphorylated or non-phosphorylated were spotted onto the nitrocellulose membrane and dried at room temperature until dried completely. The membrane was blocked using blocking buffer for 1 hour at room temperature and after blocking period, the primary antibody diluted in blocking buffer was apply to nitrocellulose membrane and incubated 1 hour at room temperature. The membrane was washed with 1x TST four times for 10 minutes each on a rocking platform. The secondary antibody diluted in blocking buffer was applied to membrane and incubated 1 hour at room temperature. Once secondary antibody incubation was finished, the membrane was washed with 1x TST four times for 10 minutes each and then ECL solution was applied to membrane for 1 minute in the dark room. The chemiluminescence was visualized on X-ray films and developed using the Compact X4 developer (Xograph Imaging Systems).

2.7. Glutathione S Transferase (GST) pulldown assay

2.7.1. Preparing GST fusion protein

Plasmids GST (pGex-4T-3), GST-ShcD-SH2-WT and GST-ShcD-SH2-S551D were transformed into competent BL21 cells for preparing GST fusion protein. The transformed BL21 were grown at 37ºC in 5ml of LB with antibiotics (GST: 100 µg/ml Ampicillin; GST-SH2-WT and GST-SH2-S551D: 50 µg/ml Kanamycin) overnight. 4ml of each culture was transferred into 200ml LB with antibiotics (final conc. of 50µg/ml for Kanamycin / 100µg/ml for Ampicillin) and grown at 37ºC for 1 hour. IPTG was added to each culture to make final concentration of 0.2 mM then grown at 30ºC for 4 hours to produce GST fusion protein. The culture was centrifuged at 4ºC with 6000rpm setting for 20 min. The supernatant was discarded, and pellet was resuspended in 4ml of Triton extraction buffer. 3 sonication cycles (Soniprep150) were performed (100%, 1 min per cycle) then 500µl of 10% TritonX100 was added to each sample followed by rolling for 40 mins at 4ºC to aid solubilisation. The cultures were then centrifuged at 4ºC at a speed of 10000rpm for 20 min. 100µl of supernatant and pellet from each extraction were kept for gel analysis. The rest of supernatant was transferred into 15ml falcon tube then 500µl of 50% glutathione-agarose slurry (centrifuged and washed with 1 x PBS prior to use) added and incubated overnight on a tumbler at 4ºC. After the tumbling the beads were washed with wash buffer 4 times then 400ul of 20% glycerol was added and stored in -20 ºC

2.7.2. Cell lysates preparation for GST-Pull down

WM266-4/DAUV cell lines were grown using 10 cm dishes for small scale GST pulldown assays, where western blotting was to be performed. One dish was serum starved for 1 hour at 37 ºC in an incubator then treated with sodium orthovanadate (100µl) for 1 hour at 37 ºC incubator. Cells were washed twice with ice-cold 1x PBS then 2ml of Triton lysis buffer was added and incubated on ice for 2 mins. The cells were scraped off on ice and transferred into cold Eppendorf tubes. The tubes were centrifuged at 4 ºC for 5 mins with maximum speed. 70µl of cell lysate was transferred to new cold Eppendorf tubes as whole cell lysates and the rest of the cell lysate was divided into 3 new cold Eppendorf tubes for incubating with purified GST, GST-SH2 or GST-SH2-S551D proteins bound to glutathione-agarose beads (3µl slurry). Sample buffer and 1M DTT was added to Whole cell lysates and boiled at 100 ºC for 5 mins then stored at -70 ºC. The tubes for the pulldown were tumbled overnight at 4ºC then washed four times with triton wash buffer. After the final wash 100µl of sample buffer and 10µl 1M DTT were added to each tube then boiled at 100 ºC for 5mins.

For large Scale pull-down experiments where precipitated proteins were to be identified by mass spectrometry the procedure was scaled up using 30 µl GST or fusion protein bound to glutathione-agarose beads and 4 x 20cm for each pull-down.

2.7.3. GST fusion protein elution

For pull-downs, purified proteins were left bound to beads, but for use as a substrate in kinase assays, the soluble proteins were required. The GST fusion protein bounded beads were washed with 10 ml of 50 mM Tris pH 8.0 and centrifuged for 500 x g for 5 minutes at 4ºC. The P1000 pipette tip was cut and used for resuspending the beads. The resuspended GST beads were transferred to 1.5ml Eppendorf tube. The 50 mM Tris pH 8.0 was added to tube to bring up to 1.5ml and centrifuged for 1000g for 1 minute at 4ºC. The tubes were placed on ice and supernatant was removed. 400 - 500 µl of elution buffer containing Glutathione) was added to resuspend the beads and tumbling for overnight at 4ºC. The beads were centrifuged at 500g for 5 minutes and 450 µl supernatant was transferred to Slide-A Lyzer G2 dialysis cassettes (Thermo Scientific). The dialysis cassette was placed in 1000ml of dialysis solution and changed to fresh solution every 4 hours with gentle stirring at 4ºC. The dialysis was carried out for 24 hours and then dialyzed samples were transferred into 1.5ml of Eppendorf tube. The samples were stored at 4ºC.

2.8. Preparing anti-HA coupled Protein G-Sepharose beads

20μl protein G-agarose Sepharose was placed in an Eppendorf tube and washed one time with 1x PBS to remove residue of ethanol. 1 μg of anti-HA (rabbit) antibody was added and left for overnight at 4 °C. The following day, the tube was placed at room temperature with occasional flickering.

2.9. Immunoprecipitation

Immunoprecipitations were generally performed using a 10 cm plate of cells. The cells were washed 2 times with pre-chilled 1x PBS and then 2ml of lysis buffer was added to each cell culture dishes. The dishes were gently swirled for covering entire cells and left 2 minutes on ice. The cells were scraped off and transferred onto pre-chilled Eppendorf tubes. The tubes were centrifuged at 4 ºC for 5 mins with maximum speed. The cell lysates were transferred to new Eppendorf tubes and 50μl of cell lysates were kept as whole cell lysate. The appropriate antibody tagged beads (either beads coupled to anti-Flag antibody (10μl) or anti-HA antibody (1 μg) bound to 20 μl protein G-agarose slurry) were introduced to cell lysates and tumbled overnight at 4 °C. The tubes were washed with wash buffer 4 times and centrifuged each wash. After the final wash 100μl of sample buffer and 10μl 1M DTT were added to each tube then boiled at 100ºC for 5mins.

2.10. Mass spectrometry analysis

Liquid Chromatography with tandem mass spectrometry (LC-MS-MS) was performed following trypsin digestion by the protein nucleic acid chemistry laboratory (PNACL), University of Leicester, using a LQT Orbitrap Velos ETD mass spectrometer.

2.11. Radioactive Kinase Assay

Hek 293 cells were transfected with either wild-type HA-PAK4, HA-PAK4-S445N or left untransfected. HA-PAK4 was immunoprecipitated with anti-HA bound to agarose beads. The immunoprecipitated HA-Pak4 was washed 3 times using cold triton wash buffer and then washed 2 times with kinase assay buffer (without addition of ATP). The beads were resuspended using in reaction mixture (consist of 2µl cold ATP (4mM), 1µl GST, 2µl GST-SH2 (2.5 µg/µl), and volume with kinase assay buffer till 56µl). The 4µl of (γ-^{32}P)-ATP was added to kinase reaction and incubated at 30°C for 20 minutes. The reactions were stopped by introducing 20µl of 3x sample buffer and boiled at 100°C heat block for 5 minutes. The samples were centrifuged for 5 minutes and run on 15% SDS-PAGE gel. The electrophoresis was continued until the bands run out of SDS-PAGE gel for removing (^{32}P)-ATP and gel was stained using Coomassie blue staining solution for overnight at room temperature. The gel was de-stained using destaining solution to visualize the bands. The gel was incubated 2 times in 20% methanol and 10% glycerol solution for 10 minutes. The gel was placed on top of 2x Whatman papers and then placed on gel drier (gel facing up) and covered with Saran wrap (prevent gel to stick to gel drier cover). The vacuum was applied for 2 hours at 50°C.The dried gel was exposed to X-ray film for 5 hours – 2 days before developing.

2.12. Microscopy analysis

2.12.1 Preparing cells for immunofluorescence microscopy

Acid washed microscopy glass coverslips were placed into wells of a 6 well-plate and cells were seeded. The seeded cells were grown until 60-80% confidence and then transfection was performed using Turbofect transfection reagent. The transfected cells were gently washed once with autoclaved 1x PBS and then 2ml of 2% paraformaldehyde fixative (3.7% formaldehyde) was applied to each coverslip then incubated at room temperature for 20 – 30 minutes. The coverslips were washed 3 times with autoclaved 1x PBS and block and permeabilization were carried out together by applying 1ml of blocking solution (3% BSA, 0.2% Triton X-100) for 1 hour. The primary antibody was diluted in blocking solution and applied to each coverslip and incubated at room temperature for 2 hours. The coverslips were washed 3 times with 2ml of PBS then appropriate secondary antibodies and Texas red-phalloidin were diluted in blocking buffer then applied to each coverslip. The incubation was carried out in the dark at room temperature at least 1 hour and then washed 3 times using 2ml of PBS. 500µl of diluted Hoechst 33342 was applied to each coverslip and left for 10 minutes at room temperature and then washed 3 times using 2ml of PBS. The coverslips were transferred and attached to glass slides with 5µl of mounting media then sealed with clear nail polish. The coverslip was stored in the dark at -4°C.

Chapter 3

INVESTIGATION OF THE INTERACTION BEWTEEN SHCD AND GAB1 PROTEIN IN MAMMALIAN CELL

3.1. Introduction

Shc family members regulate the cellular signaling responses upon stimulation of receptor tyrosine kinase (RTK) resulting in cell proliferation, migration and survival via different types of signaling pathways. ShcD adaptor protein is the most recently discovered Shc family member. The role of ShcD protein in cell signaling is not well characterized compared to other Shc family members but it was identified to play role in melanoma cell migration (Fagiani *et al*., 2007). The Shc family members may have different biological functions in cellular responses by forming protein complexes however in melanoma cell, only ShcD protein was shown to be overexpressed and to play a crucial role in melanoma cell migration and invasion, presumably through its interactions with other proteins. The SH2 domain is well categorized as a mediator of interaction with tyrosine phosphorylated proteins and this domain may possibly play a crucial role for ShcD protein to interact with other proteins. Using a modified yeast two-hybrid library screen which enabled detection of phosphotyrosine dependent interactions, previous studies in this lab identified the signaling scaffold protein, Gab1, as a binding partner for the SH2 domain of ShcD (Chung, D-H, 2013). As discussed in Chapter 1, Gab1 is a member of a family of a multi-site docking scaffolding protein involved in cell signaling (Bongartz *et al*., 2019). comprising Gab1, Gab2 and Gab3. Of these Gab1 is the best characterized, however its function in relation to ShcD is not known. Interestingly, the studies of Gab protein family members identified that Gab2 protein signaling play a critical role in melanoma. The Gab2 expression level closely related with melanoma progression as silencing Gab2 resulted decreasing cell invasion and metastasis rate while overexpression of Gab2 protein increasing melanoma progression (Horst *et al*., 2009).

In addition to identification of ShcD binding partners to explain its unique role in melanoma migration, another focus of previous experiments in this lab has been to identify sites of phosphorylation on ShcD, and the effect of phosphorylation on ShcD function. In these experiments FLAG-tagged ShcD protein was precipitated from EGF treated Hek293 cells constitutively expressing this protein. Multiple sites of phosphorylation were identified using mass spectrometry as indicated in Figure 3.5 and 3.6. Of particular significance to this project was the phosphorylation of serine 551. The original mass spectrometry data is shown in Figure 3.5. This site was of particular interest because it is located in the ligand binding pocket of the SH2 domain and hypothesized that the addition of a phosphate group to serine 551 would prevent access to a tyrosine phosphorylated ligand and may represent a negative feedback mechanism in response to growth factor stimulation as shown in Figure 1.2. Preliminary studies using the yeast two-hybrid assay to test the interaction between the SH2 domain of ShcD and the fragment of Gab1 identified in the yeast two-hybrid library screen suggested that replacement of serine 551 with aspartic acid to mimic the negative charge of phosphoserine inhibited the interaction.

In this chapter, I will investigate the interaction between a GSTShcD-SH2 fusion protein purified from bacteria and full-length Gab1 protein expressed endogenously in melanoma cells. I will also investigate the effect of changing serine 551 in the SH2 domain to aspartic acid to mimic phosphorylation of S551.

3.2. Result

3.2.1. Investigation of ShcD and Gab1 interaction in mammalian cell

3.2.1.1 Construction of the ShcD-SH2-S551D mutant construct

Previously, in this lab performed yeast two hybrid analysis to determine whether mutation of S551 to aspartic acid would affect the binding ability of ShcD in yeast and confirmed that ShcD-SH2-S551D mutation prevent binding of other proteins. This time I wanted to investigate the effect of phosphorylation at ShcD-SH2-S551 in mammalian cells. In order to do this mutagenesis was performed on (ShcD)-SH2-S551 to aspartic acid for mimic the phosphorylation. The SH2 DNA construct then sent to DNA sequencing to confirm the mutation was correctly introduced to ShcD DNA construct. The result of DNA sequencing confirmed mutation has been introduced to S551 to S551D as shown at Figure 3.1.

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CLUSTAL O(1.2.4) multiple sequence alignment


ShcA        WFHGKLSRREAEALLQLNGDFLVRESTTTPGQYVLTGLQSGQPKHLLLVDPEGVVRTKDH
ShcD        CYHGKLSRKAAESLLVKDGDFLVRESATSPGQYVLSGLQGGQAKHLLLVDPEGKVRTKDH
ShcB        WYHGRMSRRAAERMLRADGDFLVRDSVTNPGQYVLTGMHAGQPKHLLLVDPEGVVRTKDV
ShcC        WYQGEMSRKEAEGLLEKDGDFLVRKSTTNPGSFVLTGMHNGQAKHLLLVDPEGTIRTKDR
             ::*.:**: ** :*  :******.*.*.**.:**:*:: ** ********** :****

ShcA        RFESVSHLISYHMDNHLPIISAGSELCLQQPV
ShcD        VFDNVGHLIRYHMDNSLPIISSGSEVSLKQPV
ShcB        LFESISHLIDHHLQNGQPIVAAESELHLRGVV
ShcC        VFDSISHLINHHLESSLPIVSAGSELCLQQPV
             *:.:.*** :*::.  **::: **: *:  *
```

SHC-transforming protein 4 isoform X1 [Homo sapiens]

Sequence ID: XP_005254432.1 Length: 447 Number of Matches: 1

Range 1: 333 to 447 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps	Frame
238 bits(607)	2e-71	Compositional matrix adjust.	114/115(99%)	114/115(99%)	0/115(0%)	+1

```
Query  31   HIKQQLWSEECYHGKLSRKAAESLLVKDGDFLVRDATSPGQYVLSGLQGGQAKHLLLVD  210
            HIKQQLWSEECYHGKLSRKAAESLLVKDGDFLVR ATSPGQYVLSGLQGGQAKHLLLVD
Sbjct  333  HIKQQLWSEECYHGKLSRKAAESLLVKDGDFLVRESATSPGQYVLSGLQGGQAKHLLLVD  392

Query  211  PEGKVRTKDHVFDNVGHLIRYHMDNSLPIISSGSEVSLKQPVRKDNNPALLHSNK  375
            PEGKVRTKDHVFDNVGHLIRYHMDNSLPIISSGSEVSLKQPVRKDNNPALLHSNK
Sbjct  393  PEGKVRTKDHVFDNVGHLIRYHMDNSLPIISSGSEVSLKQPVRKDNNPALLHSNK  447
```

Figure 3.1: Analysis of ShcD-SH2-S551D DNA blastx search result and multiple alignment for ShcA to D. The DNA sequence was analyzed using blastx to check SH2-S551D mutation is introduced at S551 position for investigating ShcD-SH2 in mammalian cell. The multiple alignment was analyzed using Clustal O search

3.2.1.2 Determination of ShcD-SH2 interaction with Gab1 protein in mammalian cells

To compare the interaction between ShcD-SH2 and ShcD-SH2-S551D with Gab1 protein in mammalian cells, GST and GST fusion protein was produced using pGEX4T3, ShcD-SH2-WT and ShcD-SH2-S551D plasmid DNA. The glutathione beads containing purified GST or fusion protein were run on the 15% SDS-PAGE gel and stained with filtered Coomasie Blue stain and then destained to determine the protein yield by comparing to BSA standard as shown on Figure 3.2. From the results, it was observed that 10 µl of beads with bound GST or fusion proteins contained more than 20 µg protein (Figure 3.2). The amount of fusion protein or GST bound to glutathione beads was approximately equal.

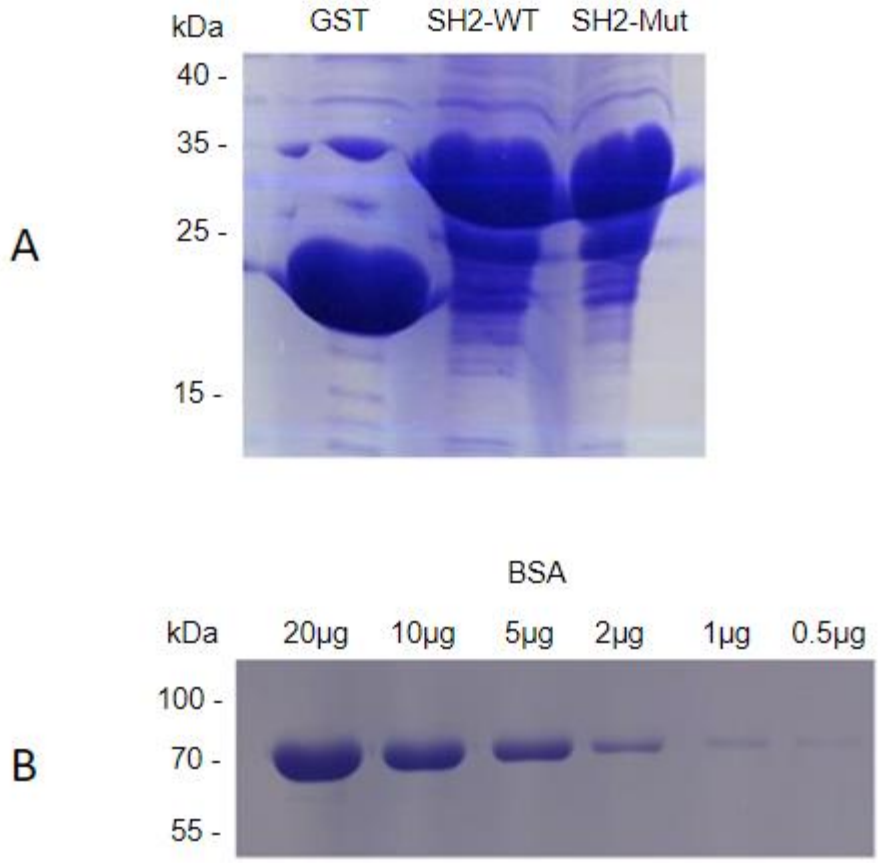

Figure 3.2: Analysis of purified GST, GST-SH2 and GST-SH2-S551D by 15% SDS-PAGE gel. 10µl of suspension of purified GST, GST-SH2 and GST-SH2-S551D bound to glutathione sepharose beads was boiled in sample buffer and analyzed on 15% SDS-PAGE gel (A). To enable approximation of amount of bound protein, defined amounts of BSA were analyzed on a separate gel. Both gels were stained with Coomasie Blue R250 stain (B). This experiment was repeated 3 times and this result is representative.

Having obtained a good yield of purified GST fusion proteins, pulldown assays were performed to investigate protein-protein interaction between ShcD-SH2 and Gab1 protein in DAUV melanoma cells. Cells were grown to 100% confluence cells were incubated in serum free medium for 1 hour then cells were treated with 3 different conditions. The first group of melanoma cells were left untreated and act as negative control, the second group of melanoma cells were treated with EGF for 5 minutes for EGF stimulation and third group of melanoma cells were treated with sodium orthovanadate to induce widespread tyrosine phosphorylation. Cell lysates were divided into three tubes and incubated with 3µg GST, GST-ShcD-SH2 or GST-ShcD-SH2-S551D for overnight with tumbling. Following overnight incubation beads were washed 4 times and protein eluted by boiling in sample buffer. Samples of the whole cell lysate and precipitated proteins were analyzed by SDS-PAGE and western blotting to detect the presence of Gab1 (Figure 3.3). The pulldown assay shows that ShcD-SH2 was able to pull down endogenous Gab1 from both EGF(25ng/ml)-treated and vanadate (1mM) treated DAUV cells, but not untreated cells. Stimulation with vanadate produced a marked shift in the molecular weight of Gab1 in whole-cell lysates, suggesting enhanced tyrosine phosphorylation of the protein compared to EGF treatment. In contrast the ShcD-SH2-S551D mutant was unable to precipitate Gab1 with a result corresponding to the negative control pull-down with GST alone. These results suggest that introduction of a negative charge into the SH2 domain binding pocket of ShcD inhibits interactions with effector proteins, and hence phosphorylation of S551 would also have a negative effect on interactions and may represent a negative feedback signaling mechanism.

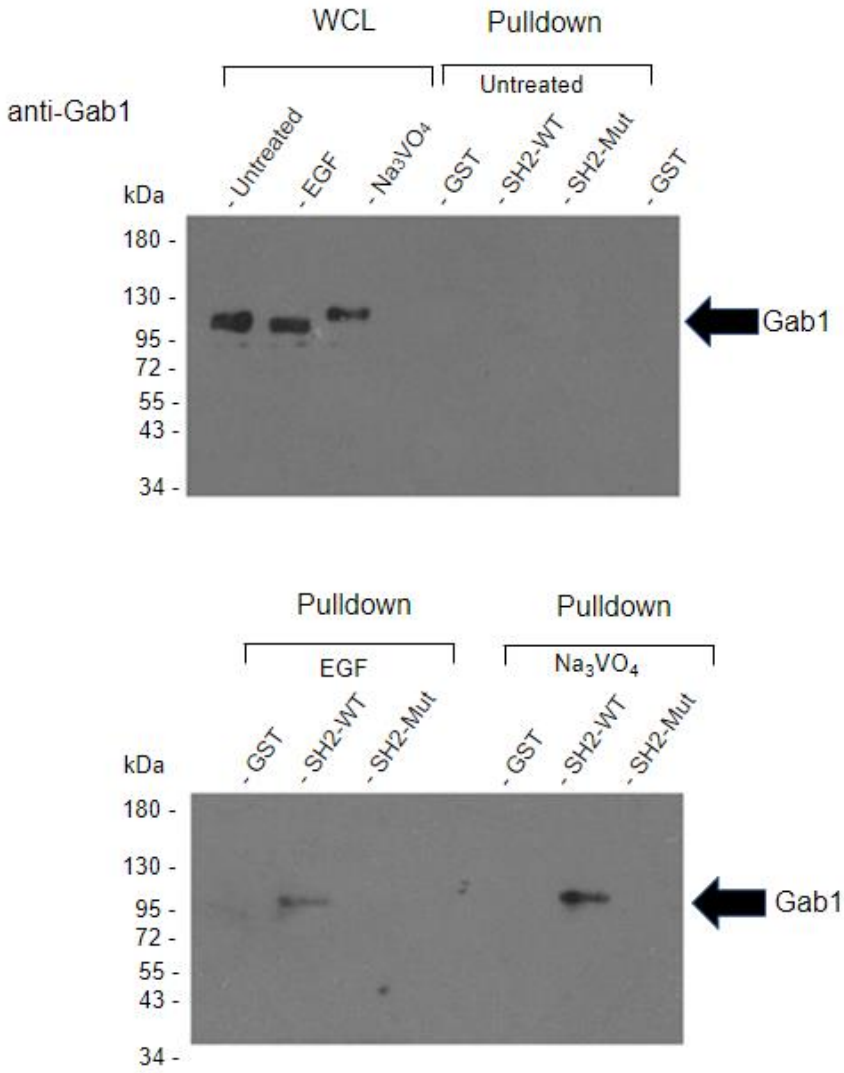

Figure 3.3: Pull-down of Gab1 from DAUV melanoma cell lysates using purified GST, GST-ShcD-SH2 and GST-ShcD-SH2-S551D bound to glutathione agarose beads. DAUV cells were serum starved for 1 hour then either left untreated or treated with EGF for 5 mins or sodium vanadate (Na_3VO_4) for 1 hour. Cell lysates were prepared, and proteins were pulled down with purified GST, GST-ShcD-SH2 (SH2-WT) and GST-ShcD-SH2-S551D (SH2-Mut) bound to glutathione-agarose beads. Whole cell lysates (WCL) and pull-down samples were analyzed on an 8% SDS-PAGE gel followed by western blotting with anti-Gab1 antibody. The Na_3VO_4 treated sample bands ran at a higher molecular weight compared to EGF treated and WCL bands possibly due to hyperphosphorylation. This experiment was performed 3 times and this result is representative.

3.2.2. Characterization of ShcD-SH2 and identification of interacting proteins

3.2.2.1. Identification of other protein candidates that may possibly interact with ShcD-SH2-domain

Having confirmed protein-protein interaction between Gab1 and ShcD-SH2 domain in mammalian cell, I wanted to investigate more about ShcD-SH2 domain and other proteins that may possibly bind to it. Since good yield of purified GST fusion proteins were available, I decided to use GST fusion protein to perform pulldown assay to detect the tyrosine phosphorylation.

Firstly, since the phosphorylation site in Gab1 (Y183) to which ShcD SH2 domain binds is conserved in Gab2, blots were also probed with anti-Gab2 antibodies. As was the case with Gab1, ShcD-SH2 domain was able to precipitate Gab2 from vanadate treated cell extracts (Figure 3.4.A).

Secondly, in order to increase amount of tyrosine phosphorylated proteins, the DAUV cell were serum starved for 1 hour then either left untreated or treated with 1mM sodium vanadate for 1 hour. Following sodium vanadate treatment, cells were lysed and incubated either with GST, GST-SH2-WT or GST-SH2-S551D fusion proteins bound to glutathione sepharose beads for overnight with tumbling at 4°C. The western blotting was performed with anti-PY99 antibody. In whole cell lysate samples, sodium vanadate treated samples from both DAUV cell showing precipitated protein bands throughout, but major bands were at around 55 and 130 kDa meanwhile untreated samples from both cells showing small amount of precipitated protein bands at 130 kDa as shown at Figure (3.4.B). In pulldown assay samples similar results at only GST-SH2-WT samples, but no bands observed at GST and GST-SH2-S551D which indicating that phosphorylation of ShcD-SH2-S551 prevent interaction between SH2 domain and other proteins as shown at Figure (3.4.B). The same blots were stripped and probe with anti-GST antibody to confirm same amount of GST fusion proteins were introduced to each sample for pulldown assay. The anti-GST antibody blot confirms similar amount of GST fusion proteins were given to each sample but GST-SH2-WT and GST-SH2-Mut have degraded product as shown on Figure (3.4.C).

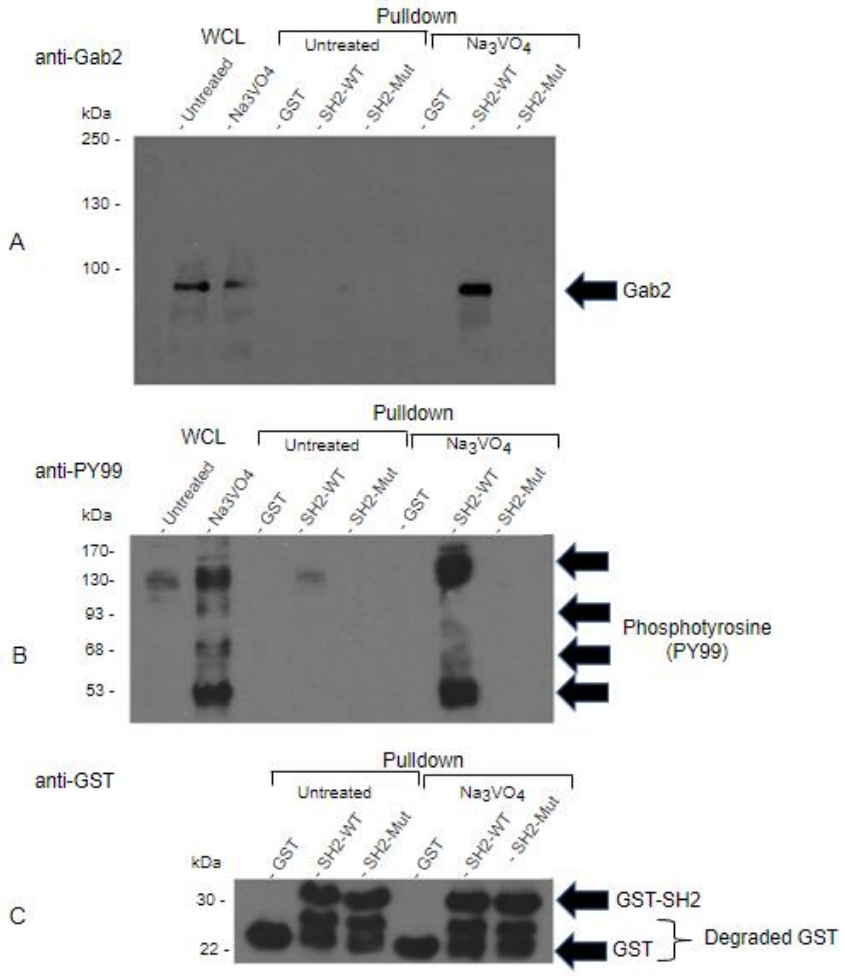

Figure 3.4: Pull-down of Gab2 and tyrosine phosphorylated proteins from DAUV melanoma cell lysates using purified GST, GST-ShcD-SH2 and GST-ShcD-SH2-S551D bound to glutathione agarose beads. DAUV cells were serum starved for 1 hour then either left untreated or treated with sodium vanadate (Na_3VO_4) for 1 hour. Cell lysates were prepared, and proteins were pulled down with purified GST, GST-ShcD-SH2 (SH2-WT) and GST-ShcD-SH2-S551D (SH2-Mut) bound to glutathione-agarose beads. Whole cell lysates (WCL) and pull-down samples were analyzed on an 8% SDS-PAGE gel followed by western blotting with antibodies recognizing Gab2 (A), phosphotyrosine (B) or GST (C). This experiment was repeated 3 times and this result is representative.

3.2.2.2. Identification of precipitated proteins from GST pulldown assay

The investigation of serine 551 position of ShcD-SH2 domain via GST pull down assay using anti-PY99 antibody confirmed that there are precipitated proteins indicating presence of tyrosine phosphorylated proteins binding to ShcD-SH2-WT as shown Figure 3.4 B. To identify those precipitated proteins, I scaled up the GST pulldown assay using DAUV cell lysates. Samples were analysed on an SDS-PAGE gel followed by staining with instant blue. Once the presence of precipitated proteins was confirmed, GST-SH2-WT's specific protein bands around 70 and 130 kDa were sliced out from gel then sent for mass spectrometry analysis. The result of mass spectrometry confirms precipitated protein as Gab1 and ShcD protein as shown at Figure (3.5). In addition, the same experiment was repeated using GST-ShcD-SH2 and GST-ShcD-SH2-S551D to determine which specific proteins only bind to the ShcD-SH2 and not to the mutant. In order to determine protein that specifically binds to only ShcD-SH2-WT, both GST-SH2-WT and GST-SH2-Mut lanes were extracted range between 130-250 kDa from the gel then sent to mass spectrometry analysis. Comparing the result of mass spectrometry analysis between GST-ShcD-WT and GST-ShcD-S551D confirmed that Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 (SHIP2) selectively binds to the WT-SH2 domain, but not to the mutant suggesting that this binds directly to the SH2 domain as shown at Figure (3.6).

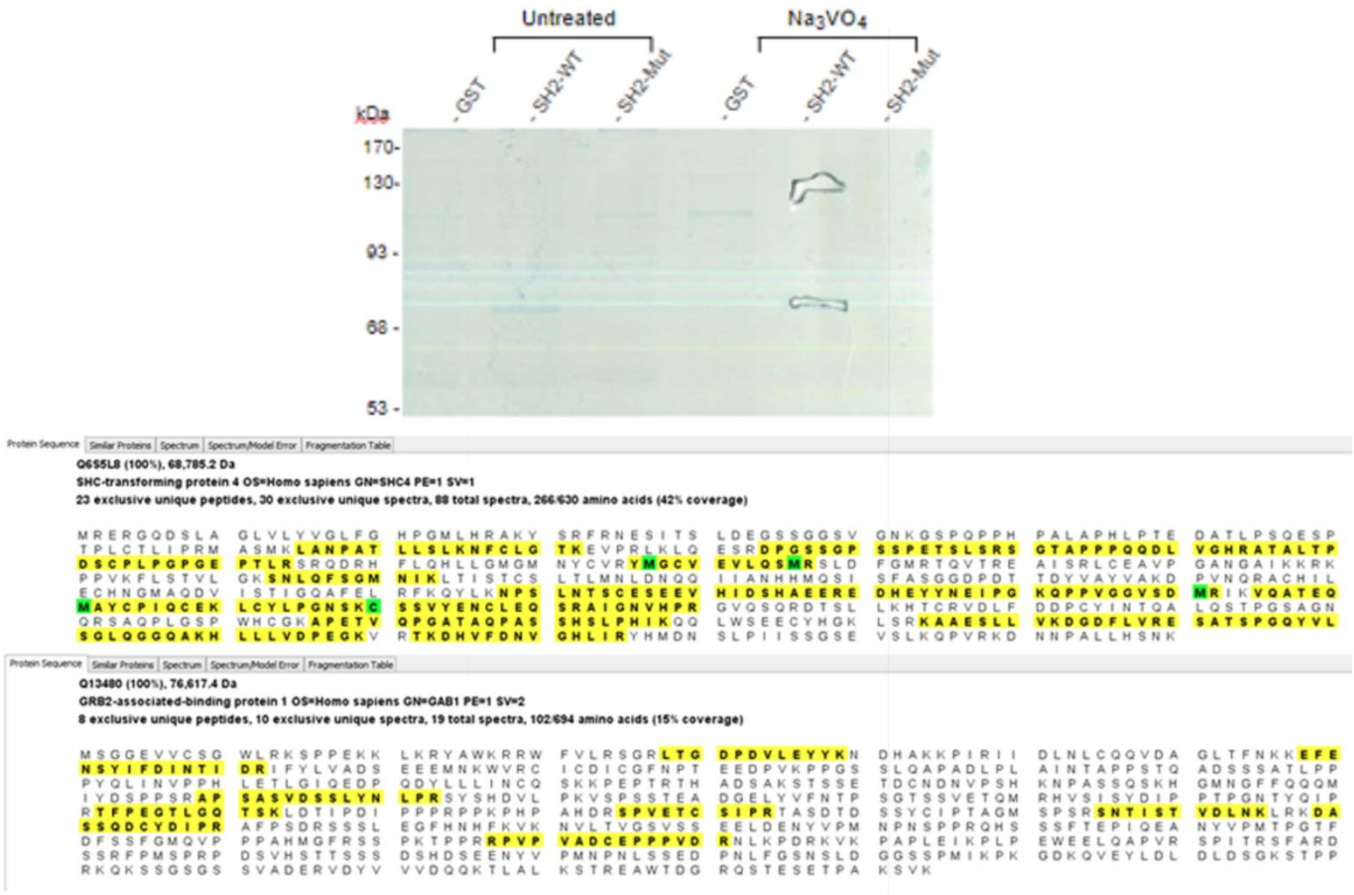

Figure 3.5: Analysis of proteins binding to GST-ShcD-SH2 fusion protein. WM266-4 melanoma cells were serum starved for 1 hour then either left untreated or treated with Na3VO4 for 1 hour. Cell lysates were prepared and proteins from were pulled down with purified GST, GST-ShcD-SH2 (SH2-WT) and GST-SH2-S551D (SH2-Mut) immobilised on glutathione-agarose beads. The Pull-down samples were analyzed on an 8% SDS-PAGE gel by staining with InstantBlue (expedeon). Two bands were excised, combined and sent for analysis by mass spectrometry. The peptides identified for the two highest scoring hits, ShcD and Gab1 are shown.

Figure 3.6: Analysis of proteins binding to GST-ShcD-SH2 fusion protein. Samples prepared as described in Figure 5 were analysed on an SDS-PAGE gel and a slice of gel was excised between 50-180 kDa from both the pull-down with the GST-ShcD-SH2 and the pull-down with GST-SH2-S551D mutant. Gel slices were sent for analysis by mass spectrometry and proteins precipitating only with the wild-type SH2 domain and not the mutant was identified.

3.2.2.3. Yeast two hybrid assay to determine whether the ShcD SH2 domain can bind directly to another ShcD domain

The mass spectrometry analysis showed very interesting results indicating ShcD-SH2 domain precipitated full length ShcD from cell lysates. The peptides identified included regions outside the SH2 domain so were not derived from the GST-SH2 domain fusion protein used for the pull-down. It is not clear whether ShcD-SH2 domain can interact directly with other domains of ShcD to form dimers or whether ShcD binds indirectly in a protein complex, possibly via Gab1. In order to determine whether, ShcD SH2 can directly bind to another domain I took advantage of yeast two-hybrid constructs that had been made previously in the lab. These included each of the ShcD domains cloned into the pBTM116-PDGFR vector (ShcD-CH2, PTB, CH1, SH2). The ShcD SH2 domain was cloned into the pVP16 derived 'prey' vector, pLEICS82, by Protex. Since the tyrosine phosphorylation sites are in the CH1 domain, if there was a direct interaction with the ShcD SH2 domain, I would expect this to be the interaction site. Therefore, I included a positive control binding partner for the CH1 domain, Grb2 SH2 domain. Beta galactosidase assays were performed on yeast transformed with combinations of bait and prey vectors (Fig. 3.7 A). As can be seen from the results, none of the individual Shc domains (expressed from the 'bait' vector) interacted with the ShcD SH2 domain (expressed in the 'prey' vector) as demonstrated by absence of blue colour. The CH1 domain interacted with the Grb2 SH2 domain indicating that it was phosphorylated and functional. To confirm that the ShcD SH2 domain in the 'prey' vector was functional, a Ret-PTC2 construct was used as 'bait' (Durick et el., 1996). This encodes an activated Ret kinase found as a fusion protein in papillary thyroid carcinomas. As can be seen, the ShcD SH2 domain interacts with active Ret, but not a kinase negative mutant which is unable to bind ATP, confirming that the ShcD SH2 domain is intact.

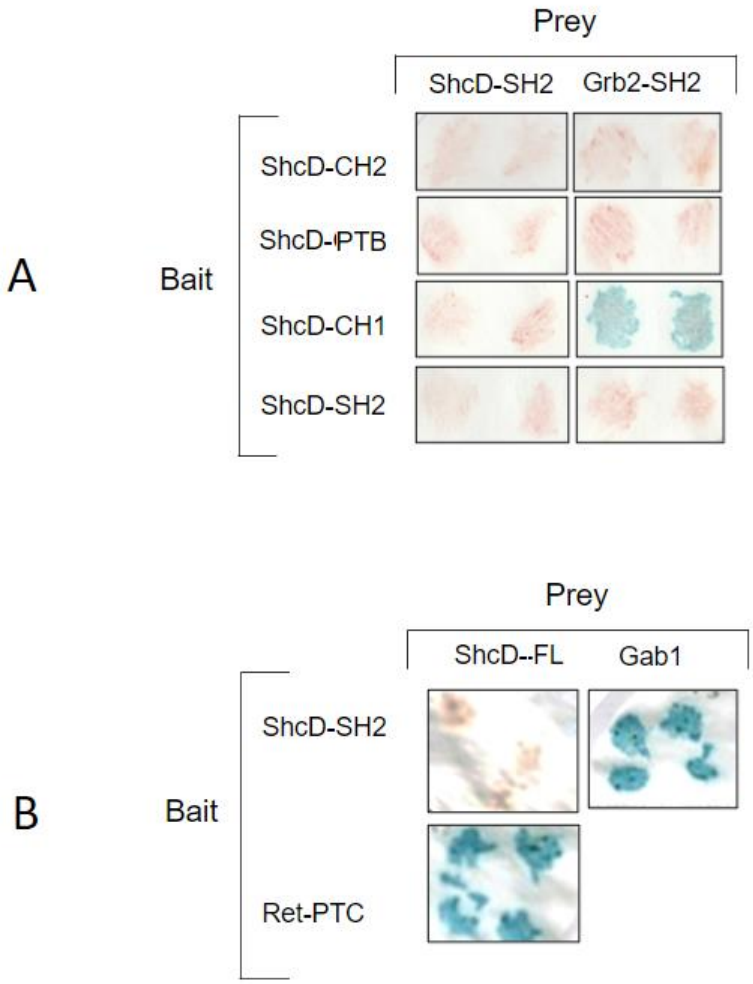

Figure 3.7 Yeast two hybrid analysis of ShcD to determine when ShcD SH2 domain facilitates formation of dimers or higher order complexes. Individual domains of ShcD (CH2, PTB, CH1, SH2) were expressed in L40 yeast as 'bait' proteins expressed from the pBTM116-PDGFR vector, together with the SH2 domain of ShcD or Grb2 in the pVP16 'prey' vector (A). Detection of LacZ reporter activation was detected by filter assays using X-Gal as substrate. Following generation of a full-length ShcD 'prey' construct (ShcD-FL), the assay was repeated using ShcD-SH2 domain as the bait construct. Gab1 was used as a positive control to confirm expression of the functional SH2 domain, whereas a Ret-PTC fusion protein was used as an alternative 'bait' protein to confirm correct functioning of the ShcD full-length protein. The experiment shown in B was performed by undergraduate project student, Alexander Haglund.

All the results suggested that there is no direct interaction between the ShcD SH2 domain and another ShcD domain, however it is possible that the SH2 domain only recognizes the correctly folded full-length protein. I therefore generated the full length ShcD sequence in the pLEICS82 vector. As can be seen from the data presented (Figure 3.7 B), the full length ShcD also failed to interact with the ShcD SH2 domain but interacted with active Ret-PTC2. The ShcD SH2 domain interacted with Gab1 as I expected. Experiments performed with full length ShcD construct and Ret-PTC2 were carried out by an undergraduate project student, Alex Haglund.

3.2.2.4. Investigating SHIP2 interaction with ShcD-SH2 domain

The mass spectrometry result for comparing between GST-SH2-WT and GST-SH2-Mut precipitated protein from lanes indicating that the phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 (SHIP2) only shown to bind selectively to WT-SH2 domain, but not to the mutant suggesting that this binds directly to SH2 domain. This result was very interesting as SHIP2 play crucial role in PI3K signaling pathways and maybe one of signaling pathway that require ShcD-SH2 domain. The phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2 (SHIP2) Is a phosphatase that act as catalyst which can dephosphorylation at 5' position of phosphinositidies or inositol phosphates and play critical roles at insulin signaling, cytoskeleton functions and regulate various biological process such as cell proliferation, apoptosis and cell adhesion (Thomas *et al*., 2017). In addition, SHIP2 has been shown to expressed in various cancer cells such as non-small cell lung cancer, gastric cancer and breast cancer but expression level of SHIP2 shown at breast cancer where upregulated SHIP2 increase rate of cell migration of breast cancer while silencing this phosphatase lead to inhibition of breast cancer cell proliferation and promote apoptosis (Zhou *et al*., 2019).

To investigate the protein-protein interaction between ShcD-SH2 and SHIP2, the pulldown assay was performed using both untreated and stimulated with Na_3VO_4 (1 hour) DAUV cell lysates then tumbling overnight with purified GST fusion proteins. The western blotting with anti-SHIP2 antibody confirmed the protein-protein interaction between SH2 domain of ShcD and SHIP2 bands at 150 kDa for both untreated and Na_3VO_4 treated whole cell lysates and for pulldown only SH2-WT as shown on Figure 3.8. The result shown that protein bands were only selectively binding to the GST-SH2-WT on both untreated and treated with Na_3VO_4 (1 hour) which supporting back the mass spectrometry analysis result where it only bound to GST-SH2-WT.

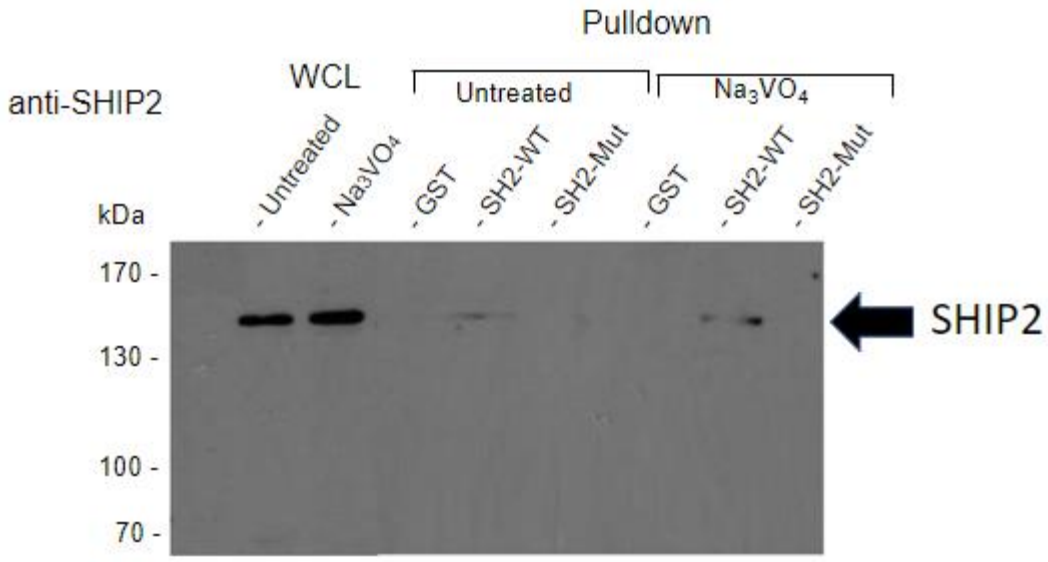

Figure 3.8: Pull-down of SHIP2 from DAUV melanoma cell lysates using purified GST, GST-ShcD-SH2 and GST-ShcD-SH2-S551D bound to glutathione agarose beads. DAUV cells were serum starved for 1 hour then either left untreated or treated with sodium vanadate (Na_3VO_4) for 1 hour. Cell lysates were prepared, and proteins were pulled down with purified GST, GST-ShcD-SH2 (SH2-WT) and GST-ShcD-SH2-S551D (SH2-Mut) bound to glutathione-agarose beads. Whole cell lysates (WCL) and pull-down samples were analyzed on an 8% SDS-PAGE gel followed by western blotting with anti-SHIP2 antibody. The bands were only binding to GST-SH2-WT which indicating that there is protein-protein interaction between ShcD-SH2 and SHIP2. This experiment was performed 3 times and this result is representative.

3.2.2.5. *Investigating SH-PTP2 interact with ShcD-SH2 domain*

The one of research journal from Wills and research team stated that SH-PTP2 may possibly interact with ShcD-SH2. The affinity purification-mass spectrometry analysis was performed to identify the proteins that interact with ShcD protein and SH-PTP2 was identified to be binding to ShcD-SH2 (Wills *et al*., 2017). The SH-PTP2 has been shown to involved in various cancers (breast, leukemia, lung, liver and gastric) and cancer related process such as cell invasion, metastasis and apoptosis (Zhang *et al*., 2015). I wanted to investigate this SH-PTP2 interaction with ShcD-SH2 because our mass spectrometry analysis didn't detect it and SH-PTP2 was widely involved in both cancers and cancer cell process.

To investigate the interaction between ShcD-SH2 and SH-PTP2, the pulldown assay was performed using both untreated and stimulated with Na_3VO_4 (1 hour) WM-226 cell lysates then tumbling overnight with purified GST fusion proteins. The western blotting with anti-SHP2 antibody confirms clear bands around 70 kDa on both untreated and Na_3VO_4 treated whole cell lysate but no bands observed on pulldown which indicating there are no protein-protein interaction between ShcD-SH2 and SH-PTP2 as shown on Figure (3.9).

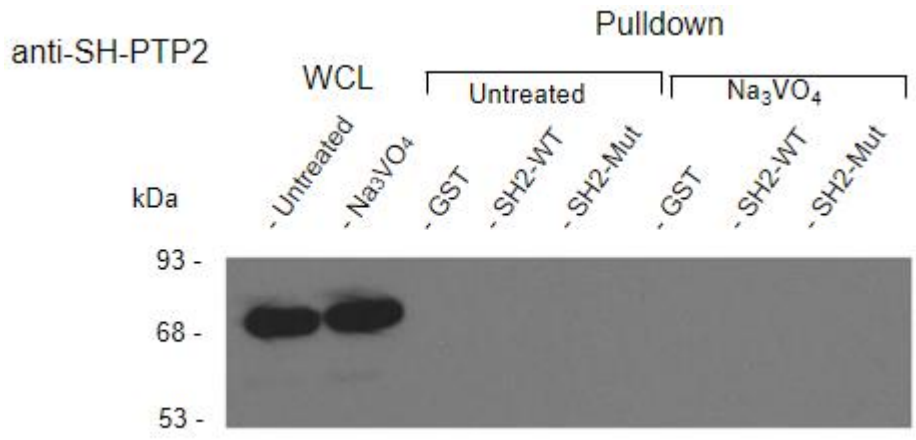

Figure 3.9: Pull-down of SH-PTP2 from DAUV melanoma cell lysates using purified GST, GST-ShcD-SH2 and GST-ShcD-SH2-S551D bound to glutathione agarose beads. WM266-4 cells were serum starved for 1 hour then either left untreated or treated with sodium vanadate (Na_3VO_4) for 1 hour. Cell lysates were prepared, and proteins were pulled down with purified GST, GST-ShcD-SH2 (SH2-WT) and GST-ShcD-SH2-S551D (SH2-Mut) bound to glutathione-agarose beads. Whole cell lysates (WCL) and pull-down samples were analyzed on an 8% SDS-PAGE gel followed by western blotting with anti-SH-PTP2 antibody. Unlike WCL, there were no bands at GST-SH2-WT on both untreated and Na_3VO_4 which indicated that there is no protein-protein interaction between SH-PTP2 and ShcD-SH2. This experiment was performed 3 times and this result is representative.

3.3. Discussion

3.3.1. Gab1 protein is major binding partner for ShcD in mammalian cells

Previously, in this lab it was shown that Gab1 can interact with ShcD via SH2 domain in yeast two hybrid assay. In this project, I managed to determine that Gab1 protein can interact with ShcD-SH2 using GST pulldown assay and phosphorylation of ShcD-SH2-S551 prevent ability to interact with other proteins. The Gab1 interaction with ShcD-SH2 only occurs when Gab1 is phosphorylated upon EGF or Na_3VO_4 treatment. In addition, the phosphorylation site in Gab1 (Y183) to which ShcD SH2 domain binds is conserved in Gab2; the western blot analysis confirmed that Gab2 also can interact with ShcD-SH2. This finding suggests that upon growth factor such as EGF stimulation, EGFR, Gab1 and ShcD form a complex. However, this signaling pathway still need further investigation to determine the result of this process.

There are many studies suggesting that Gab1 protein can interact with Shc protein family members like ShcA and ShcC. The process of ShcA interaction with Gab1 protein start with phosphorylation of ShcA recruiting Grb2 and Gab1 protein can interact with Grb2 to form a protein complex that can regulate the breast cancer progressions such as cancer cell survival, angiogenesis and metastasis (Ursini-Seigel and Muller., 2008) while phosphorylated ShcC can interact with Gab1 protein to form a protein complex which can recruit PI3K subunit P85 to induce the AKT signaling pathway for neuronal and thyroid cancer survival (Ferro *et al*., 2011).

In contrast ShcD has not been shown to activate AKT (Wills *et al*., 2014) and has been reported to inhibit AKT activation downstream of Ret receptor (Mabruk *et al*., 2018). The role of ShcD in activation of the Ras/MAPK pathway also suggests a distinct function to other Shc proteins. There are some studies suggesting that ShcD plays a crucial role in Ras/MAPK signaling pathway in response to EGF and IGF1 treatment when overexpressed in normal non-metastatic cell lines melanocytes to regulate cell migration. However, in metastatic melanoma cell lines, upon inhibiting Ras/MAPK pathway, it appears ShcD overexpressing cells are still able to mediate the cell migration, so other pathways are involved (Fagiani *et al*., 2007). In several studies ShcD has been shown to suppress downstream activation of MAPK/Erk in response to growth factor treatment and has also been shown to enhance EGFR phosphorylation at specific sites in a ligand independent fashion.

The growth factor stimulation such as EGF, PDGF, IL-3, IL-6 or HGF can lead to phosphorylation of Gab1 protein and lead to PI3K/AKT and Ras/MAPK signaling (Lamothe *et al.*, 2004) and in this lab confirmed SH2 domain of ShcD protein can directly bind to Gab1 via tyrosine 183 in mammalian cells. In addition, ShcD can directly bind to activated RTKs via its PTB domain (You *et al*., 2010, Wills *et al*., 2017) and recruit either the Grb2-SOS or Grb2-Gab1/2 complex through its CH2 domain while another ShcD protein binds to Gab1 via its SH2 domain. This interaction between Gab1 and ShcD possibly results in increasing rate of cell migration but may require other domains (PTB, CH1 and CH2) to form a protein complex as these domains are free to associate with other signaling proteins. In addition, to identify biological process of ShcD, it still requires to investigating other ShcD domain (PTB, CH1 and CH2) interaction with other signaling proteins.

3.3.2. ShcD-SH2 possibly interacting with other ShcD protein.

The result of mass spectrometry analysis confirmed Gab1 protein is the major binding partner for ShcD-SH2 domain. Interestingly, mass spectrometry analysis also identified ShcD protein as possible binding partner for the ShcD-SH2 domain. Yeast two hybrid assay indicate ShcD-SH2 does not directly interact with neither individual modular ShcD domains nor full ShcD protein. Since Shc family members are scaffolding protein that involved in various signaling pathway via forming a protein complex, it is likely that ShcD was associated in a bigger protein complex, involving Gab1. In the model depicted in Figure 3.10 large complexes could assemble where Gab1/2 and ShcD are present in equal amounts which could explain why these were the only two proteins visible as distinct bands in the original mass spectrometry analysis (Grb2 would have run off the bottom of the gel). The ability of both proteins to interact with each other by two distinct mechanisms (via Grb2 or directly via Gab1 (Y183) and ShcD SH2 domain) also raises the possibility that they could be involved in stabilizing oligomers of receptors. This complex may protect the receptors from dephosphorylation and contribute to the increase in tyrosine phosphorylation of the EGFR observed when ShcD is overexpressed (Wills *et al*., 2014).

3.3.3. Identification of other proteins that interacting with ShcD-SH2

The finding of SHIP2 from mass spectrometry was very interesting due to this enzyme acting as catalyst to induce hydrolysis of PIP3 to form PI(3,4)P2. PIP3 is the product of the reaction catalyzed by PI3K that results in the activation of AKT, therefore SHIP2 is effectively negatively regulates PI3 kinase. (Agollah *et al*., 2014). SHIP2 is involved in cell proliferation and metabolic effects of insulin via decrease the level of phosphatidylinositol 3,4,5-trisphosphate while increasing level of phosphatidylinositol 3,4-bisphosphate (White *et al*., 2020). SHIP2 has also been shown to play critical role in cancer cells where SHIP2 is overexpressed in breast cancer and depletion of SHIP2 activity led to inhibition of cell migration and metastasis in breast cancer (Ghosh *et al*., 2018). In gastric cancer interestingly SHIP2 showed reduced expression and inhibition of this enzyme led to increased proliferation and tumorigenesis via the PI3K/AKT signaling pathway (Ye *et al*., 2017). In addition, SHIP2 overexpression was observed in melanoma cell lines but did not shown any regulatory function, possibly due to cell type specific but overexpression of phosphatidylinositol 4,5-bisphosphate 5-phosphtase inhibited the AKT activation and siRNA knock-down promoted activation of AKT in melanoma (Ye *et al*., 2013).

The ability to recruit SHIP2 to the RTK-Grb2-Gab1/2-ShcD complex could explain why overexpression of ShcD has not been lead to AKT activation since it would lead to hydrolysis of PIP3.

The SH-PTP2 was also a candidate that possibly interacts with the SH2 domain of ShcD as one study showed in their Flag immunoprecipitation experiments with individual domains of ShcD that SH-PTP2 binds to ShcD-SH2 domain (Wills *et al*., 2017). In their model looking at TrkB signaling, Grb2 binding to ShcD sequesters it in a non-productive signaling complex that does not activate MAPK/Erk. MAPK activity is restored by overexpression of Grb2. They propose that SH-PTP2 induced dephosphorylation of the CH2 domain releases Grb2 so it can bind directly to TrkB

to activate the MAPK/Erk pathway. (Wills *et al*., 2017). However, in our experiment with the GST-ShcD pulldown assays I did not detect assays-PTP2 using WM-266-4 melanoma cell lysate. The SH-PTP2 is an enzyme encoded by PTPN11 gene that consist of two SH2 domains and a phosphatase domain. Both SH2 and phosphatase domains are required for SH-PTP2's biological function (Agazie and Hayman, 2003). The SH-PTP2 was identified to be associated with different types of cancers and upregulated SH-PTP2 has been shown to be involved in cell invasion of breast and lung cancer, tumour differentiation in gastric cancer and in leukemia inactivating mutation of tyrosine phosphatase have been identified (Zhang *et al*., 2015). In addition, The SH-PTP2 is being researched as cancer drug target.

While SH-PTP2 may well be a component of a signaling complex involving ShcD, as previous studies in this lab have precipitate SH-PTP2 in complexes with full length ShcD, I have not been able to demonstrate that it binds directly to the SH2 domain. Yeast two-hybrid studies may be able to test for direct interaction.

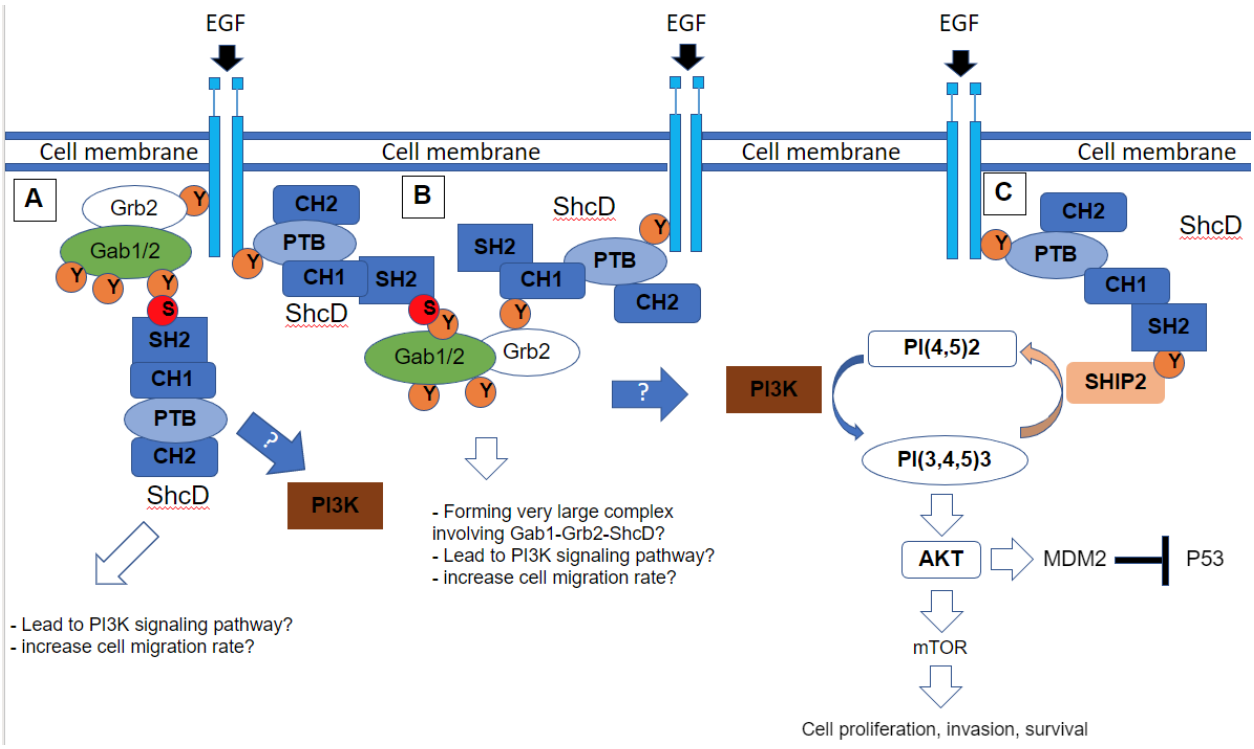

Figure 3.10: Diagram of possible ShcD and Gab1/2 protein/SHIP2 enzyme involvement in RTK signaling pathways. The Grb2/Gab1 protein complex can bind to phosphorylated EGF receptor and interact with the SH2 domain of ShcD which possibly lead to PI3K signaling pathway (A). The other involvement is via the PTB domain of ShcD that binds directly to phosphorylated EGF receptor then recruits the Grb2/Gab1 protein complex recruited through CH1 domain and the ability of ShcD to associate with Gab1 via two different domains could result in the formation of very large complex involving Gab1-Grb2 and ShcD (B). The PTB domain of ShcD that binds directly to phosphorylated EGF receptor then recruits the Grb2/Gab1 protein complex recruited through CH1 domain while SHIP2 recruited through SH2 domain and SHIP2 lead to hydrolysis of PIP3 (C)

Chapter 4

INVESTIGATING THE KINASES THAT PHOSPHORYLATE AT SHCD S551

4.1. Introduction

In the previous chapter I confirmed that Gab1 protein is a major binding partner for the ShcD-SH2 domain in mammalian cells and mass spectrometry analysis of ShcD SH2 domain associating proteins revealed that SHIP2 may also interact with ShcD-SH2 domain It was also shown that S551 is a phosphorylation site in the ShcD SH2 domain, and that mimicking this phosphorylation site by changing it to aspartic acid inhibited the function of the SH2 domain and prevented it from binding to its binding partner Gab1. In this chapter I sought to identify possible kinases that could be responsible for this phosphorylation event that could negatively regulate pathways involving ShcD interactions.

Using phosphorylation site prediction software kinases of the AGC family (PKA, PKG and PKC) were identified as potential kinases that could potentially phosphorylate ShcD S551. (Table 4.1). In addition, analysis of the literature identified one study that identified a PAK4 phosphorylation site motif which corresponded to the S551 site in ShcD. The identification of PAK4 motifs in this study was performed using degenerate peptides as substrates (Rennefahrt *et al*., 2007). This was of particular interest since the PAK4 kinase has also been shown to interact with Gab1 protein in the Met signaling pathway forming a complex that regulates cell migration and invasion (Paliouras *et al*.,2009). Interestingly the PAK4 interaction site on Gab1 was mapped to a previously uncharacterized region of Gab1 involving amino acids 116-234; a region that has not been previously reported to interact with any proteins. This region contains Y183, which is the tyrosine phosphorylation site in Gab1 to which ShcD SH2 domain binds. This suggests that there may be a possible competition for binding to Gab1 and this may be regulated by phosphorylation of the SH2 domain.

P21-Activated Kinase 4 (PAK4) is enzyme from PAK family group II that plays a critical role in cell signaling. PAK4 regulates many different types of biological signaling functions such as cell migration, proliferation, and survival but when PAK4 fails to regulate its own expression and activity, this leads to the development of pathological conditions (Won et al., 2019). PAK4 is a major effector that links between Rho-GTPases and the actin cytoskeleton by interacting with CDC42 (cell division control protein 42 homolog) and this interaction allows PAK4 to regulate the cytoskeleton organization, cell cycle and cell mobility (Ha *et al*., 2018). PAK4 has also been shown to be involved in cancer development and overexpression of PAK4 in cancer leads to cancer cell migration, adhesion, proliferation, survival and metastasis (Wang *et al*., 2019). PAK4 in ovarian cancer regulates the cancer cell migration and metastasis by activation of at least two PAK4 pathways which are PAK4 to c-Src and PAK4 to MEK-1/MEK1/2 to MMP2. In cell proliferation of ovarian cancer PAK4 activation of c-Src together with activation of the EGFR pathway co-operate to regulate the expression level of cyclin D1 and CDC25A (Siu *et al*., 2010). As a result, PAK4 is intensively studied as a potential drug target or marker for cancer or PAK4 related disease.

In this chapter, I will explore whether PAK4 can phosphorylate ShcD- S551 by using phospho-specific antibodies raised in rabbits to perform western blotting and in-vitro kinase assay. In addition, I would like to confirm whether activation of other kinases, PKC, PKA and Jnk could similarly cause phosphorylation of ShcD-S551.

4.2. Results

4.2.1. Characterization and finding optimal conditions for antibodies raised against a ShcD- S551 phosphopeptide

In order to investigate which kinases are able to induce phosphorylation of ShcD-S551, custom phosphospecific antibodies were raised against the synthetic phosphopeptide of the following sequence: VKDGDFLVRE[pS]ATSPGQYVLC. The antibody was characterized in the following studies from the final bleed. The antibodies Pep S551 and Pep S551(P) does not have information regarding their antibody sensitivity, optimal antibody dilution ratio and blocking buffer type. In order to find the optimal condition for those antibodies, Dot blotting was used to investigate the specificity of the antibody towards phosphorylated versus non-phosphorylated peptide and to determine the sensitivity of the antibody. The different peptide quantity from 0.01 - 100ng of Pep S551 (non-phosphorylated) and Pep S551(P) (phosphorylated were spotted on to nitrocellulose then dried. Once dried the blot was blocked in 5% BSA blocking buffer and probed with crude serum, or antibody that was either purified by affinity chromatography using the phosphopeptide or on the non-phosphorylated peptide (Figure 4.1). Furthermore, additional dot blotting was performed with different dilution ratio of antibodies and 1:2000 dilution was determined to be optimal dilution factor for those antibodies.

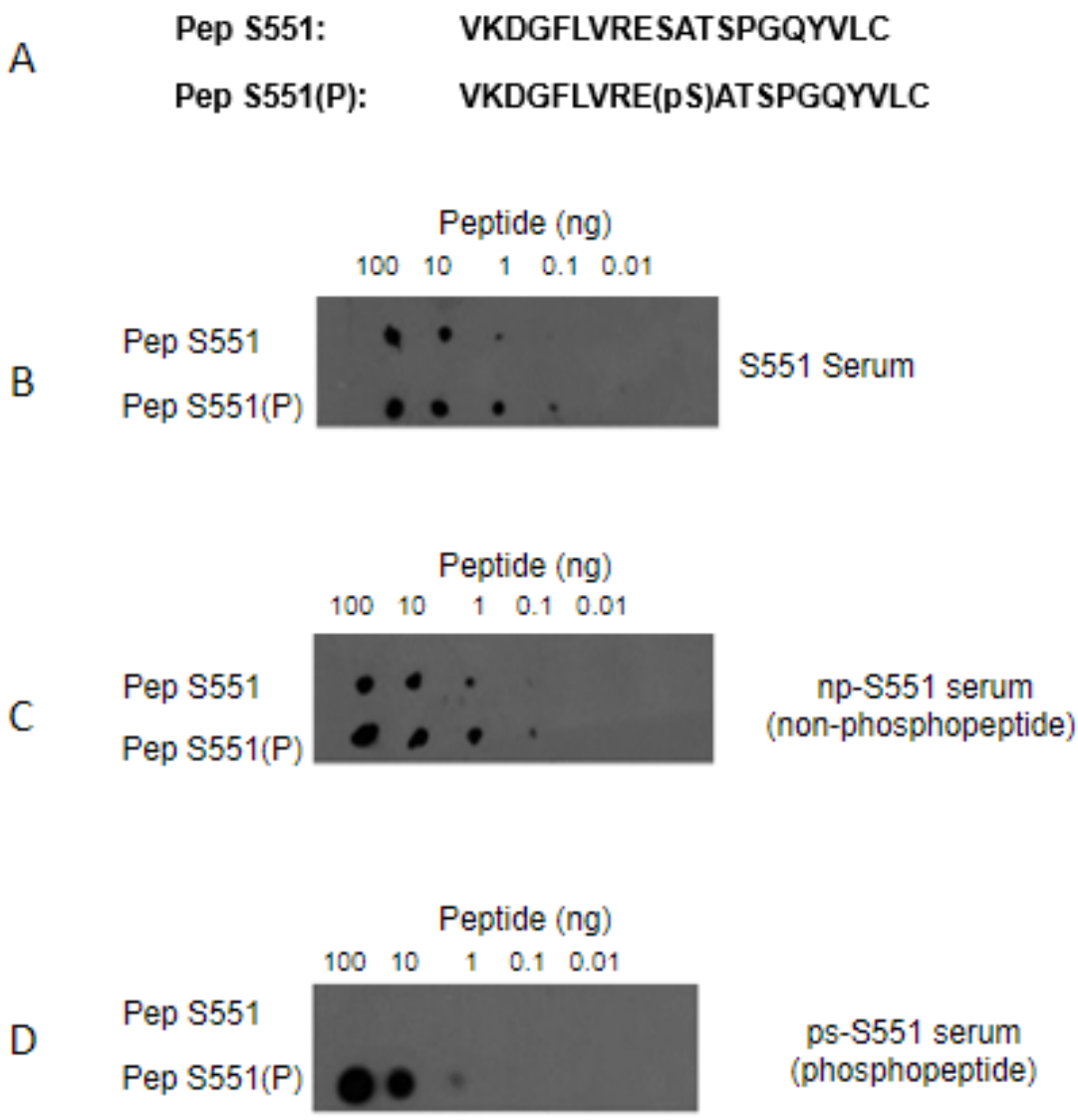

Figure 4.1: Characterization of an antibody raised against a ShcD phosphopeptide corresponding to pS551, Pep S551(P), using dot blotting. The sequence of the ShcD phosphopeptide, Pep S551(P), and corresponding non-phosphorylated peptide, Pep S551, are indicated (A). Different quantities (0.01-100 ng) of Pep S551(P) and PepS551 were spotted onto nitrocellulose and allowed to dry. After blocking, blots were probed with antibody serum raised against Pep S551(P) (A), antibody purified using the non-phosphorylate peptide (B) or antibody purified using the phosphorylated peptide (C).

As can be seen in Figure 4.1, both the crude serum and antibody purified using the non-phosphorylated peptide reacted with both the phosphopeptide (Pep S551(P)) and non-phosphorylated peptide (Pep S551). However, rabbit serum purified using the phosphopeptide was highly specific for the phosphopeptide in the dot blot and did not react with the unphosphorylated peptide.

4.2.2. Testing expression and immunoprecipitation of HA-PAK4 for use in studies to investigate phosphorylation of ShcD S551

Having optimised conditions for using the phospho-specific antibody, I now wanted to determine whether PAK4 can phosphorylate ShcD S551. I had available a HA-PAK4 DNA construct generated previously in the lab and first wanted to test its expression in HEK293 cells. To do this, HEK293 cells were transfected with HA-PAK4 or left untransfected for negative control then lysed with sample buffer. The western blotting was performed using whole cell lysate samples then probed with anti-HA antibody to confirm the HA-PAK4 (HA-Pak4) expression. As can be seem in Figure 4.2, a single band was observed at the reported molecular mass for PAK4 at around 64 kDa in the transfected cell lysates, but not the control cell lysates

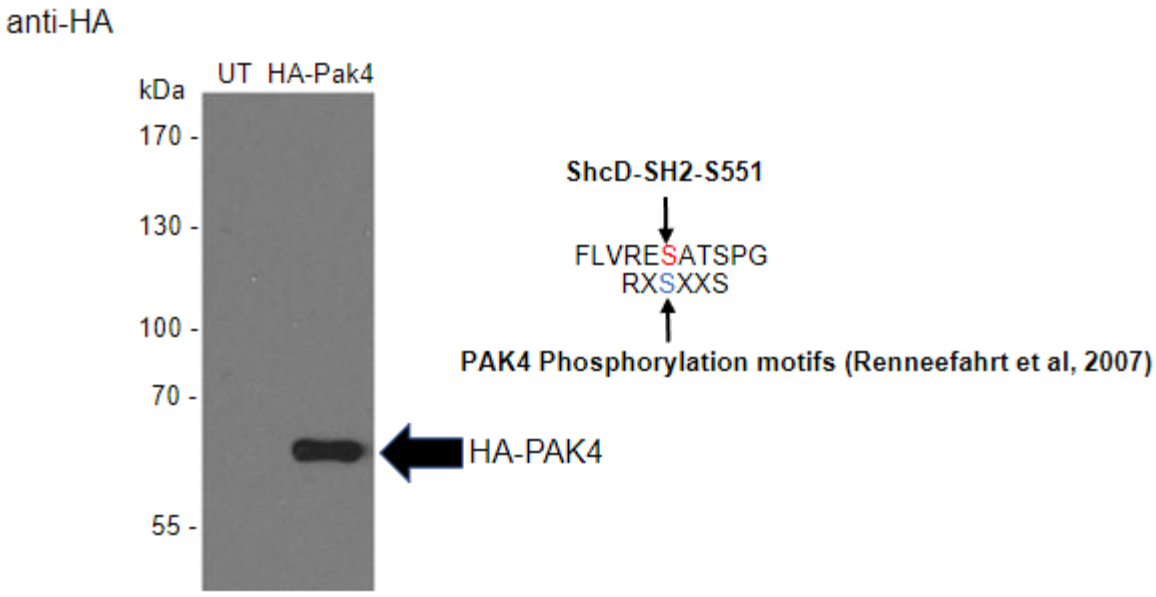

Figure 4.2: Western blot analysis to test the expression of HA-Pak4 using anti-HA antibody. HEK293 cells were transfected using HA-PAK4 plasmid (HA-Pak4), or left untransfected (UT) then lysed in sample buffer. Whole cell lysate was analyzed on an 8% SDS-PAGE gel followed by western blotting with an anti-HA antibody

In order to determine whether PAK4 can phosphorylate ShcD S551, I wanted to generate a constitutively active form of the kinase. It has previously been reported that mutation of serine 445 in PAK4 to asparagine results in a constitutively active kinase (Renneefahrt *et al*., 2007). Site directed mutagenesis was used to generate the HA-PAK4-S445N mutant and its sequence confirmed by DNA sequencing. The expression of the construct was confirmed by transfection into HEK293 cells alongside the wild-type HA-PAK4 construct, and cell lysates were analysed by western blotting with anti-HA antibody (Figure 4.3). Since future experiments would require immunoprecipitation of HA-PAK4 protein, an immunoprecipitation was performed in parallel using HA-immunobeads and immunoprecipitates were analysed on the same blot (Figure 4.3).

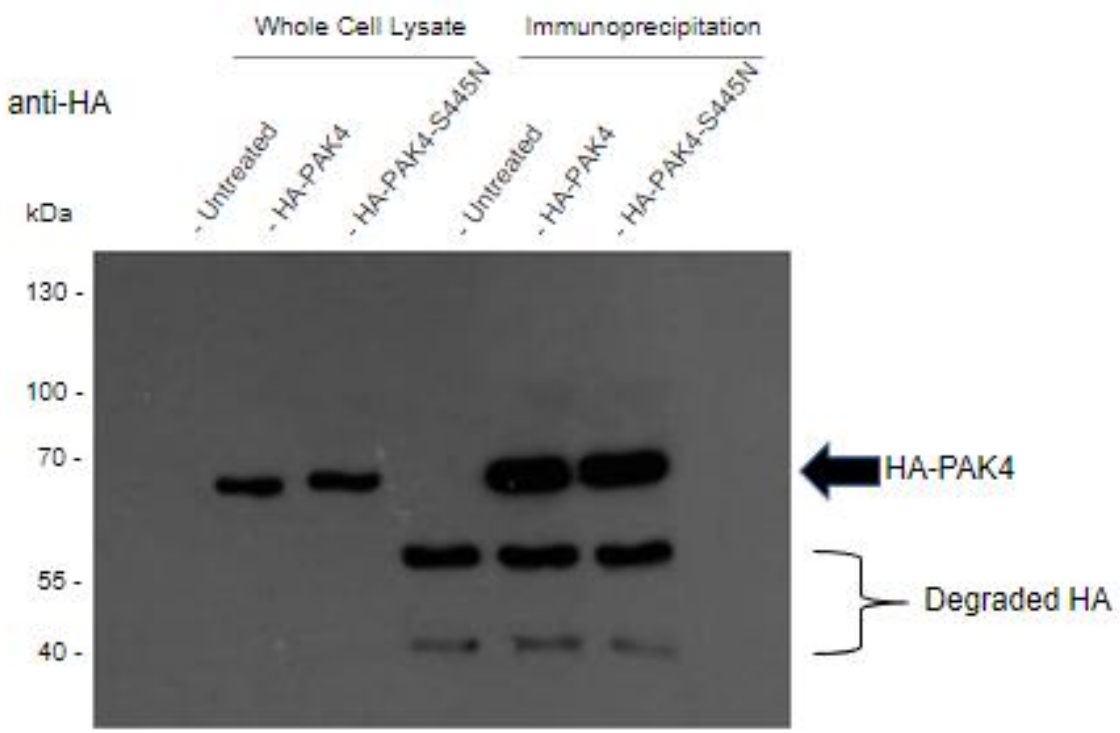

Figure 4.3: Western blotting analysis to test immunoprecipitation with anti-HA antibody. HEK293 Cells were transfected with HA-PAK4 and HA-PAK4-S445N DNA then cells were lysed. Lysed samples were immunoprecipitated with HA immuno-beads followed by analysis on 8% SDS-PAGE gel and western blotting with anti-HA antibody. This experiment was repeated 3 times and this result is representative.

As can be seen from Figure 4.3, the HA-PAK4-S445N and wild-type construct are expressed at similar levels. Both wild-type and mutant PAK4 could be efficiently immunoprecipitated from transfected HEK293 cells.

4.2.3. Identifying kinases that possibly phosphorylate at ShcD-SH2-S551 phosphorylation site prediction software

Previously, in this lab performed mass spectrometry analysis on ShcD-SH2 and identified S551 as a possible phosphorylation site. in order to identify the kinases that possibly phosphorylate at ShcD-S551, the ShcD protein or amino acid sequence was analysed by kinase prediction websites. Three kinase prediction websites GPS5.0, PhosphoNet and NetPhos3.1 were used to identify kinases that could possibly phosphorylate ShcD-S551. The kinases with highest evaluated possible phosphorylation were selected from each prediction websites.

GPS 5.0	
Kinase name	Evaluated possible phosphorylation (Score)
AGC/PKA	22.143
AGC/PKC/PKCa/PRKCB	2.556
PhosphoNet	
Kinase name	Evaluated possible phosphorylation (Score)
MSK1	372
AKT1	359
NetPhos 3.1	
Kinase name	Evaluated possible phosphorylation (Score)
PKA	0.670
PKG	0.497
Commonly predicted kinases from 3 prediction websites	
PKA, PKG, PKC	

Table 4.1: Predicted kinases that possibly phosphorylate with ShcD-S551.

4.2.4. Investigating phosphorylation of ShcD-S551 via western blotting

Characterization of phospho-specific antibodies and generation of a constitutively active mutant of PAK4 provided the tools to investigate the phosphorylation of ShcD-S551 by PAK4. The activation of other kinase pathways was also explored: PKC and PKA that had been identified as potential kinases that could phosphorylate S551 using predictive software, and JNK, which would not be expected to phosphorylate this site as it requires a proline residue C-terminal to the serine residue. HEK293 cells stably expressing Flag-ShcD were either transfected with HA-PAK4-S445N or left untransfected. Cells were serum starved 1 hour prior to treatment with forskolin, PDBu or sorbitol to stimulate the PKA, PKC and JNK respectively, or left in serum-free medium. Each sample was lysed with lysis buffer. The samples were analyzed on an 8% SDS-PAGE gel then western blotting was performed using ps-S551 antibody.

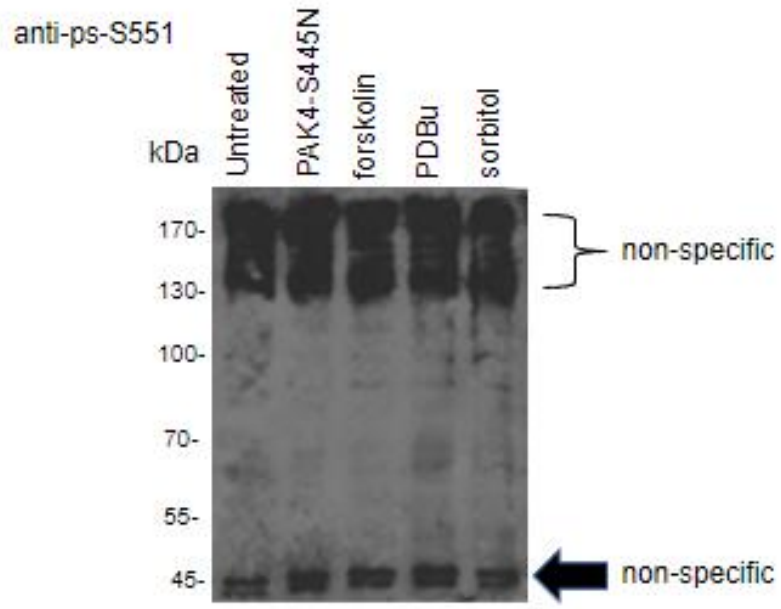

Figure 4.4: Western blotting analysis of using whole cell lysate samples to identify kinases that phosphorylate ShcD-SH2-S551 using ps-S551 antibody. HEK293 cells expressing Flag-ShcD were either transfected with PAK4-S445N or treated with forskolin, PDBu or sorbitol to activate PKA, PKC and JNK respectively. Cells were serum starved 1 hour prior to stimulation. The cells were lysed with lysis buffer and samples were analysed on an 8% SDS-PAGE gel followed by western blotting with ps-S551 antibody. This experiment was repeated 3 times and this result is representative.

However, due to unspecific binding was preventing to determine the phosphorylation of ShcD-S551(Figure 4.4), The lysed samples were immunoprecipitated with anti-FLAG antibody (mouse) agarose beads then the samples were analyzed on an 8% SDS-PAGE gel. The western blotting was performed using ps-S551 antibody. The same blots were stripped and reblotted with anti-Flag antibody (rabbit) to confirm that the same amount of anti-FLAG agarose beads were introduced to each stimulated/transfected sample. In addition, the imageJ was used to quantify and calculate the mean of three independent experiments data. The results are shown in Figure 4.5.

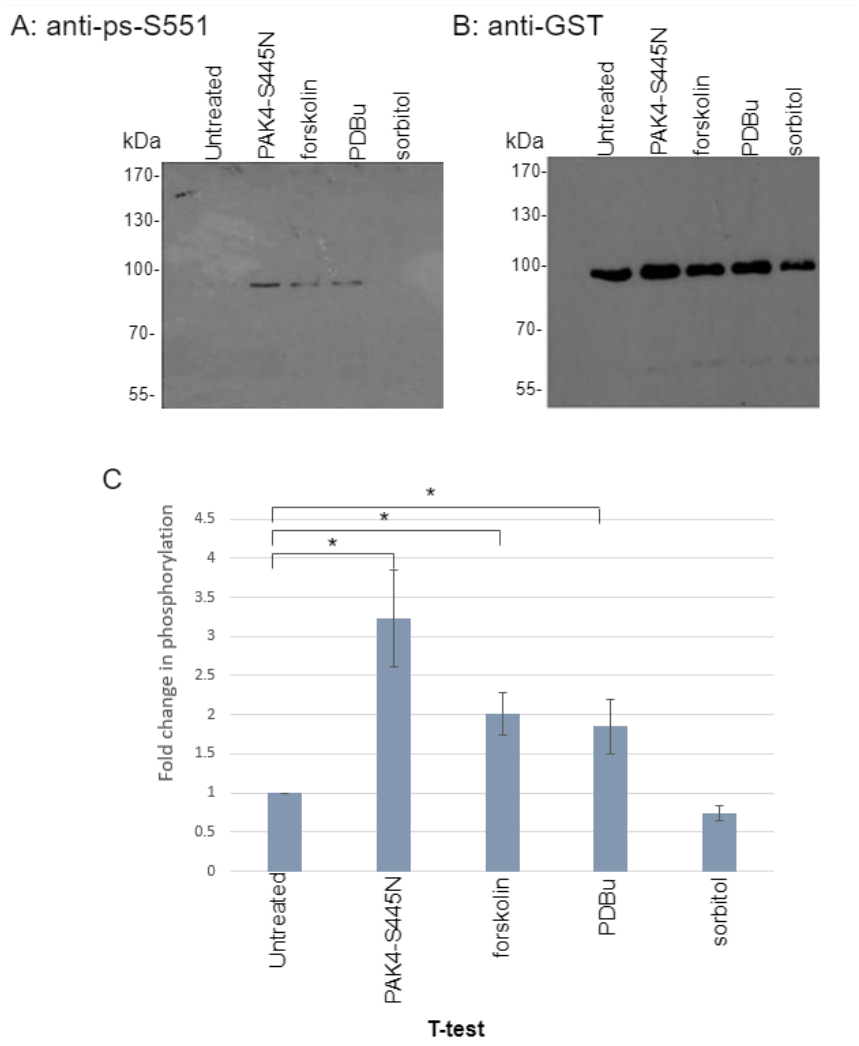

Figure 4.5: Western blotting analysis to identify kinases that phosphorylate ShcD-SH2-S551 using ps-S551 antibody. HEK293 cells expressing Flag-ShcD were either transfected with PAK4-S445N or treated with forskolin, PDBu or sorbitol to activate PKA, PKC and JNK respectively. Cells were serum starved 1 hour prior to stimulation. Immunoprecipitation was performed with anti-FLAG antibody (mouse) coupled to agarose beads. The samples were analysed on an 8% SDS-PAGE gel followed by western blotting with ps-S551 antibody (A). Blots were then stripped and re-probed with rabbit anti-FLAG (B). The data from three experiments was quantified using ImageJ and the means are presented +/- SEM. * indicates $p< 0.05$.(C).

The western blotting analysis suggests that constitutively active PAK4 can phosphorylate ShcD-S551 as seen by an increase in the band intensity obtained with the phosphospecific antibody in cells transfected with the HA-PAK4-S445N construct (Figure 4.5). In addition, chemical agents commonly used to activate PKA and PKC also promoted phosphorylation of ShcD to a lesser extent, while activation of JNK appears to have no effect. The same blot was stripped and reprobed with anti-FLAG antibody to confirm that a similar amount of FLAG-ShcD was present in each of the immunoprecipitates. ImageJ software was used to quantify the three experiments. Paired t-test analysis comparing individual treatments with the untreated cells revealed a significant difference ($p<0.05$) between untreated cells and those transfected with constitutively active PAK4 or treated with forskolin or PDBu.

4.2.4.1. Confirmation of activation of PAK4, PKC, PKA and JNK pathways

In order to confirm that PAK4, PKA, PKC and JNK had been activated in the experiment shown in Figure 4.5, whole cell lysates were analysed from the samples used for the immunoprecipitations and western blotting was performed to detect phosphorylated proteins associated with each of the pathways. Cofilin is a substrate of LIM kinase 1 (LIMK1) which is a downstream effector of PAK4 (Ahmed *et al*. 2008). LIMK1 phosphorylate and reducing the cofilin to depolymerize actin when HGF-PAK4 phosphorylate LIMK1 (Won et al., 2019). Forskolin activates adenylate cyclase, increasing cAMP levels resulting in PKA activation. Creb1 is a well characterized substrate of PKA (Tacke *et al*.,2005), and other kinases. pDBU is a phorbol ester and potent activator of PKC. Erk is a well characterized substrate of PKC (Basu and Tu., 2005). Sorbitol induces osmotic stress resulting in activation and phosphorylation of JNK. Probing for phospho-colfilin, phospho-creb1, phospho-Erk and phospho-JNK was therefore used to confirm activation of PAK4, PKA, PKC and JNK pathways respectively (Figure 4.6). In addition, the blots were stripped and reblotted with anti-Flag antibody to confirm same amount of samples were introduced each lane.

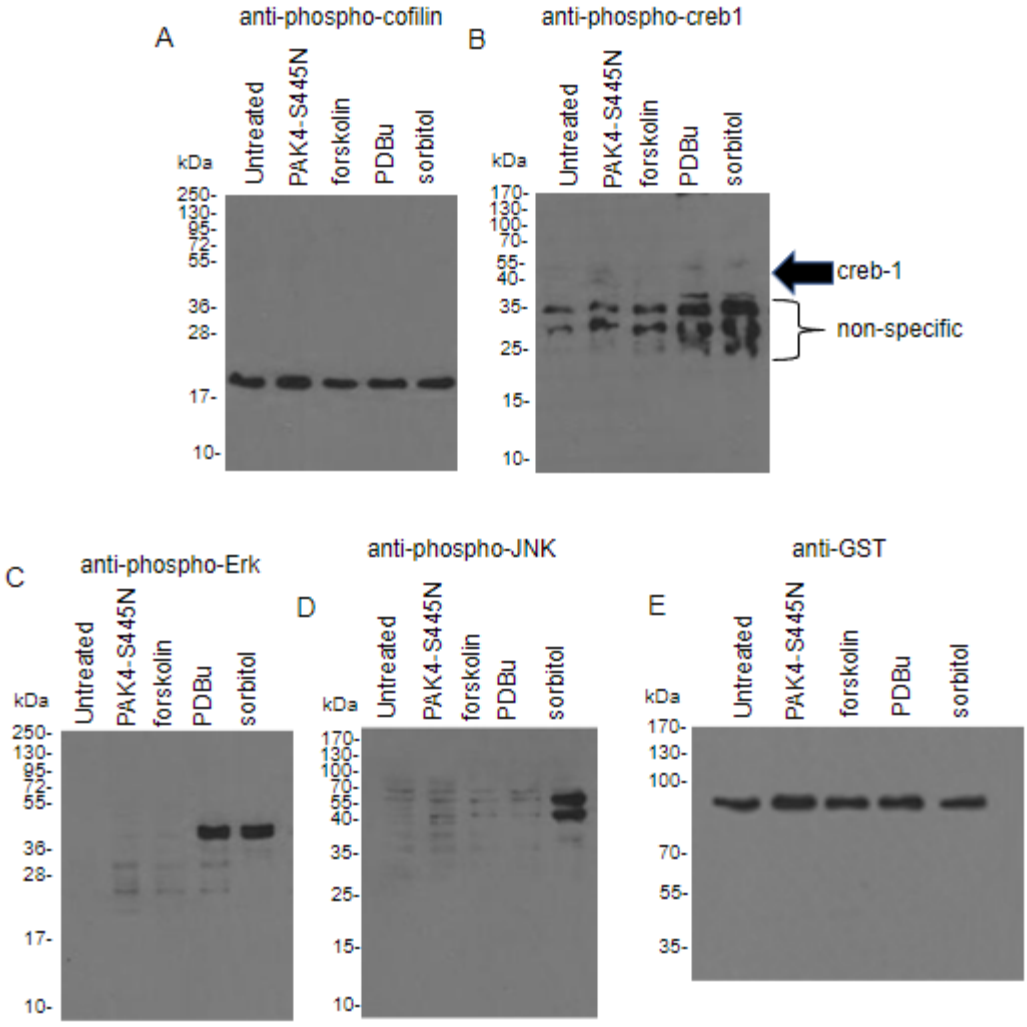

Figure 4.6: Western blotting analysis to determine activation of PAK4, PKA, PKC and JNK. HEK293 cells expressing Flag-ShcD were either transfected with PAK4-S445N or treated with forskolin, PDBu or sorbitol to activate PKA, PKC and JNK respectively. Cells were serum starved 1 hour prior to stimulation. Cell lysates were analysed by western blotting using anti-phospho-cofilin (A), anti-phospho-creb1 (B), anti-phospho-Erk (C), anti-phospho-JNK antibodies (D) and blots were stripped and reblotted with anti-Flag antibody (E). This experiment was repeated 3 times and this result is representative.

From the results presented in Figure 4.6, JNK was specifically activated by sorbitol treatment. PKC was activated by PDBu, as indicated by phosphorylation of Erk. Sorbitol treatment also resulted in Erk phosphorylation. This has been reported previously and is independent of PKC (Fusello *et al*., 2006). Cells transfected with active PAK4 showed a modest increase in phospho-cofilin levels. It should be noted however that phospho-cofilin levels were already high in the untransfected cells, and not all the cells will have been transfected. Although transfection efficiency was not determined in this experiment previous immunofluorescence experiments using these cells, with different constructs, would not usually exceed 50%. Although a band at 37kDa corresponding to phospho-creb1 (S133) is increased in intensity by forskolin treatment, PDBu and sorbitol apparently induced an enhanced phosphorylation, while active PAK4 also caused a slight increase. Creb1 phosphorylation at serine 133 via RSK2, a downstream effector of the Ras/MAP kinase pathway, has been reported previously (Xing *et al*., 1996). Since Erk is activated by PDBu and sorbitol, it is not surprising the creb1 phosphorylation is induced. Pak4 has also been reported to phosphorylate creb1, although at a different site (S115), so direct phosphorylation would not be detected by this antibody. However, Pak4 also activates the PI3K/AKT pathway, and AKT has been shown to phosphorylate S133, so this is a possible mechanism (Won *et al*., 2019).

4.2.5. In vitro kinase assay to detect direct phosphorylation of purified GST-ShD-SH2 domain.

The western blots with the phosphospecific antibody suggested that PAK4, and possibly other kinases are able to phosphorylate S551 in the SH2 domain. I wished to determine whether PAK4 can directly phosphorylate purified GST-SH2 domain expressed in bacteria. To do this, HEK293 cells were transfected with either HA-PAK4, HA-PAK4-S445N or left non-transfected. Cells were lysed with lysis buffer on ice then PAK4 was immunoprecipitated with HA antibody coupled to beads. after immunoprecipitation was done, the beads were washed two times with lysis buffer then washed once with kinase buffer then purified GST or GST-(ShcD)-SH2 was added to the samples. The kinase assay was initiated by adding kinase buffer containing P^{32} labeled γATP and samples were incubated 30^{o}C for 30mins. Tubes were then centrifuged and 3X sample buffer was added to stop the reaction. The tubes were immediately boiled for 5 mins and analysed on a 15% SDS-PAGE gel. The gel was stained with Coomassie Blue R staining solution overnight then destained. The destained gel was dried down and exposed to x-ray film overnight. Results are shown in Figure 4.7.

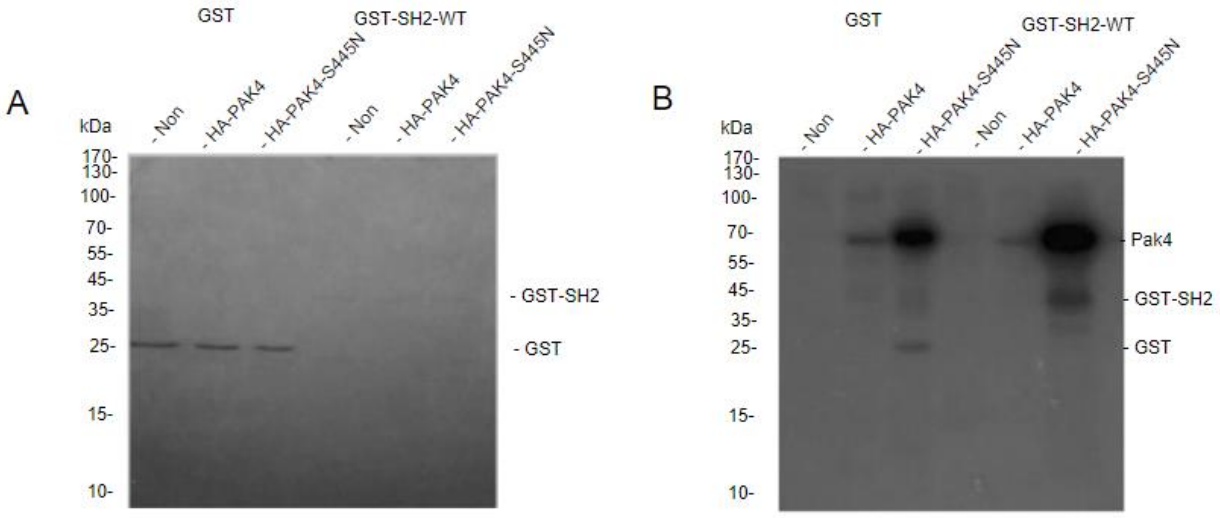

Figure 4.7: Analysis of radioactive kinase assay to determine whether ShcD SH2 domain is a substrate for Pak4. HEK293 cells were transfected with plasmids encoding HA-tagged wild type Pak4 or an activated mutant, HA-Pak4-S445N, or were left non-transfected (Non). Cells were lysed with lysis buffer then immunoprecipitations performed using HA antibody coupled to beads. Purified GST or GST-SH2 was added to the immunoprecipitates and kinase assays were performed using P^{32} labeled γATP. The samples were analysed on 15% SDS-PAGE gel and stained with Coomasie Blue R250 dye solution followed by destaining (A). After destaining, gels were dried and exposed to x-ray film overnight (B). This experiment was repeated 2 times and this result is representative.

The results of the assay showed very convincingly that the mutation in PAK4 resulted in greatly increased kinase activity as shown by the enhanced autophosphorylation of the band at 70 kDa compared to the wild-type protein (Figure 4.7 B). This band is absent in the immunoprecipitated samples from untransfected cells. Of interest, there is a band at approximately 36 kDa corresponding to the phosphorylated GST-ShcD-SH2 domain in the kinase assay with mutant PAK4. However, a faint band is also visible corresponding to GST, when GST was used as a control substrate, suggesting that in this *in vitro* assay, PAK4 is able to phosphorylate GST to some extent. It is however notable that the amount of GST-ShcD-SH2 protein used in the assay was much less than GST, with GST-ShcD-SH2 being barely visible on the coomassie stained gel (Figure 4.7 A). If equivalent molar quantities of substrate had been used, a more striking and quantifiable result may have been achieved.

4.3 Discussion

The concept that SH2 domain interactions can be negatively regulated by phosphorylation of the SH2 domain is an interesting one. One such phosphorylation event was reported previously where the SH2 domain of the p85 subunit of PI3 kinase was reported to be phosphorylated by PKD, activated downstream of PKC (Lee *et al*., 2011). The phosphorylation site was in the ligand binding pocket, in the sequence F**L**V**R**ES**S**, where the identified phosphorylation site motif is shown in large bold font. Interestingly the site in ShcD is present in the same position in the binding pocket, but the serine which is phosphorylated is the preceding one, and the serine identified in p85 is not present in the ShcD SH2 domain. From our study it is possible that PKC similarly mediates phosphorylation of ShcD at the sequence FLVRE**S**A, as PKC activation resulted in an increased band detected on a western blot with the S551-phosphospecific antibody.

The identification of phosphorylation site specific kinases is a challenging task given the large number of different kinases. Studies by Manning in 2002 reported the kinase complement of the human genome (the kinome) and identified 518 putative kinases, 71 of which had not been previously identified. For some of these, their specific phosphorylation site motif is well documented, but for a majority, a specific phosphorylation motif has not been identified. Very recently, a novel approach for wide scale identification of kinase phosphorylation motifs has been described, given the name; kinase inhibitor profiling to identify kinases (KiPIK) (Watson *et al*., 2020).

To facilitate some degree of screening for kinases that could phosphorylate ShcD S551 I generated a phosphspecific antibody for the site. The antibody purified by affinity chromatography on immobilised phosphopeptide specifically bound to the

phosphopeptide on dot blots, but not the non-phosphorylated peptide. By performing western blots of immunoprecipitated FLAG-ShcD from HEK293 cells stably expressing this protein I identified PAK4, PKC and PKA as possible kinases that could phosphorylate ShcD S551, either directly or through activation of another kinase. While the results are clearly of interest further studies need to be performed to validate these observations. Although I have shown that the purified antibody is specific for the S551 phosphopeptide, rather than the non-phosphorylated peptide, I have not tested whether it can bind to other serine phosphorylated sequences. Ideally, I would like to test the antibody on a selection of synthetic phosphopeptides by dot blotting for direct comparison. In the absence of such peptides, I could acutely activate Erk and JNK by sorbitol treatment and see if the antibody can detect an increase in phosphorylated proteins, which would suggest the antibody is recognizing phosphoserine in a context independent fashion.

While there is an indication from the *in vitro* kinase assay that PAK4 may directly phosphorylate the ShcD SH2 domain, the experiment needs to be refined. Clearly the amount of substrate added needs to be carefully quantified so the same concentration of GST and GST-ShcD-SH2 are used in the assay. An additional control would be the GST-ShcD-SH2-S551D mutant which cannot be phosphorylated in the *in vitro* assay. In addition, a widely used ‘universal’ kinase substrate should be included such as myelin basic protein (MBP). The kinase assay should also be repeated using commercially available purified PKC and PKA to determine whether they directly phosphorylate the ShcD SH2 domain or whether a downstream effector kinase is involved. For future research, kinase active assay can help to determine the phosphorylation between the ShcD SH2 domain with PKA/PKC/PAK4 or other downstream effector kinases.

Chapter 5

INVESTIGATION OF SHCD PROTEIN SUBCELLULAR LOCALISATION

5.1. Introduction

In the previous chapters I described the phosphorylation of the the ShcD-SH2-domain at position S551 and the likely inhibitory effect of this phosphorylation on binding to other signaling proteins like Gab1.In this chapter I aimed to explore the subcellular localization of ShcD protein and compare this to ShcD mutants that could not be phosphorylated at this site (S551A) or contained a phosphomimetic mutation (S551D).

ShcD is generally reported to be distributed throughout the cytoplasm (Fagiani et al., 2007, Ahmed and Prigent., 2014). However, in a number of studies its subcellular localization is altered. In COS-1 cells overexpressing GFP-ShcD, ShcD protein is found to colocalise with the EGFR in a recycling endosomal compartment and to promote phosphorylation and ubiquitylation of the EGF receptor. (Wills *et al*., 2017). This ability to sequester EGFR in the cell in the absence of stimulation was reported to be cell type dependent, but the phenomenon was also reported in other cell types such as CHO (Chinese hamster ovary) cells and U87 glioma cells (Wills *et al*., 2017). In another study, co-expression of GFP-ShcD and Myc-Ret resulted in their endosomal localization and their interaction prevented Erk/AKT signaling pathway leading to reduced cell migration and survival rates (Mabruk et al., 2018). Studies in DAUV melanoma cells demonstrated that the CH2 domain of ShcD contains a nuclear export sequence ‘^{83}LCTLIPRM^{90}’ that allows cytoplasmic localization of ShcD however under conditions of extreme oxidative stress, a small proportion of ShcD protein accumulates in the nucleus (Ahmed and Prigent., 2014).

Since ShcD is reported to have a main role in promoting cell migration, I postulated that overexpression of ShcD might alter the organization of the actin cytoskeleton. I also planned to look at the effect of overexpression on cell migration. For these studies I chose U2OS osteosarcoma cells, as they are a well-established cell type used for immunofluorescence microscopy and colleagues in the department had

already established migration assays using these cells and been able to demonstrate changes in morphology and migration by transfection of EML4-Alkv3 (O'Regan *et al*., 2020). They also express very low levels of endogenous ShcD (The Protein Atlas). In this chapter, I will be investigating the subcellular localization of Flag-ShcD and its effect on the actin cytoskeleton. The effect of ShcD-SH2-S551A/S551D on subcellular localization will be explored. Previous studies have shown that U2OS cells express PDGFR-β and that PDGFR-β signaling is essential for U2OS cell migration (Glen *et al*.,2011). Given that U2OS cells express PDGFR-β receptor, and this receptor was identified in our mass spectrometry analysis of proteins interacting with the ShcD GST-SH2 domain, I also looked at subcellular localization in response to PDGF stimulation.

5.2. Results

5.2.1. Investigating the subcellular localization of Flag-ShcD and the effect of overexpression on the actin cytoskeleton

U2OS cells grown on coverslips were transfected with Flag-ShcD and serum starved for 1 hour then either left untreated or treated with PDGF (15mins). The cells were analyzed by immunofluorescence staining using anti-Flag and Texas red-phalloidin antibodies followed by goat anti-mouse secondary antibody coupled to Alexa Fluor 488. Flag-ShcD was mainly localized throughout the cytoplasm while actin was localized to actin stress fibres spanning the cytoplasm and at the cell periphery (Figure 5.1). The result indicated that there were no significant differences in actin cytoskeleton localization between the un-transfected and Flag-ShcD transfected cell.

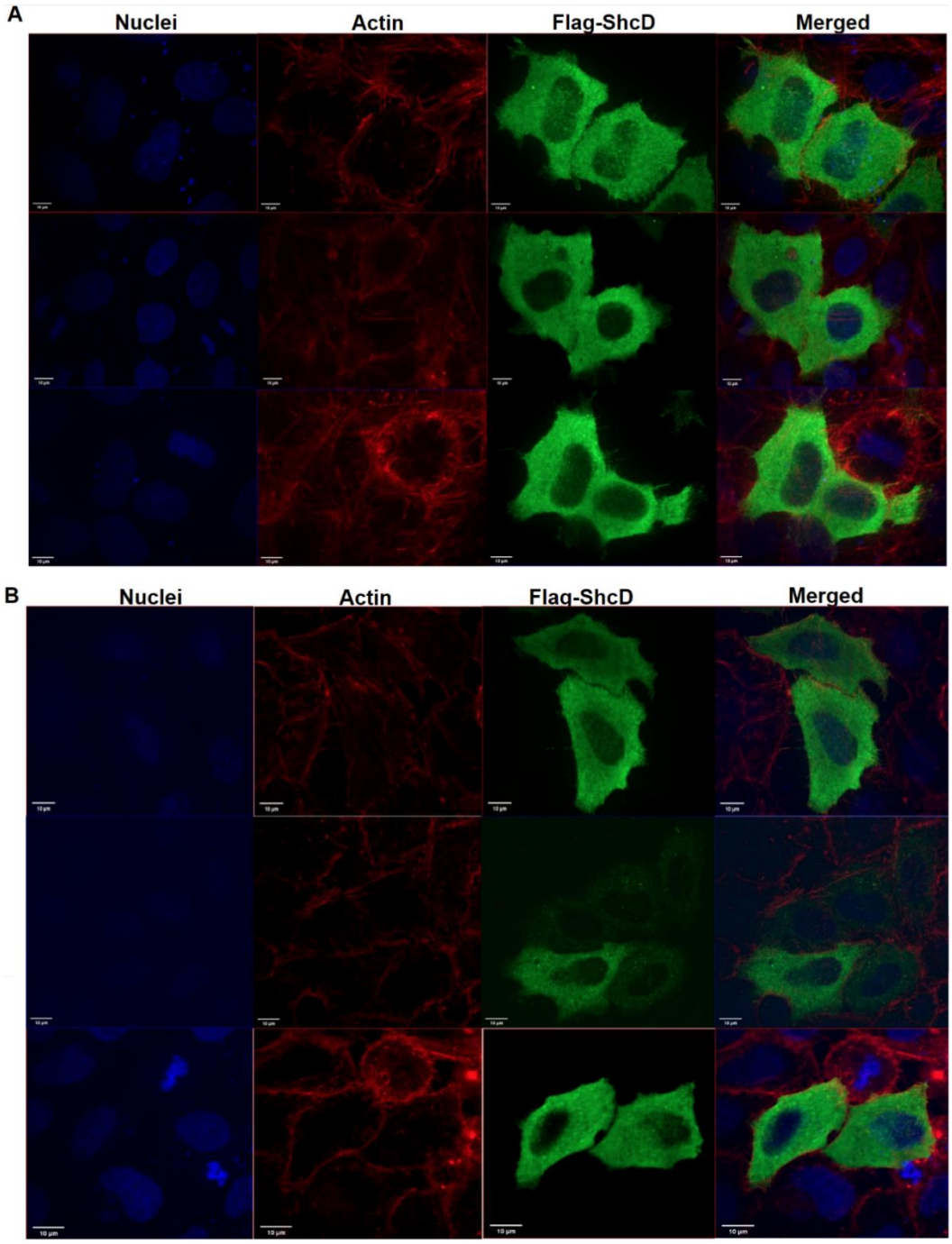

Figure 5.1: Determination of Flag-ShcD subcellular localization and effect of Flag-ShcD overexpression on the actin cytoskeleton. U2OS cells were either transfected with Flag-ShcD and then serum starved for 1 hour followed by either stimulation with PDGF (15 mins) [B] or left untreated [A]. The fixed cells were analyzed by confocal microscopy. The scale bar presented in the bottom left corner corresponds to 10 μm.

5.2.2. Investigating the subcellular localization of Flag-ShcD-S551A and Flag-ShcD-S551D in the presence or absence of PDGF

Mutations were generated in the wild-type Flag-ShcD contruct to either prevent phosphorylation Flag-ShcD-S551A, or mimic phosphorylation Flag-ShcD-S551A. The U2OS cells grown on coverslips were transfected with either Flag-ShcD-S551A or Flag-ShcD-S551D then serum starved for 1 hour followed by stimulation with PDGF (15 mins) or left untreated. The transfected U2OS cells were stained with anti-Flag antibody (Figures 5.2 and 5.3). The stained cells were analyzed by using VisiTech Infinity3 confocal laser microscope

. The cells transfected with Flag-ShcD-S551A or Flag-ShcD-S551D (Figures 5.2 and 5.3) had a very different appearance from those transfected with the Flag-ShcD wild-type (Figure 5.1) as they contained many distinct punctae all over the cell. These structures were less prominent in the PDGF treated cells. There were insufficient PDGF-treated cells to score but the number of untreated cells showing this distinct phenotype were counted. This experiment was performed one time.

	Number of cells with distinct punctae	Percentage score
Flag-ShcD-wild-type	5/72	6.9%
Flag-ShcD-S551A	51/87	58.6%
Flag-ShcD-S551D	78/120	58.3%

Table 5.1: Number of cells with distinct punctae for each transfected samples and score.

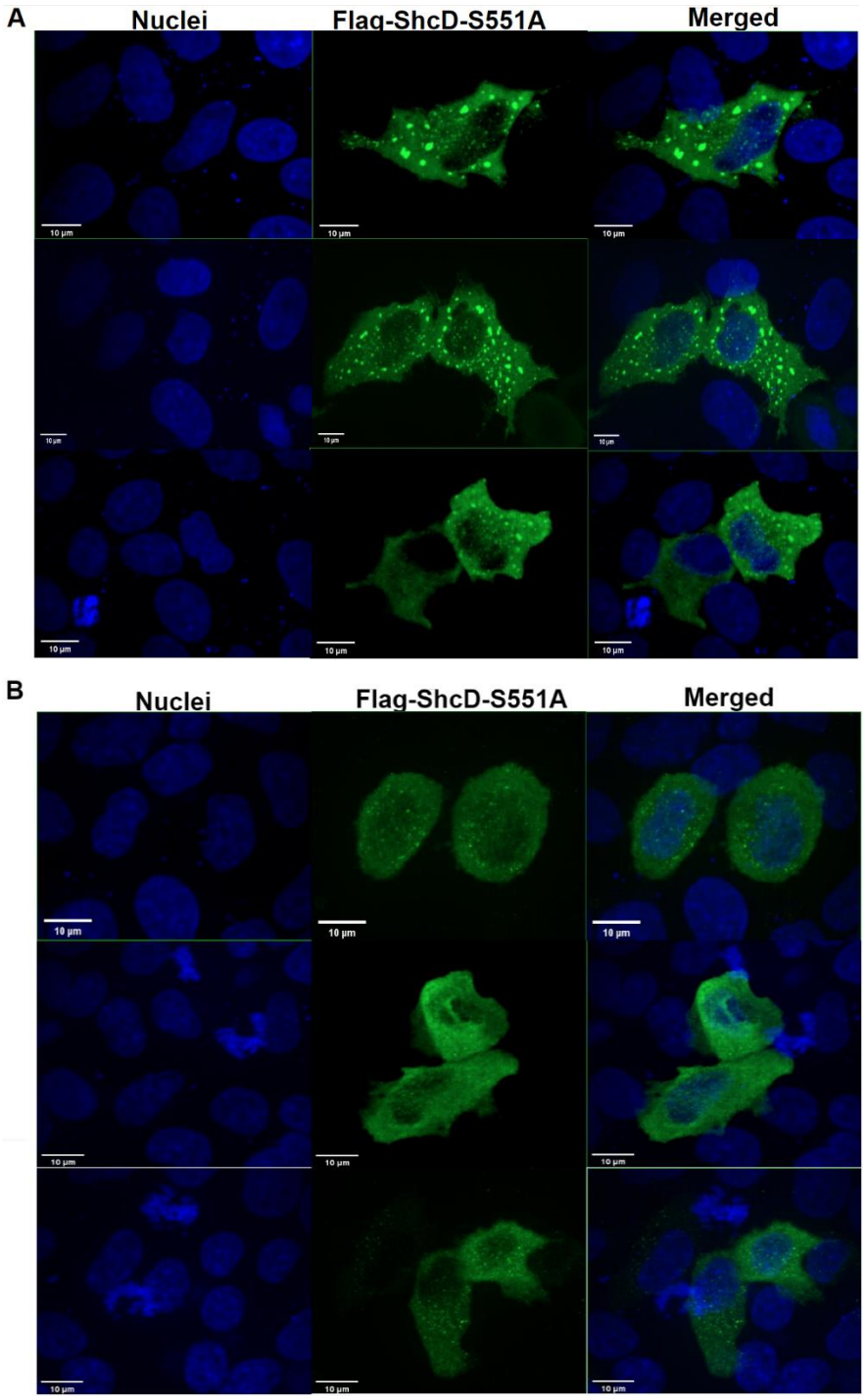

Figure 5.2: Investigating subcellular localisation of the Flag-ShcD-SH2-S551A. The U2OS cells were either transfected with Flag-ShcD-SH2-S551A and then serum starved for 1 hour followed by either stimulating PDGF (15 mins) [B] or left untreated [A]. The stained cells were analyzed by confocal microscopy. The scale bar at the bottom left corner of each image represents 10 μm.

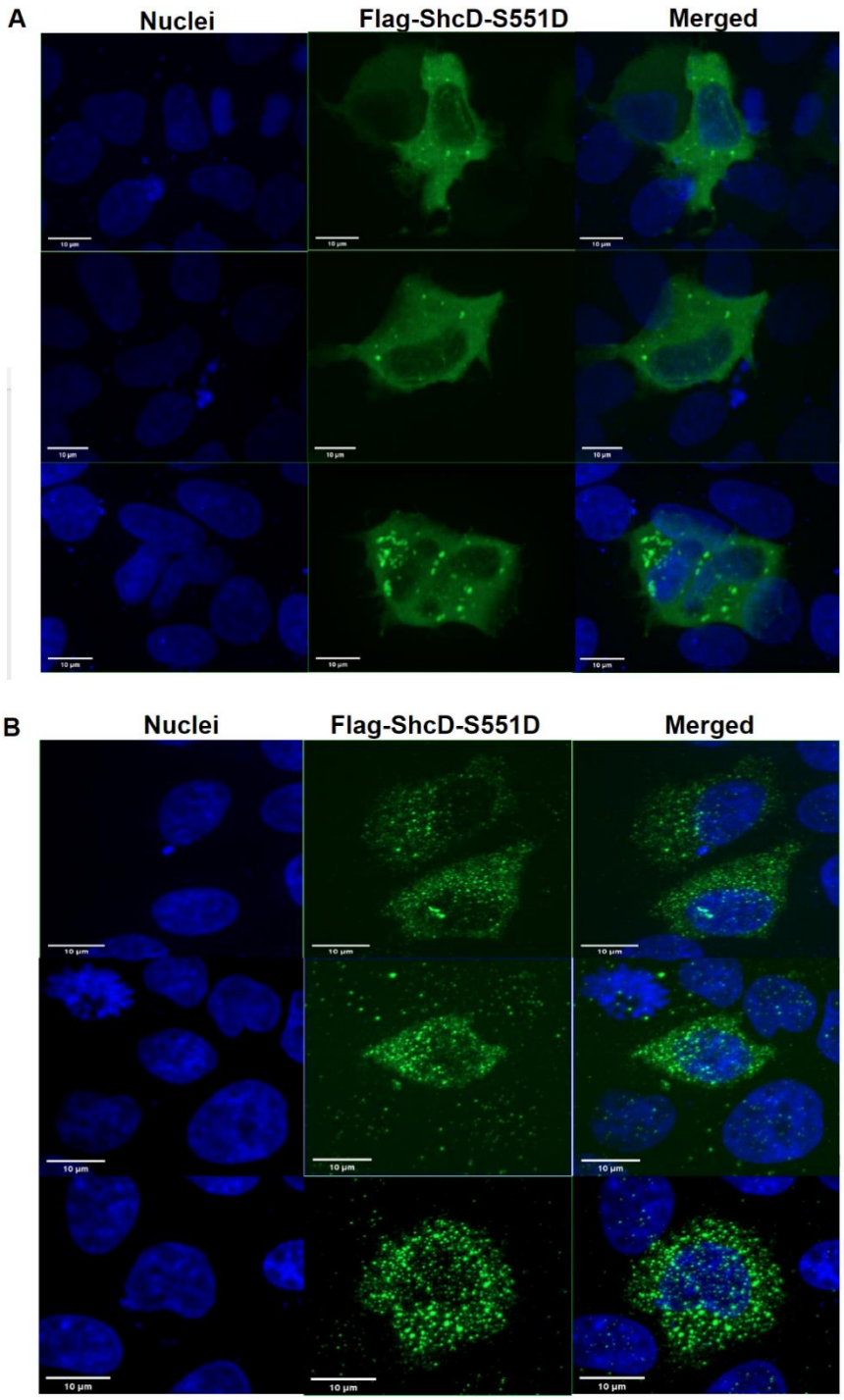

Figure 5.3: Investigating the subcellular localization of ShcD-SH2-S551D. The U2OS cells were either transfected with Flag-ShcD-SH2-S551D and then serum starved for 1 hour followed by either stimulating PDGF (15 mins) [B] or left untreated [A]. The stained cells were analyzed by confocal microscopy. The scale bar at the bottom left corner of each image represents 10 μm.

5.3. Discussion

The ShcD proteins were observed predominately in cytoplasm without much difference regardless of the PDGF presence or not. This is perhaps surprising as I would expect a proportion of ShcD to localise to PDGFR when it becomes phosphorylated. In order to confirm that PDGFR is indeed activated in the U2OS cells I need to perform a time-course experiment and perform western blotting with an anti-phosphotyrosine antibody.

The phosphomimetic (S551D) and loss of function (S551A) ShcD-SH2 domain mutants have a significantly different localization as multiple punctae/dots were observed in the cytosol. The multiple punctae/dots observed in the cytosol are possibly either endosomal compartments or mitochondria. ShcD has previously been reported to accumulate with EGFR in an endosomal recycling compartment (Wills *et al*., 2017). In order to determine whether the structures I observed are re-cycling endosomes I could co-stain with an antibody to Rab11. MitoTracker staining dye could be used to visualize mitochondria in future experiments. P66ShcA has previously been reported in mitochondria (Galimov., 2010). In addition, the multiple punctae/dots possibly protein granules localising as similar immunomicroscopic images were confirmed as the echinoderm microtubule-associated protein like 4 (EML4) ALK fusion oncoprotein localising upon RAS GTPase activation (Tupule *et al*., 2021). It is also possible that both S551A and S551D mutations interfere with interactions with SH2 domain. The further research needs to be done to check whether the S551A mutation interferes with the interaction with Gab1. It may be the case that interactions with the SH2 domain sequester ShcD in the cytoplasm and when these are prevented, ShcD localizes to a distinct compartment. This experiment also needs to have time course PDGF stimulation experiments and western blotting with anti-PY99 antibodies to test whether PDGF is actually stimulating U2OS cells.

The actin cytoskeleton is one of the cytoskeletal systems in mammalian cells along with the intermediate filament network and microtubules. It plays a critical role in

maintaining cell structure and is important for controlling cell migration (Tang and Gerlach., 2017). Cell migration, as part of metastasis process, requires specific alterations in actin skeleton dynamics. Very recently ShcD has been shown to inhibit the Rac1 activation in melanoma cells, and lead to a RhoA dependent increase amoeboid mobility (Aladowicz *et al*., 2020). The structure of the actin cytoskeleton showed no obvious difference between transfected and un-transfected cells in our studies and was not affected by PDGF treatment, despite the known involvement of PDGF in U2OS migration. However, this experiment lacked cell density optimization as U2OS cells were very compacted and overgrown, which may have influenced the cells' ability to respond to PDGF and alter morphology.

Chapter 6

Discussion

6.1. Discussion

This research confirmed that Gab1 protein is a major binding partner of ShcD-SH2 in mammalian cells and phosphorylation at ShcD-SH2-S551 inhibits the binding ability. In addition, there are also other proteins that associate with ShcD-SH2 and those proteins were analysed with mass spectrometry. The mass spectrometry result identified ShcD, SHIP2 and Gab1 as binding partners for ShcD-SH2. The yeast two hybrid assay determined that there is no protein-protein interaction between ShcD and individual ShcD domains which indicates ShcD protein is possibly forming a large protein complex to interact with other ShcD protein. Also, confirmation of SHIP2 interaction with ShcD-SH2 suggests that the ShcD protein is involved in modifying signaling pathways mediated specifically by PI(3,4)P2 produced by the action of SHIP2 on PI(3, 4, 5)P3. . The PKA, PKC and PAK4 can phosphorylate ShcD and possibly play a role in melanoma cell signaling. The ShcD protein predominantly localised at cytoplasm but phosphorylation at S551 and knockout (S551A) have punctae/dots in the cytosol.

Cell signaling via receptor tyrosine kinases mediates communication between cells and involves protein complex formation between proteins containing either SH2, PTB or both domains like Shc adaptor proteins. The ShcD protein is most recently identified and least characterized among Shc adaptor protein family members. The main objective of this project was to investigate protein-protein interactions between ShcD-SH2 and Gab1 and other proteins in mammalian cell with identifying possible involvement in cell signaling pathways.

The result of this project, ongoing research in the lab and along with published research data suggest that ShcD protein is involved in forming a protein complex with other proteins as shown Figure 1.4 and 3.10. Kinases that could potentially phosphorylate this site include PAK4, PKA and PKC. Also, interestingly SHIP2 was identified to interact with ShcD-SH2. Previous studies showed that the ShcD PTB domain binds to a NPXY motif which allows ShcD to interact with EGFR, TrkB, Met, Ret and Musk (Smith *et al*., 2006). However, the biological function of ShcD protein and its involvement in signal transduction is still poorly understood.

The interaction between ShcD-SH2 and Gab1/Gab2 suggests that ShcD protein may possibly play a role in Ras/Erk or PI3K/AKT signaling pathways. The Gab1/2 proteins have been identified to mediate Ras/Erk and PI3K/AKT signaling but more interestingly Gab2 protein shown to amplify the progression of melanoma metastasis via AKT signaling (Ding et al., 2015; Chernoff et al., 2009). Since ShcD protein regulates the cell migration and metastasis of melanoma (Fagiani et al., 2007) it is possible to hypothesize that ShcD protein is possibly involved either Ras/Erk or PI3K/AKT signaling pathways via forming a protein complex. This finding allows to understand involvement of ShcD protein in mammalian cell signaling through Gab proteins as well as melanoma progression. In addition, ShcD can be possible biomarker for melanoma, due to ShcD protein associate with metastatic melanoma and Gab proteins which amplify the melanoma progression. However, further research will require to characterize the ShcD signaling pathway and determine effect of the ShcD interaction with Gab protein in migration such as using wound healing assay.

A mass spectrometry analysis identified that SHIP2 interacts with SH2 domain of ShcD and which it is possible that ShcD protein plays a role in negative feedback via forming a complex with SHIP2. The SHIP2 act as negative regulator of PI3K/AKT signaling by dephosphorylating the 5-position phosphate from PI(3, 4, 5)P3 to form PI(3, 4)P2 (Azzi A., 2020). It is very interesting as this lab have demonstrated interaction between ShcD-SH2 and Gab1/2 by pulldown assay and considered ShcD possibly involved in PI3K/AKT signaling but SHIP2 also interacts with ShcD-SH2 which known for negative modulator of AKT. Those findings suggest that ShcD adaptor protein forming a complicated protein complex that interacting both Gab proteins and SHIP2 to mediate the negative feedback in signaling. In addition, ShcD protein was also pulled down by the ShcD-SH2 domain and identified by mass spectrometry analysis but yeast two hybrid assay revealed that there are no direct interactions between each individual ShcD modular domains nor full length ShcD with ShcD-SH2 domain which indicates that ShcD may require other adaptor proteins such as Gab1/2 to form a higher order of complex to interact each other. It is possible that ShcD interaction with Gab1 and SHIP2 could contribute to activation of Akt, but this has yet to be confirmed. If the interaction does contribute to melanoma invasiveness, then it may be possible to develop inhibitors to prevent interaction. Although protein-protein interaction (PPI) inhibitors have proved challenging to develop, recent progress has seen a number of PPI inhibitors enter clinical trials or be approved for treatment of diseases (Lu *et al*., 2020).

It is not clear whether PAK4, PKA and PKC can phosphorylate ShcD-S551 directly or via activation of other kinases. Firstly, PAK4 is member of the PAK family that regulates many biological functions such as cell migration, adhesion, proliferation and survival but also play a critical role in many different types of melanoma progression via PAK4-CREB axis (Won et al., 2019). Secondly, PKA and PKC are family of protein kinase that both play a critical role in cell signaling and functions such as cell proliferation, migration and apoptosis. The PKC in melanoma activate the Erk/MAPK pathway to induce melanoma proliferation and also involved in

several self-enhanced mitogenic loop via positive feedback mediated PI3K/AKT signaling (Denning., 2012). Those findings indicate that ShcD protein does play a role in mediating biological functions in mammalian cell as PAK4/PKA/PKC regulate the cell signaling and functions. Also, interestingly PAK4 and PKC are known to play a role in melanoma and ShcD protein also has evidence to relate with same type of cancer. However, future experiments should aim at determining direct phosphorylation between PKA/PKC/PAK4 or any other kinases and ShcD SH2 domain. The kinase active assay with phosphor-specific antibody allows to detect the phosphorylation but it will require high specificity antibody.

ShcD can directly interact with Gab1/2 proteins via its SH2 domain upon both EGF and Na_3VO_4 stimulation. I demonstrated ShcD interaction with Gab1/2 in mammalian cells using a pull-down assay (Figure 3.3 and 3.4.A). I also showed that phosphorylation of ShcD-SH2-S551 (S551D mutant) inhibited the ability to interact with other proteins including Gab1/2 (Figure 3.3 and 3.4).

The phosphorylation between PAK4/PKA/PKC and ShcD-S551 was confirmed by western blotting using ps-S551 antibody (Figure 4.5). I also showed that PAK4 may directly phosphorylate to ShcD-SH2 via *in vitro* kinase assay (Figure 4.7). However, this *vitro* kinase assay experiment needs to be refined. The amount of substrate needs to be quantified carefully for introducing same amount of substrate into each reaction. An additional control is required as ShcD-SH2-S551D cannot be phosphorylated in the *in vitro* assay. Furthermore, PKA and PKC also need to be determined whether they can phosphorylate ShcD SH2 domain in *in vitro* kinase assays.

The immunofluorescence analysis of ShcD localisation confirmed that ShcD proteins were predominately localised in cytoplasm (Figure 5.1). However, I expected to see increased membrane localisation in presence of PDGF. The phosphomimetic (S551D) and loss of function (S551A) ShcD-SH2 mutants showed punctae/dots in

the cytosol. Furthermore, PDGF stimulation of cells expressing both mutants interestingly have speckles (Figure 5.2 and Figure 5.3). Those speckles might be either endosomal compartments or mitochondria. To confirm the identity of these structures I need to repeat the experiment with endosomal marker antibody such as Rab11 and MitoTracker staining dye to determine it. ShcD has been reported to sequesters EGFR in a recycling endosomal compartment (Wills *et al*., 2017) and p66ShcA localizes to mitochondria following the oxidative stress (Pinton and Rizzuto., 2008). In addition, those speckles from both S551D and S551A mutations are possibly protein granules forming cluster under RAS GTPase activation (Tupule *et al*., 2021).

In addition, I need to check PDGF stimulation is phosphorylating PDGFR in U2OS cells and whether S551A mutant can interact with Gab1 protein or unable to interact it. The structure of actin cytoskeleton showed no significant difference between un-transfected and transfected cells. Interestingly, I expected to see difference in PDGF stimulation, but actin cytoskeleton was not affected despite the known effect of PDGF stimulation on U2OS cell migration. But again, this experiment needs to repeated as it lacked cell density optimization. The result showed that ShcD protein mainly localised in cytoplasm and phosphomimic (S551D)/knock-out(S551A) have speckles which indicate disruption at ShcD-SH2-S551 affect the biological function or forming protein granules under specific conditions.

In conclusion, although I have confirmed several novel binding partners for ShcD, further research needs to be performed to determine the effect of ShcD interactions on cell signaling and function.

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