REGULATION OF RHOGAP DLC1 BY FAK, PP2A AND MEK/ERK IN CELL DYNAMICS

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DECLARATION

I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Archna Ravi

20 August 2013

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Though I'm the only one getting my name in print for the work done on this thesis, it could not have been completed without the help of others. So, in no particular order, here is a thank you to all those people who in some way or the other helped me get here.

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In the words of Page and Plant

"Leaves are falling all around, It's time I was on my way. Thanks to you, I'm much obliged for such a pleasant stay....Ramble on, now's the time, the time is now, to sing my song."

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SUMMARY

Actin remodelling is essential to many dynamic cellular processes such as morphogenesis, motility, differentiation and endocytosis. These changes are controlled by Rho GTPases that cycle between the active GTP- and inactive GDP-bound forms, which in turn are tightly regulated by guanine nucleotide exchange factors (GEFs), GTPase activating protein (GAPs) and the guanine nucleotide dissociation inhibitor (GDIs). Deleted in Liver Cancer-1 (DLC1), is a bona fide tumor suppressor GTPase activating protein (GAP) acting preferentially on Rho. It is a multi-domain protein, consisting of N-terminal SAM domain, C-terminal START domain and the catalytic RhoGAP domain. This allows for its interaction with diverse cellular proteins, including FAK, Tensins and Talin, all of which are focal adhesion-associated proteins, as well as other scaffolding, regulatory proteins such as 14-3-3, EF1A1, and S100A10. As such, the tumor suppressive function of DLC1 can be mediated in a GAP-dependent or GAP-independent manner. Interestingly, DLC1 also contains a serine-rich region which is a phosphorylation hot-spot and is thought to be modified downstream of several potential kinases such as Akt, RSK and PKC/PKD. Despite all these, the nature of DLC1s activation and inactivation remains largely unknown. Here we elucidate a novel pathway involving the concerted action of Ras/Mek/Erk pathway, Focal adhesion kinase (FAK) and Protein phosphatase-2A (PP2A) to activate DLC1s GAP function. EGF stimulation not only leads to the phosphorylation of DLC1 but also that of FAK to inactivate it, thus allowing PP2A-mediated dephosphorylation at a secondary site on DLC1. This signalling cascade directly affects DLC1s effect on cell spreading and migration, which can be correlated to the reduced RhoA levels.

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LIST OF ABBREVIATIONS

Ala(A): Alanine

Arp2/3: Actin-related protein 2/3

BSA: bovine serum albumin

C-terminus: Carboxy-terminus

Ca^{2+}: Calcium ions

Cdc42: Cell division control protein 42 homolog

DAG: diacylglycerol

DLC1: Deleted in Liver Cancer1

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethylsulfoxide

DNA: deoxyribonucleic acid

DTT: Dithiothreitol

EGF: Epidermal growth factor

ERK: Extracellular signal-regulated kinases

FA: Focal adhesions

FAK: Focal adhesion kinase

FAT: Focal adhesion targeting

FERM: erythrocyte band four.1-ezrin-radixin-moesin

FI: FAK inhibitor

FRNK: FAK-related-non-kinase

FBS: Fetal Bovine Serum

GAPs: GTPase Activating Proteins

GDIs: Guanine nucleotide dissociation inhibitors

GDP:Guanosine Diphosphate

GEFs: Guanine nucleotide exchange factors

GFP: Green Fluorescent Protein

GST: Glutathion S-transferase

GTP: Guanosine Triphosphate

HCC: hepatocellular carcinoma

Hr: hours

IAA: Iodoacetamide

IP: immunoprecipitation

IP3, inositol-3,4,5-triphosphate

IPTG: isopropyl-thiogalactoside

Kb: kilobase

kDa: kilodalton

l: litre

M: molarity, moles/dm3

MAPK: Mitogen-activated protein kinase

mDia: mammalian Diaphanous

MEK: MAPK/ERK kinase

mg: milligram

min: minute

ml: millilitre

MLCK: Myosin light chain kinase

MLCP: Myosin light chain phosphatase

mM: molarity, millimoles/dm3

MW: molecular weight

N-terminus: Amino-terminus

NLS: Nuclear localization signal

OA: Okadaic acid

OD: optical density

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

PC: phosphatidylcholine

PCR: polymerase chain reaction

PD: pull-down

PDGF: Platelet-derived growth factor

PI(4,5)*P*2: phosphatidylinositol-4,5-bisphopshate

PLC: phospholipase C

PP2A: Protein phosphatase 2A

$PP2A_C$: PP2A catalytic domain

PTB: Phosphotyrosine binding

Rac1: Ras-related C3 Botulinum Toxin Substrate 1

RBD: Rho binding domain

RhoA: Ras homologous member A

ROCK: Rho Kinase

rpm: rotation per minute

RPMI: Roswell Park Memorial Institute

SAM: Sterile Alpha Motif

SDS: sodium dodecyl sulphate

Sec: second

Ser (S): Serine

SH2/SH3: Src homology 2/3

START: STAR-related lipid-Transfer

Thr (T): Threonine

Tyr (Y): Tyrosine

U: unit

µg: microgram

µl: microlitre

v/v: volume by volume

w/v: weight by volume

WCL: whole cell lysate

CHAPTER 1

INTRODUCTION

1 Introduction

Cell migration in all multicellular organisms, is a process that is essential starting from development and playing a role in later stages during processes such as immune surveillance and wound healing. Migration is controlled by extracellular cues which direct the movement of the cell. These cues control the process by eliciting a multitude of cellular changes such as actin cytoskeletal reorganization, gene transcription and vesicular transport [Raftopoulou and Hall, 2004]. Not only is cell migration important in physiological processes, it also plays a role in cancer progression. The migratory process is similar in both physiological conditions and cancer. What is different is that in cancer cells the signals activating migration are dominant over the ones controlling its inhibition and it is this imbalance that allow the tumor cells to metastasize [Friedl and Wolf, 2003].

Many signalling pathways are involved in cell migration and small GTPases are one of the key molecules. These molecules are under tight spatio-temporal regulation [Pertz, 2010]. Upon dysregulation, they increase the migratory behaviour of the cells and are also seen to be up-regulated in Epithelial-Mesenchymal Transition (EMT) which is a necessary step for a tumor cell to become invasive [Friedl and Wolf, 2003; Yamaguchi et al., 2005]. In the coming sections we will discuss a sub-family of small GTPases, namely, RhoGTPases their regulation and role in cancer as well as a tumor suppressor which has been identified as a regulator of RhoGTPases.

1.1 Ras Superfamily:

The Ras superfamily of proteins is a group of small guanosine triphosphatases (GTPases). These proteins are similar in their functions and biochemistry to the heteromeric G proteins α subunit but they function as monomeric G proteins [Wennerberg et al., 2005]. This superfamily comprises

of about 150 members in the humans and has orthologues in *Drosophila*, *C. elegans*, *S. cerevisiae*, *S. pombe*, *Dictyostelium* and plants, all of which are evolutionarily conserved [Colicelli, 2004]. The Ras, identified as an oncoprotein in Rat sarcoma, is the founding member of the family that is divided into five subfamilies based on their sequence, structural and functional similarities, namely: Ras, Rho (Ras homology), Ran (Ras-like nuclear proteins), Arf (ADP-ribosylation factor) and Rab (Ras-like proteins in the brain) (Fig 1.1).

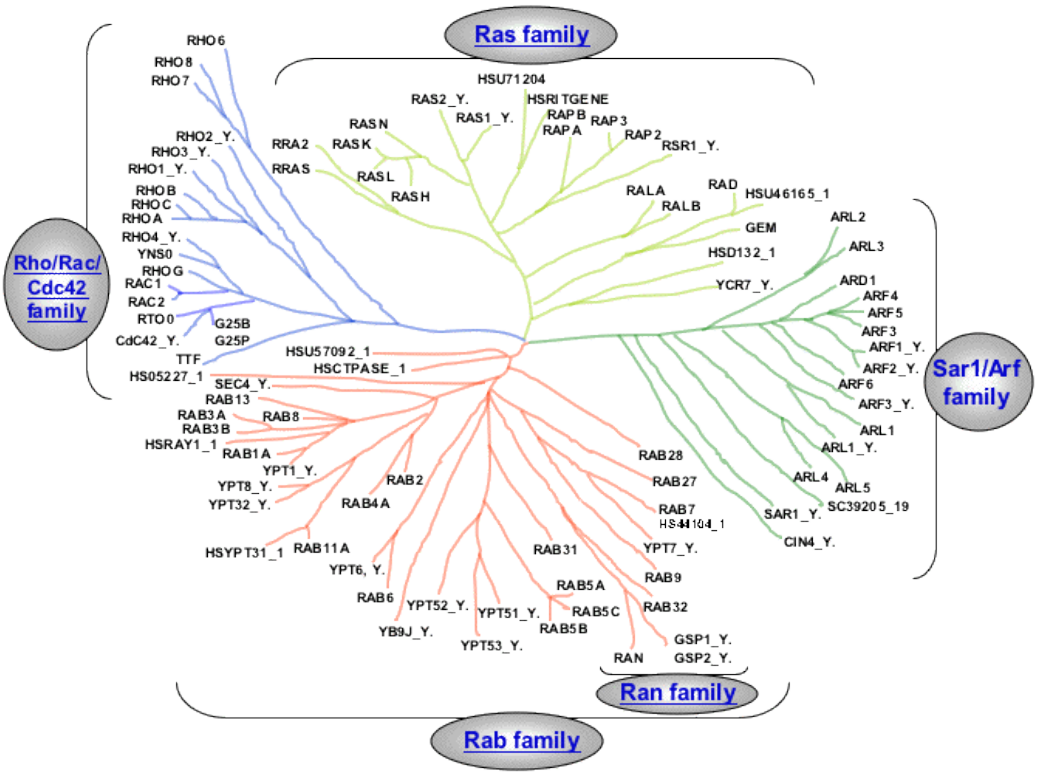

Figure 1.1: Ras superfamily of proteins [Takai et al., 2001]

This group of proteins act as binary molecular switches and based on the structural differences and post-translational modifications, these proteins localize to different sub-cellular compartments, where they exert their functions to regulate a multitude of cellular processes, such as proliferation

and cell survival in the case of Ras, actin-cytoskeleton remodelling by Rho, intracellular vesicular transport and protein trafficking by the Rab and Arf subfamily, nucleocytoplasmic transport RanGTPases and mitochondrial integrity in the case of Miro.

Ras superfamily GTPases, as molecular switches, alternate between GDP-bound and GTP-bound states. The G domain of the superfamily is about 20 kDa and is not only conserved amongst the Ras superfamily but also in Gα and other GTPases. At the N-terminus they have a set of G box with GTP/GDP-binding motifs: G1 (GXXXXGKS/T), G2 (T), G3 (DXXGQ/H/T), G4 (T/NKXD) and G5 (C/SAK/L/T).

1.2 Rho-GTPase family

Rho was initially discovered as a Ras-related protein in 1985 in *Aplysia* [Hall, 2012] and to date about 20 human proteins have identified in this family, with Rho, Rac and Cdc42 being the best characterized [Wennerberg et al., 2005]. The Rho subfamily itself can be further divided into 5 groups: Rho-like, Rac-like, Cdc42-like, Rnd, and RhoBTB [Burridge and Wennerberg, 2004]. To this classification a 6^{th} group, known as Miro can be added, which is an atypical GTPase [Wennerberg and Der, 2004]. Figure 1.2 shows the Rho subfamily of proteins.

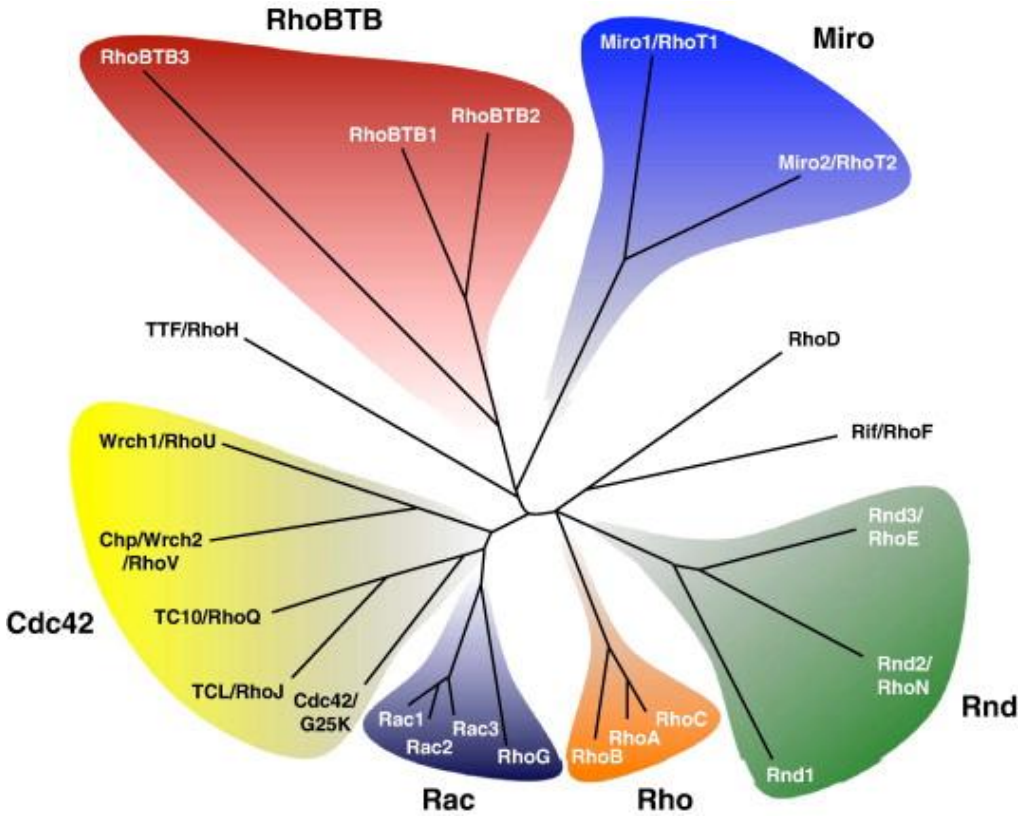

Figure 1.2: Rho subfamily of proteins [Grise et al., 2009].

1.2.1 RhoGTPases: Binary molecular switches

The RhoGTPases like most of the members of the Ras superfamily function as binary molecular switches cycling between the active GTP-bound form and the inactive GDP bound form [Vetter and Wittinghofer, 2001]. Compared to the other members of the Ras superfamily, the RhoGTPases have an insertion of 13 amino acid motif into its G-domain [Wennerberg and Der, 2004]. This G-domain forms a conserved α/β structure, folding into a shallow pocket at the surface to accommodate the guanine nucleotide [Scheffzek and Ahmadian, 2005]. For mediating the binding with the guanine nucleotides, the G-domain contains two switch regions (Switch I and Switch II) and a

phosphate binding loop or the P-loop, which allow for interactions with the γ-phosphates of the guanosine nucleotides [Vetter and Wittinghofer, 2001].

In the "ON" state, GDP gets exchanged for GTP [Vetter and Wittinghofer, 2001] which results in a conformational change in the G-domain as both the Switch I and II regions directly make contact with the γ-phosphate, which presents a binding surface that allows for recognition and binding of the downstream effectors, leading to their activation. Whereas in the "OFF" state, there is an irreversible hydrolysis of GTP to GDP, leading to the release of the γ-phosphate. This leads to a conformational change, releasing the effector proteins which now have reduced affinity for this state [Scheffzek and Ahmadian, 2005; Vetter and Wittinghofer, 2001]. The hydrolysis to bring about inactivation of RhoGTPases is mediated by the intrinsic, albeit slow, GTPase activity of the G-domain. The exchange of the GDP for GTP starts off the next cycle, allowing for a control of the downstream signalling.

Since the RhoGTPases regulate various important cellular processes, there has to be a tight and efficient control of their switching between the two states. For this regulation, there are three classes of molecules, namely: GEFs (guanine nucleotide exchange factors), GAPs (GTPase-activating proteins) and GDIs (guanine nucleotide dissociation inhibitors). Figure 1.3 summarizes the RhoGTPase cycle.

1.2.2 RhoGTPases: Regulators

To ensure signalling specificity and timely turning ON and OFF of RhoGTPase cycle, not only do RhoGTPases themselves get modified but there are other regulators of RhoGTPases as mentioned earlier. There are 82 GEFs, 67 GAPs and 3 GDIs which control the RhoGTPases [Lahoz and Hall, 2008]. Each RhoGTPase can be regulated by multiple GAPs and GEFs, while GAPs

and GEFs are very specific in their function [Wennerberg et al., 2005]. GDI sequesters RhoGTPases in the cytosol, keeping it inactive under quiescent

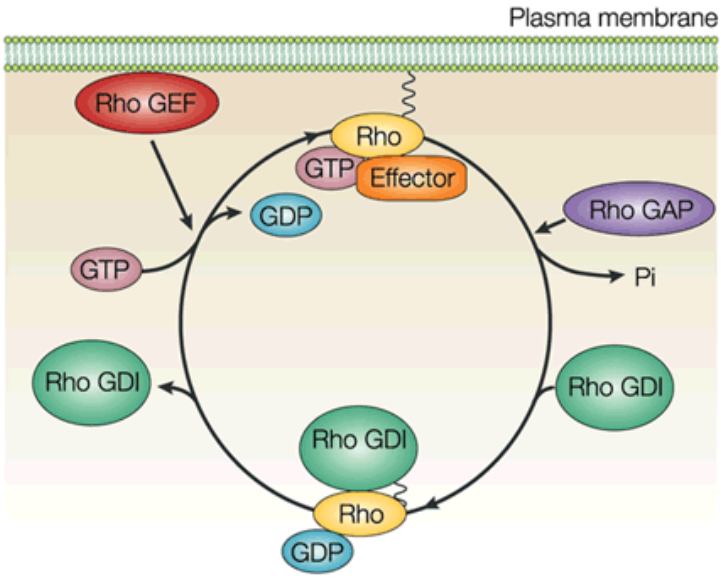

Figure 1.3: RhoGTPase as a binary switch and its regulators [Fukata and Kaibuchi, 2001].

conditions. In the event of a stimulus, GDIs release the RhoGTPases, leading to interaction with GEFs which catalyse the activation by allowing for exchange of GDP for GTP. To enhance the intrinsic GTPase function and attenuate the signal, GAPs get recruited to the RhoGTPase. This GDP-bound RhoGTPase now binds the GDI again and remains in the cytosol until the next round of signalling events start off the cycle [Tcherkezian and Lamarche-Vane, 2007].

1.2.2.1 Rho GDI

There are three GDIs expressed in humans namely, RhoGDI-1, -2 and -3. While RhoGDI-1 is ubiquitous in its expression pattern, RhoGDI-2 is hematopoietic-specific and RhoGDI-3 are found to be expressed only in the testis, lung and brain [DerMardirossian and Bokoch, 2005]. The switch region of RhoGTPase get prenylated, leading to its binding with GDI, which leads to the sequestration of RhoGTPase in the cytosol by preventing the C-terminal lipid modifications needed for its translocation to the plasma membrane [Seabra and Wasmeier, 2004]. GDIs carry out their inhibition of RhoGTPase function in three main ways. First of all, they prevent the dissociation of GDP from RhoGTPases by inhibiting the activation by GEFs. Next, they interact with the GTP-bound form, preventing the action of the internal as well as GAP mediated hydrolysis, interrupting the active binding with the downstream effectors. Finally, they modulate the RhoGTPase translocation from the cytosol to their site of action [DerMardirossian and Bokoch, 2005].

GTPases, to a large extent, exist in their inactive form as suggested by the existence of comparable amount of GDIs in relation to the RhoGTPase concentration in the cells. Hence, GDI are a key regulation component of the GTPase functions [Michaelson et al., 2001].

1.2.2.2 Rho GEFs

GTP hydrolysis to GDP is an irreversible step. For the next round of activation, GDP has to be dissociated from the RhoGTPase before it can be loaded with GTP, making this the rate limiting step in the RhoGTPase cycle [Erickson and Cerione, 2004]. GEFs catalyse this step, allowing the timely activation of the RhoGTPases. In humans, there are 85 GEFs present. These are activated downstream of growth factor receptor stimulation. They are

usually found as a part of signalling complexes brought together by scaffolding proteins, allowing for specificity [Bos et al., 2007]. The affinity of RhoGTPase is the same for GTP and GDP and GEF does not work by favouring the binding of either over the other. Instead, GEFs function by modifying the nucleotide binding site that consists of the two switch regions and the P-loop, weakening the affinity of that site to bind nucleotide. This exchange is also mediated by the fact that the affinity of the binary complex (GTPase for either the nucleotide or the GEF) is much higher than the affinity of the ternary complex (GEF for a nucleotide-bound G protein or nucleotide for a GEF-bound G protein). Hence, the nucleotide gets displaced upon GEF binding to the GTPase and the replacing nucleotide displaces the GEF from it. Since, GEF does not favour the binding of either GDP or GTP, the GTP loading on the GTPase is determined by the fact that there is ten times higher concentration of GTP in the cell [Bos et al., 2007; Vetter and Wittinghofer, 2001].

GEFs are also in turn controlled by regulatory mechanisms which control their translocation to site of GTPase regulation, removal of the auto-inhibition and bring about changes in their catalytic domain. Factors that usually control this are: post-translational modifications, interaction with second messengers, other proteins as well as lipids [Bos et al., 2007]. GEFs have been identified as oncogenes, not surprisingly, as they up-regulate RhoGTPases which have a role to play in cancer [Hall, 2005].

1.2.2.3 Rho GAPs

Since the intrinsic GTPase of the G protein is extremely slow to be able to catalyse the hydrolysis of GTP to GDP by itself, it requires RhoGAP as a catalyst. The GAPs mainly function by stabilizing the intrinsic, highly mobile, catalytic domain of the GTPases as well as inserting a catalytic

residue *in trans*. GAP mediated hydrolysis is composed of various steps, which involves orienting the water molecule for a nucleophilic attack [Vetter and Wittinghofer, 2001], obstructing the water from entering the active site as well as stabilizing the transition state [Bos et al., 2007]. GAPs contain a conserved arginine residue, known as the arginine finger, that causes the neutralization of the negative charge on the γ-phosphate, which stabilizes the transition state [Rittinger et al., 1997]. Mutating the arginine residue renders the GAP inactive, demonstrating the importance of the residue to GAP function. Also, the stabilizing of the glutamine 61 residue by the GAPs, allows for the optimal positioning for the attack by the water molecule [Scheffzek and Ahmadian, 2005]. Also, this restricts the movement of the water molecule lowering the energy barrier for hydrolysis of GTP [Nassar et al., 1998].

GAPs are regulated by mechanisms similar to that of GEFs via binding of secondary messengers, other proteins and lipids and post-translational modifications [Bos et al., 2007]. GAPs act as tumor suppressor since they function to inhibit RhoGTPase mediated cellular processes. They are seen to be more frequently mutated in cancers as compared to the GEFs.

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1.2.3 Rho GTPases: Downstream effectors

Conformational change brought about by the activation of RhoGTPases, leads to binding of the downstream effector targets, which in turn get activated to bring about cellular changes. Of the 23 Rho family proteins known, the best studied ones are Rho, Rac and Cdc42. There are over 50 effectors that have been identified for them, including serine/threonine kinases, tyrosine kinases, lipid kinases, lipases, oxidases and scaffold proteins [Jaffe and Hall, 2005]. The most common mechanism of activation by RhoGTPases is by disrupting the intramolecular autoinhibitory interactions in the effector proteins. Also, it is seen that quite a few of the effector proteins of

RhoGTPases contain a coiled-coil region which allows for oligomerization [Bishop and Hall, 2000].

The main target proteins for Rho are serine/threonine kinases like ROCK (Rho Kinase) and scaffold proteins (Dia). Through their coiled-coil regions, these effectors recognize and bind active Rho [Bishop and Hall, 2000]. Rho-mediated downstream functions mainly include actin cytoskeletal reorganization. The Ser/Thr kinases, ROCK1 and ROCK2, are both ubiquitously expressed, and have 64% identity with their kinase domains having the maximum similarity. ROCK promotes Rho-mediated increase in cellular contractility by cross-linking actin and myosin. Effect of ROCK on the actin and myosin leads to changes in cell motility, adhesion, smooth muscle contraction, neurite retraction, and phagocytosis [Riento and Ridley, 2003]. mDia is a formin molecule that promotes the actin nucleation and polymerization to form elongated actin filaments [Narumiya et al., 2009]. The coordination between ROCK and mDia leads to actin reorganization downstream of Rho [Watanabe et al., 1999].

Rac and Cdc42 on the other hand seem to have common effectors. This can be attributed to about 70% sequence identity amongst them. The downstream effectors of the these two have a common motif known as CRIB (Cdc42/Rac-interactive binding) motif [Hakoshima et al., 2003]. This motif was initially identified in p21-activated protein kinase 1 (PAK1) which belongs to the PAK family and regulate cell motility, cell survival and cell cycle progression [Bishop and Hall, 2000; Bokoch, 2003]. Crystal structures of activated Cdc42-associated kinase (ACK), Wiskott-Aldrich syndrome protein (WASP) and partition-defective protein (Par6) and PAK1 have shown that the CRIB motif is important for interaction with the RhoGTPases and it forms an intermolecular β-sheet with Rac and Cdc42 [Hakoshima et al., 2003]. WAVE2 belongs to the Wiskott–Aldrich syndrome protein (WASP) family, which consists of the WASP subfamily and the WAVE subfamily (WASP family verprolin-homologous protein). These are scaffold proteins which

directly bind to and activate the Arp2/3 complex, leading to actin polymerization and filopodia formation [Takenawa and Suetsugu, 2007].

1.2.4 RhoGTPases: Cellular functions

The RhoGTPases induce, in response to external stimuli, a cascade of synchronized changes in the actin cytoskeleton and the transcription to bring about various changes at the physiological level. These changes include morphogenesis, chemotaxis, vesicle transport, cell polarity, axonal guidance, and cell cycle progression and upon dysregulation are seen to play a role in oncogenesis.

1.2.4.1 Cell cycle regulation

In the cell cycle, the G1-S phase progression, mitosis and cytokinesis are all in some way or the other controlled by RhoGTPase activity. G1-S progression depends on the regulation of cyclin and Cdk inhibitors. Cyclin concentrations are affected by maintaining the levels of ERK and by extracellular matrix proteins. Rho proteins act at this level to regulate this phase. The RhoGTPases also trigger the transcription of cyclin D and activate the Serum Response Factor (SRF). They are important for serum-induced G1 progression as well as Ras-induced cell transformation [Hall, 1998; Jaffe and Hall, 2005]. RhoGTPases also regulate the levels of $p21^{cip1}$ and $p27^{kip1}$, which are Cdk inhibitors. Mitosis and cytokinesis regulation is affected by the ability of RhoGTPases to act on the cytoskeletal components [Jaffe and Hall, 2005]. Rho and Cdc42 also play a role in the formation of the actomyosin contractile ring in the late stages of the cell cycle [Hall, 1998].

1.2.4.2 Cytoskeletal dynamics and cell movement

The activity of RhoGTPases on the actin cytoskeleton is conserved in all eukaryotes. Involvement of RhoGTPases in cytoskeletal dynamics was first seen when Rho and Rac, in response to stimulus regulated the actin assembly and organization. In response to lysophosphatidic acid (LPA) or integrin engagement, Rho leads to the formation of stress fibers and focal adhesions. Rac on the other hand, in response to platelet-derived growth factor (PDGF) or insulin, forms lamellipodia and membrane ruffles by the virtue of promoting assembly of the peripheral actin network, while response from Cdc42 was elicited upon stimulation by bradykinin and interleukin 1 (IL-1), leading to the formation of filopodia, by actin bundling at the cell periphery. Rho brings about changes in the actin cytoskeleton through its interaction with ROCK and mDia. ROCK in turn phosphorylates myosin light chain phosphatase (MLCP) to inactivate it and hence ensuring phosphorylation of myosin by myosin light chain kinase (MLCK). This leads to actin-myosin cross-linking, triggering cell contraction. Rac and Cdc42 exert their influence by activating Arp2/3 through their interactions with WAVE and WASP respectively, which leads to the elongation of the peripheral F-actin generating a meshwork [Hall, 2012; Nobes and Hall, 1995; Ridley and Hall, 1992].

The dynamic rearrangement of the cytoskeleton drives cell migration. The coordinated effect of all three Rho GTPases is required to bring about the changes at the front and the rear end of the cell for a directed cell movement. Cdc42 determines the polarity of the cells by sensing the extracellular cues, and the direction of the cell movement. It also determines the regions of Rac accumulation. At the leading edge, Rac, by forming the membrane protrusions drives the forward movement of the cell. Rho, at the rear of the cell induces stress fibre formation causing cell body contraction, which also allows the cell to move forward. Apart from these changes, both the cell-cell adhesion as well as cell-matrix adhesions determine cell migration and are also regulated by the

RhoGTPases [Fukata et al., 1999]. In cell-matrix adhesions, Rho is required for the assembly of integrin-based focal complexes. Rho GTPases also regulate the formation and maintenance of specialised cadherin-based junctional adhesion complexes known as the tight junctions and adherens junctions. Formation of cell-matrix adhesions allows the progression of cell migration, whereas the cell-cell junctions inhibit the cell migration. Apart from controlling the actin dynamics, Rho GTPases also influence microtubule dynamics by regulating the microtubule plus end-binding proteins [Hall, 2012; Jaffe and Hall, 2005; Malliri and Collard, 2003].

1.2.5 Rho GTPases: Cancer

RhoGTPases are involved in various stages of tumorigenesis. It has been shown that Rho proteins not only regulate the cytoskeletal reorganization and hence cell morphology, but also have potent effect on cell proliferation, gene expression and apoptosis. These effects are generally mediated by over-expression of the proteins and in some cases point mutations and alternatively spliced form [Fritz et al., 2002]. This can lead to aberrant RhoGTPase signalling. Also, activated Rho mutants can independently transform cells albeit to a lesser degree as compared to the Ras mutants. They also co-operate with Raf for this purpose. The deregulation of RhoGTPases has been shown to correlate with poor cancer prognosis. As RhoGTPases are important regulators of cell migration, their deregulation will lead to metastasis in tumor cells. This also leads to loss of polarity in migrating cells and they are probably one of the factors involved in EMT. RhoGTPase dysregulation can also lead to breakdown of the cell cycle as they control CDKs which in turn control the cell cycle. Cancer cells do not have apoptotic properties and there

is evidence of Rho-proteins being involved in anti-apoptotic pathways. [Sahai and Marshall, 2002].

1.2.6 Rho GAP-containing proteins are critical regulators of diverse cellular activities

The RhoGAP family of proteins is defined by the presence of a GAP domain with activity towards RhoGTPases. It consists of 150 amino acids and shares 20% sequence identity with other GAP domains in the family. There are about 70 RhoGAPs that have been identified in the humans, compared to only 20 RhoGTPases. The high number of RhoGAPs suggests that each GAP has a very specific function and is under a very tight spatial and temporal regulation. This domain consists of nine alpha helices as well as the highly conserved "Arginine finger" that is the key to its GAP function [Moon and Zheng, 2003].

Apart from the GAP domain, RhoGAP proteins contain various other domains that help in determining their subcellular location and interacting partners. Some of the well-characterized domains are Src Homology 2 and 3 (SH2 and 3) domains that allow for protein-protein interactions, pleckstrin homology (PH) and bin-amphiphysin-rvs (BAR) domains which are lipid interaction domains and allow GAPs to be targeted to the membranes. These domains also serve as scaffolds for protein complex formation [Bos et al., 2007]. RhoGAPs also contain other catalytic domains which make them points of convergence or divergence in the RhoGTPase cycle. For example, they may contain a GEF domain, which allows for simultaneous regulation of different Rho family members [Chuang et al., 1995]. Some GAP domain-containing proteins have no known RhoGAP function, in which case they might just simply serve as a RhoGTPase binding domain like in the case of p85 [Zheng et al., 1994].

1.2.6.1 Mechanisms of Rho GAP regulation

Because the RhoGAPs outnumber their downstream effectors, they are under a strict regulation, both spatially and temporally to ensure that the RhoGTPases are not perpetually in an inactive state. Phosphorylation, protein-protein interaction, phospholipid binding and proteolytic degradation are the main events that regulate RhoGAP function.

Protein-protein interaction: The RhoGAPs have various protein interaction domains, which regulate their GAP function by either activating them or inactivating them. Examples of the interaction inactivating GAP activity are the binding of intersectin, a scaffold protein, to CdGAP and TCGAP with Fyn Kinase (Moon and Zheng, 2003; Jenna *et al.*, 2002). On the other hand, interaction of RA-RhoGAP with Rap1 activates the GAP function by removing the auto-inhibition (Yamada et al 2005). The protein interaction can also be for the purpose of targeting the GAP to a particular subcellular location without affecting the GAP function as is the case with p120RasGAP and p190RhoGAP (Bradley *et al.*, 2006). Interaction of the Ras/Rap1-associating (RA) domain in RA-RhoGAP with Rap1 was also found to release the Rho GAP from auto-inhibition, thereby inducing GAP activity (Yamada *et al.*, 2005).

Phospholipid-binding: The association of Rho GAPs with phospholipids usually leads to the translocation of the RhoGAP to the plasma membrane, bringing it in contact with the RhoGTPase it exerts its function on like in the case of phosphatidylinositol (3, 4, 5)-triphosphate (PIP3) interaction with ARAP3 RhoGAP (Krugmann *et al.*, 2004). Also, as phospholipids are associated with growth factor signalling, binding of RhoGAPs to them could lead to RhoGAP regulation by growth factors (Bernards and Settleman, 2005).

Phosphorylation: Phosphorylation is a common regulatory event seen in proteins and RhoGAPs are no exception. In p190RhoGAP, phosphorylation by insulin growth factor receptors led to its localization from the cytosol to the plasma membrane (Sordella *et al*., 2003). Whereas in MgcRacGAP, a Rac1/Cdc42 GAP, serine phosphorylation changes its effector specificity to RhoA (Lee *et al.*, 2004). Deleted in Liver Cancer1 (DLC1), a GAP for RhoA, gets inactivated upon phosphorylation by Akt [Ko et al., 2010b].

Proteolytic degradation: Rho GAPs can be regulated temporally through proteolytic degradation which controls its turnover rate. Levels of p190RhoGAP determine the cytokinesis completion. These levels are in turn determined by ubiquitin-mediated degradation of the RhoGAP (Su *et al*., 2003). Another RhoGAP, DLC1 was seen to be susceptible to degradation by the 26S proteasome [Luo et al., 2011]. DLC1 is the protein of interest in our study and will be discussed in detail in the following sections.

1.2.6.2 RhoGAPs: Effects on cellular processes

With RhoGTPases regulating cell processes such as trafficking, endocytosis, cell growth and differentiation and cytoskeletal dynamics, RhoGAPs are bound to influence these processes as well by the virtue of their control of RhoGTPases. RLIP76, a Rac/Cdc42-GAP domain containing protein is important for RalGTPase mediated endocytosis, by acting as a link between growth factor receptor signalling and protein involved in endocytosis [Jullien-Flores et al., 2000]. BPGAP1, a novel GAP identified by our group showed that upon interaction with EEN/endophilin II activate ERK signalling via EGFR mediated endocytosis [Lua and Low, 2005]. It was also shown that the BNIP-2 and Cdc42GAP Homology (BCH) domain of BPGAP1, via its interaction with K-Ras induces PC12 cell differentiation [Ravichandran and

Low, 2013]. BPGAP1 promotes cell migration through a concerted action of its BCH, proline-rich and GAP domains as well as its binding with Cortactin, a cortical actin binding protein, leading to its translocation to cell periphery [Lua and Low, 2004; Shang et al., 2003]. p190RhoGAP has been seen to play a role in axon outgrowth, guidance and fasciculation, and neuronal morphogenesis. Also, p190B RhoGAP, one of the p190RhoGAP isoforms affects cell growth and differentiation, an effect seen by the reduction in size of mice thymus in the absence of p190B. MgcRacGAP mediated down-regulation of Cdc42 also affects cell growth by affecting the spindle formation in cytokinesis [Moon and Zheng, 2003]. DLC1 also affects cell migration and brings about change in cell morphology by reducing the stress fiber formation via its activity on RhoA [Kim et al., 2008].

1.2.6.3 RhoGAPs: Tumorigenesis

RhoGTPases' role in tumorigenesis and progression, and RhoGAPs'role in suppression of RhoGTPases, logically places RhoGAPs under the class of tumor suppressors. Indeed, RhoGAPs have been seen to be down-regulated or deleted in various cancers. Our protein of interest, DLC1 was seen to deregulated in many tumors as well as the gene was deleted in more than 40% of Hepatocellular carcinoma (HCC) in which it was initially identified. Since then, work done has found loss of DLC1 correlated to various cancers other than HCC [Lahoz and Hall, 2008]. One of the first identified RhoGAPs BCR is seen to undergo chromosomal translocation to form a fusion protein with Abl, an oncogene in leukaemia. GRAF, an FAK-associated RhoGAP, has also been identified in leukaemia undergoing translocation. With cancers showing a loss of RhoGAPs, experiments were done to see if the overexpression of these proteins could reverse the effect. True to the expected role as tumor suppressors, overexpression of p190RhoGAP repressed Ras-

induced transformation in NIH3T3 fibroblast cells [Moon and Zheng, 2003]. p85-alpha subunit of PI3-Kinase has a RhoGAP domain which is seen to suppress metastasis in ovarian cancers.

1.3 Deleted in Liver Cancer-1: A RhoGAP and a Tumor suppressor

Of approximately 70 GAPs that are expressed by the human genome, only a few of these are seen to localize at the focal adhesions. Deleted in Liver Cancer1 (DLC1) is one such RhoGAP. DLC-1 transcript is 3850bp and translates to about a 125 kDa protein. It was identified in a representative difference analysis (RDA) screen as being absent in Hepatocellular Carcinoma (HCC) tissues as compared to being ubiquitously present in the non-cancerous tissues [Yuan et al., 1998].

DLC1 has since then been characterized as a bona fide tumour suppressor. 8p21.3-22, location of DLC1 on the chromosome, is a region that is subjected to high frequency of Loss of Heterozygosity (LOH). Though initially identified to be deleted in HCC, this loss now extends to various cancers including prostate, lung, breast, colon, bladder and head and neck [Lahoz and Hall, 2008]. mRNA down-regulation of DLC1 is a key feature in many of these cancers and this can be attributed to epigenetic silencing mechanisms like DNA hypermethylation and histone acetylation apart from the frequently observed LOH [Durkin et al., 2007a]. Recently, somatic mutations of DLC1 have also been identified in prostate cancer. Initial experiments demonstrated DLC1's ability to inhibit tumorigenicity in nude mice and cell growth, when carcinoma cell lines lacking DLC1 were transfected with DLC1 cDNA. [Ng et al., 2000; Yuan et al., 2004; Zhou et al., 2004]. Also, studies done in breast cancer lines indicated that the metastatic

potential could be correlated with the expression levels of DLC1 [Goodison et al., 2005].

DLC1 is a GTPase-activating protein (GAP) protein with *in vitro* activity for the small GTPases RhoA, RhoB, and RhoC, and to a lesser extent Cdc42 [Healy et al., 2008; Wong et al., 2003]. It is a multi-domain protein that contains an amino-terminal sterile α motif (SAM), a RhoGAP domain, a serine-rich unstructured region between the SAM and the RhoGAP domain and a StAR (steroidogenic acute regulatory)-related lipid transfer (START) domain at its carboxy-terminus. It belongs to a family of protein which contain the SAM-RhoGAP-START domain architecture [Durkin et al., 2007b]. DLC2 and DLC3 belong to this family. The genes encoding these three proteins are paralogues of each other, which arose by gene duplication [Durkin et al., 2007a].

1.3.1. DLC1 Domains and their functions:

1.3.1.1 SAM domain

SAM domain at the N-terminal of DLC1 is about 70 amino acids. The human genome contains about 200 proteins that contain the SAM-domain [Qiao and Bowie, 2005]. This motif has been seen to occur in many other proteins including transcription factors and signalling molecules. The tertiary

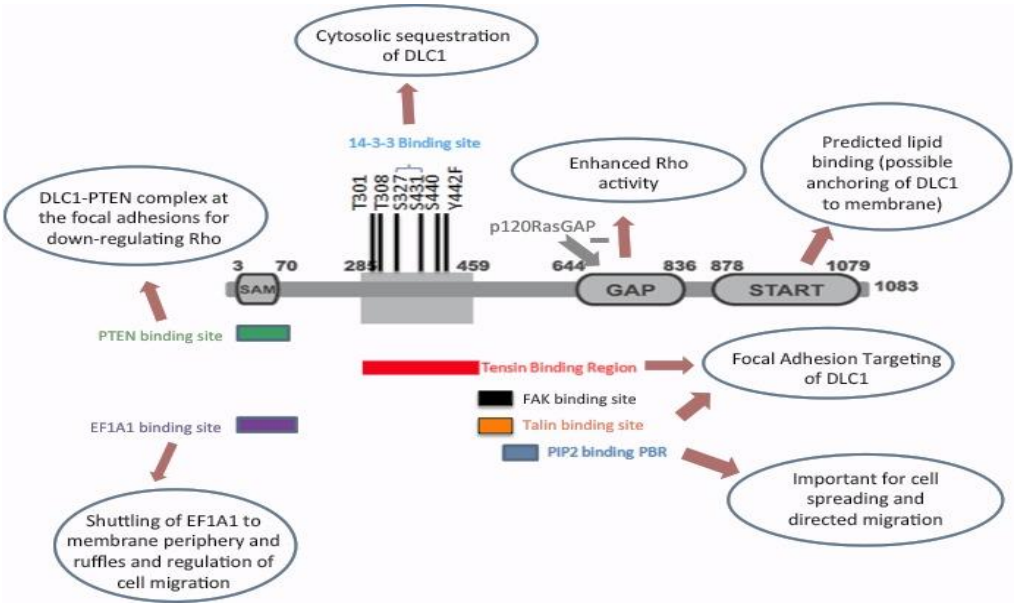

Fig 1.4: DLC1 domain architecture and its interactome (Courtesy: Shelly Kaushik)

folds in this domain are similar across the different range of proteins. SAM domain has a globular tertiary structure formed by folding of amino acids into 5 alpha helices, encasing a hydrophobic core. Despite the structural similarity in the proteins, proteins containing SAM domain have a global cellular distribution with varied interacting partners, giving the proteins diverse and unique functions [Kim and Bowie, 2003; Qiao and Bowie, 2005]. These are mainly involved in protein-protein interactions with SAM domain-containing proteins, which may be homo- and heterotypic in nature, as well as, with other proteins which do not have the SAM domain, leading to the formation of dimers, oligomers and polymers [Durkin et al., 2007b]. Apart from this they have also been shown to interact with lipids, like in the case of SAM domain of p73, a p53 homologue and RNA, in the case of Smaug and its homologue, which are translational repressors [Kim and Bowie, 2003].

So far, the work done on SAM domain of DLC1 has shown that it might not be necessary for DLC1's GAP-dependent functions. Transfection of SAM domain alone was unable to induce morphological changes in the cell,

namely cell rounding, bring about the dissolution of the actin stress fibres or inhibit colony forming ability of HCC cells, which are characteristic of DLC1 expression [Wong et al., 2005]. Also, SAM domain alone does not localize to the focal adhesion [Kim et al., 2008]. This observation was complemented by a previous observation which showed that over-expression of a DLC1 mutant lacking SAM domain, exhibited behaviour similar to that of wild-type DLC1. This confirms that SAM domain is not necessary for DLC1s GAP-mediated function or its tumor suppressive activity [Wong et al., 2005]. Work done by Kim et al. 2008, shows that SAM domain might possibly be a negative regulator of DLC1 GAP activity, by the means of auto-inhibition. Introducing DLC1 lacking SAM domain showed a reduction in the directionality of cell movement and induced a more drastic morphological change in the cells when compared to the wild type DLC1, as this mutant probably has constitutively active GAP function.

Recently, eukaryotic elongation factor 1A1 (EF1A1) was identified as a potential binding partner of DLC1s SAM domain. Upon growth factor stimulation, EF1A1 interacts with DLC1 leading to its localization at the membrane periphery and ruffles, which regulates cell migration [Zhong et al., 2009]. Migratory ability of breast cancer cells were also seen to be affected by the interaction of the SAM domain with PTEN (phosphatase and tensin homologue), a tumor suppressor [Heering et al., 2009].

An NMR study done to resolve the structure of DLC1 SAM revealed a surprising fold in the SAM domain. It showed that DLC1 has a monomeric four α-helical structure unlike the five α-helical bundles usually seen in SAM domains of other proteins. This is similar to the structure of DLC2 SAM domain [Yang et al., 2009a].

1.3.1.2 RhoGAP domain

RhoGAP domain of the DLC family of proteins is the most conserved domain amongst them, with about 70% sequence identity [Durkin et al., 2007b]. This is the functional domain of DLC family, which enhances the intrinsic GTPase activity of Rho proteins, thus regulating their cycling between the active and the inactive state. DLCs have a conserved "arginine finger" arginine residues, namely R677 and R718 in humans, which are vital for the RhoGAP function. The loop containing this residue lends a positive charge to the catalytic site of Rho, which allows the glutamine residue present there to be stabilized in a proper conformation [Li and Zhang, 2004]. This conformation makes it susceptible to nucleophilic attack by water molecule leading to hydrolysis of the γ-phosphate of the GTP [Bos et al., 2007].

It has been seen that DLC1 has *in vitro* GAP activity which is specific for RhoA, RhoB, RhoC and to a lesser extent towards Cdc42 and does not show any effect on Rac1 [Healy et al., 2008; Wong et al., 2003]. This was consistent with the observation that p122RhoGAP also showed *in vitro* GAP activity for RhoA but not Rac1 [Sekimata et al., 1999]. The *in vitro* and *in vivo* substrate specificity of the various GAPs differ from each other [Moon and Zheng, 2003]. Even though it was not conclusively said, data showed that overexpression of DLC1 and its rat homologue p122RhoGAP results in the loss of actin stress fibers, which is due to the possible down-regulation of RhoA [Sekimata et al., 1999; Wong et al., 2005]. This is confirmed by overexpression of constitutively active RhoA reverses the loss of actin stress fibers brought about by p122RhoGAP [Sekimata et al., 1999]. Introduction of GAP-inactive mutants of DLC1 and p122RhoGAP or mutants lacking the GAP domain had no effect on the cellular morphology or the actin cytoskeleton [Sekimata et al., 1999; Wong et al., 2005]. Also, active RhoA at the leading edge of protrusions of migrating cells was drastically reduced upon

ectopic expression of DLC1 [Healy et al., 2008]. Furthermore, RhoA knockdown in two independent experiments with murine hematomas which lacked DLC1resulted in suppressed tumor growth proved that hyperactivation of RhoA upon loss of DLC1 was a key factor in tumorigenesis [Xue et al., 2008].

The effect of DLC1 GAP domain on RhoA activation was confirmed by the use of RhoA-Raichu biosensor. It was seen that wild-type DLC1 but not the GAP-negative mutant was responsible for a decrease in the emission ratio of the biosensor during fluorescence resonance energy transfer (FRET), which is in response to the hydrolysis of RhoA-GTP to RhoA-GDP [Holeiter et al., 2008]. Also, RhoA mediated change in the actin cytoskeleton was affected by expression of wild-type DLC1 and not GAP-inactive mutants. Hence the rounding up of cells, cortical retraction and other cytoskeletal changes can be attributed to the GAP activity of DLC1 [Wong et al., 2005; Yuan et al., 2007]. Actomyosin contractility is controlled by phosphorylation of myosin light chain (MLC2) by Rho Kinase (ROCK), a RhoA effector. Wong et al (2008) showed that the deregulation of this pathway leading to the dissolution of the stress fibers and disassembly of the focal adhesions was a GAP dependent function of DLC1. The Rho/ROCK/MLC2 regulation by DLC1 GAP function was confirmed with the increase in local RhoA activation, which lead to the strengthening of the focal adhesions as well as the actomyosin contractility, upon DLC1 displacement from the focal adhesions [Wong et al., 2008]. As a consequence of DLC1 mediated decrease in actomyosin contractility, HCC cells' capability to migrate and metastasize was greatly impaired [Kim et al., 2008]. Another example of DLC1s specificity to Rho is the inability of DLC1 to affect the formation of actin protrusions via Rac1 dependent pathway forming actin-related protein 2/3 (Arp 2/3) actin nucleation complex [Kim et al., 2008]. Evidence of direct modification of GAP domain of DLC1 was seen recently when S807 was identified as a target for Protein Kinase D (PKD). The authors showed that a phospho-defective mutant S807A inhibited colony

formation more potently that the wild type DLC1, suggesting that phosphorylation of these residues acts to negatively regulate DLC1s tumor suppressive function [Scholz et al., 2011].

Small GTPases, Ras and Rho, crosstalk has been a point of interest for a long time as both the pathways play significant roles in carcinogenesis. One such point where the pathways seem to come together was seen when p120RasGAP was identified as an interacting partner for the GAP domain of DLC1 [Yang et al., 2009b]. This interaction upon overexpression in colon carcinoma cells completely nullified the tumor suppressive function of DLC1 by inhibiting its GAP activity and thus increasing active RhoA levels at the focal adhesions [Yang et al., 2009b].

Although, there are reports that show that GAP-negative mutant of DLC1 is sufficient to inactivate DLC1s tumor suppressive functions, recent findings suggest otherwise. DLC1-K714E was seen to lose its ability to suppress colony forming capabilities of HCC cell [Wong et al., 2005]. At the same time, it was seen that formation of stress fibers could not be suppressed alone by the GAP domain of DLC1 and probably requires others domains or the immediate regions that flank the GAP domain at the C- and N-terminal [Wong et al., 2005]. Healy et al (2007) reported that DLC1s tumor suppressive functions were mediated in both GAP-dependent and GAP-independent fashion, leading to the conclusion that the DLC1 GAP activity is important but not sufficient to carry out the tumor suppression.

1.3.1.3 START domain

This domain forms the C-terminus of DLC. These domains are typically found in lipid metabolizing proteins or lipid transfer proteins, in which they form a hydrophobic pocket to capture the lipid molecule for

transport between membranes [Ponting and Aravind, 1999]. Initially indentified as a domain being conserved in two lipid-binding proteins namely, steroidogenic acute regulatory protein (STAR) and metastatic lymph node 64 (MLN64) at their C-termini, now about 15 human proteins have been seen to contain this 200-210 amino acid containing domain, which can be divided into six subfamilies [Alpy and Tomasetto, 2005].

Crystal structures solved thus far show that START domains contain a conserved fold forming a tunnel-like structure consisting of 9 β-strands giving rise to a β-sheet which is capped at the C- and N- termini by α-helices [Alpy and Tomasetto, 2005]. This β-sheet tunnel is large enough to fit in lipophilic molecules such as cholesterol as seen in the case of STAR and MLN64. The C-terminal α-helix acts as a lid for this structure allowing the entry of the molecules [Soccio and Breslow, 2003]. One would expect with such conserved structures, that the proteins containing these domains would have similar interacting partners. But these proteins not only have varied binding partners but also are very specific in their interactions for example STARD5 binds only 25-hydroxycholesterol whereas STARD1/MLN64/STARD5 binds only cholesterol [Alpy and Tomasetto, 2005]. This binding property is indicative of the fact that these proteins are irreplaceable in their functions. The ability of START-domain contain protein to bind lipids make them key players in cellular mechanisms which includes trafficking, metabolism, regulation of transcription and signal transduction involving lipids. In fact, START domain-containing proteins are involved in various lipid-related cellular processes, including lipid trafficking, lipid metabolism, lipid-modulated signal transduction and transcriptional regulation.

In a START domain containing proteins review by Alpy and Tomasetto (2005), they have summarized various mechanisms by which these proteins play a role in the cellular processes by means of acting as an interface for lipid-sensing or lipid transfer. MLN64 is an example for a protein that via its lipid-transferring capabilities plays a role in cholesterol homeostasis by

transporting endosomal cholesterol. STARD6 through lipid-interaction, regulates transcription during the process of spermatogenesis. On the other hand, START domain STARD11 plays a role in signal transduction by regulating its own kinase activity towards the Goodpasture antigen in the Goodpasture disease. STARD10 is seen to localize to the sperm flagellum and is a transporter for phosphatidylcholine (PC) and phosphatidylethanolamine. It is suggested that STARD10 might be involved in the energy metabolism in the sperm flagellum by providing PC as potential substrate.

Despite the vast knowledge about the various START domains, the role of DLC1s START domain still remains to be elucidated to a great extent. There has been no evidence of lipid-binding property of START domain of DLC1. Sequence alignment with START domains of other proteins show similarity with the regions flanking the PC binding site as seen in the PC-transfer protein, which points towards the possibility of DLC1 binding PC and other similar lipids [Ponting and Aravind, 1999]. The rat homologue of DLC1, p122RhoGAP, is seen to interact with phospholipase C delta 1 (PLC-δ1) leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns (4,5)*P*2] which leads to the formation of inositol 1,4,5-triphosphate, finally releasing Ca^{2+} from the intracellular stores [Homma and Emori, 1995]. This interaction is carried out via its C-terminus encompassing both the START and the GAP domain [Sekimata et al., 1999]. p122RhoGAP is seen to localize to the plasma membrane of the caveolae, binding to caveolin there, potentially through the lipid-binding property of the START domain [Yamaga et al., 2004]. Recent work has showed that START domain is important but not sufficient in itself for carrying out DLC1s tumor suppressive. START domain is not necessary for the focal adhesion targeting function of DLC1 [Kim et al., 2008], but loss of this domain affects DLC1s ability in inhibiting the colony forming nature of HCC cells as well inhibition of stress fiber formation leading to morphological changes in the cell [Wong et al., 2005]. Another recent study showed that the START domain of DLC1 is necessary for DLC1

interaction and localization with caveolin-1. Without the presence of the START domain, DLC1 failed to interact with caveolin-1 and this directly affected DLC1s ability to suppress neoplastic growth [Du et al., 2012].

Though there is a lack of knowledge, we can extrapolate that the START domain might help in determining the subcellular localization of DLC in the cell and might also be involved in regulating its GAP function, like in the case of other RhoGAP proteins such as Cdc42GAP which contains the Sec14 domain [Alpy and Tomasetto, 2005; Moon and Zheng, 2003].

1.3.1.4 Serine-rich region

The region between SAM and GAP domains is an unstructured region whose tertiary structure is unknown and shows the least sequence conservation amongst the three DLCs, showing only short stretches of high similarity separated by large gaps and insertions [Durkin et al., 2007a]. This region is rich in amino acids S, P, Q, E and K, which are known to be enriched in unstructured proteins as they disrupt an orderly arrangement. Also, it lacks hydrophobic amino acids, which potentially give proteins a globular structure by forming the core. The serine-rich region is interspersed with proline-rich regions, thereby allowing binding to proline recognition motifs like the WW motif and the SH3 motif [Durkin et al., 2007b]. It also contains LD motif (LDXLLXXL) that is seen in paxillin and its binding partners [Brown et al., 1998]. The unstructured region gives the protein flexibility, thereby allowing interaction with multiple proteins which could aid in spatial and temporal regulation as well as increasing DLC1s susceptibility to proteolysis thereby by controlling the protein turnover. These regions are also hot spots for post-translational modifications like phosphorylation especially by serine/threonine kinases, which regulate the activity of the protein itself [Durkin et al., 2007b].

One such finding was the S567 phosphorylation by Akt, which negatively regulates DLC1s GAP-dependent suppression of tumorigenesis and metastasis [Ko et al., 2010b].

DLC1 is known to cause morphological change in cells [Sekimata et al., 1999]. This function of DLC1 which led to rounding up of cells was attributed to its localization to focal adhesions and not only its GAP activity [Kawai et al., 2009a]. There have been studies that show DLC1 interaction with a group of proteins called Tensins which consist of tensin1, tensin2, tensin3 and cten and are important for DLC1s localization to focal adhesions [Liao et al., 2007; Qian et al., 2007; Yam et al., 2006]. Tensins have a highly conserved C-terminus, which consist of a Src-homology 2 (SH2) domain and a phosphotyrosine binding (PTB) domain [Lo and S., 2004]. A yeast two hybrid screening of human liver cDNA revealed Tensin 2 as a novel interacting partner and they were seen to colocalize to vinculin-associated focal adhesions via the phosphotyrosine-binding domain (PTB) on Tensin2 [Chan et al., 2009; Yam et al., 2006]. DLC1 interacts with cten via the SH2 domain in a phosphotyrosine independent manner although requiring the sites S440 and Y442 and the mutation of these sites causes DLC1 to be cytoplasmic diffused [Liao et al., 2007; Qian et al., 2007]. The two sites which were seen to be important for cten interaction with DLC1 were also found to be necessary for DLC1 interaction with the PTB and SH2 domain of tensin1 [Qian et al., 2007]. Independently conducted studies have shown that DLC1 mutants which lose their binding ability with tensin do not localize to focal adhesion, also tend to lose their ability to suppress colony formation. This indicates that DLC1 localization to focal adhesions is necessary for its GAP activity [Liao et al., 2007; Qian et al., 2007]. Region between 359-397 amino acids on DLC1, termed as Focal Adhesion Targeting (FAT) region mediates its binding to the PTB domain of tensin, which was later narrowed down to amino acids 375-385 [Kawai et al., 2010; Kawai et al., 2004]. In addition to localization at the focal adhesions, tensin2 and DLC1 were found to be in a complex with

caveolin-1 at the caveolae of hepatocytes and were involved in the Ras signalling inhibition [Yam et al., 2006]. LD-like motif consisting of amino acids 469-476 in DLC1 is necessary for its binding to two molecules namely, talin and focal adhesion kinase (FAK). This motif is homologous to the LD motif seen in paxillin. DLC1 mutants lacking residues from this region or carrying amino acid substitutions were seen not to be able to bind either of the molecules. Though, both FAK and talin were targeted to the same region on DLC1, their binding was independent of each other. Mutants lacking the ability to bind talin and FAK though did not show any suppressed GAP activity, they were less potent in tumor suppression. This binding was also seen to be required for DLC1s focal adhesion localization [Li et al., 2011].

The serine-rich region of DLC1 contains a bipartite nuclear localizing signal sequence which lies within amino acids 415 – 430 the regulatory region for which is located within the region of amino acids 209 – 291, which controls the duration of nuclear residency of DLC1 as well as its stability [Yuan et al., 2007]. DLC1 also undergoes a phosphorylation-dependent interaction with 14-3-3 adaptor proteins. This interaction, stimulated by protein kinase C and protein kinase D, inhibits the nucleocytoplasmic shuttling of DLC1 and its cellular functions by blocking its nuclear localization signal sequence [Scholz et al., 2008]. Another region between amino acid 600-700 was identified as being important for the purpose nuclear localization of DLC1. It was seen that in the fusion protein variant of DLC1 (NLS-DLC1) which predominantly localizes to the nucleus, the tumor suppression mediated by DLC1 was greatly reduced as seen by the failure to suppress colony formation and formation of stress fibers in SMMC-7721 cells. *In vivo* it was seen that nude mice injected with the NLS-DLC1 expressing hepatoblasts, these cells formed tumors much more effectively as compared to the wild-type DLC1 [Chan et al., 2011]. DLC1 contains 5 conserved PEST sequences in its serine-rich region, which increases its stability, without which DLC1 was seen to be susceptible to degradation by the 26S proteasome [Luo et al., 2011].

Protein Kinase A (PKA) mediates the phosphorylation of S431 and S549. It was seen that the phosphorylation of S549 was important for the event of S431 phosphorylation. The importance of S549 phosphorylation is not limited to that but is also seen to be required for DLC1s tumor suppressive functions as S549A fails to inhibit colony formation is SMMC7721 cells. It also had an effect on the migratory and invasive properties of hepatoma cells. Phosphorylation of this residue also induced dimerization of DLC1, in turn enhancing its RhoGAP activity [Ko et al., 2013]. α-catenin was identified as novel binding partner for DLC1. This requires amino acids 340-345 in the serin-rich region and the C-terminus of α-catenin. This binding led to the accumulation of the complex at the plasma membrane and stabilized the Adherent Junctions (AJ). This was carried out by reduction in the active RhoA levels, which also increased the mobility of E-cadherin as well as affected the actin cytoskeleton. In metastatic prostate cancer cells it was seen that this association led to a more potent DLC1 activity [Tripathi et al., 2012].

Apart from protein based-interactions, a study revealed that adjacent to the GAP domain of DLC1, there is highly conserved region consisting mainly of basic amino acids termed as Poly Basic Region (PBR), which facilitates the binding of DLC1 with phosphatidylinositol-4,5-bisphosphate or PI(4,5)P2. This interaction greatly enhances the GAP activity of DLC1, leading to inactivation of Rho signalling [Erlmann et al., 2009]. It has also been shown that Erk5 expression in Src-transformed cells, increases DLC1 expression, which in turn leads to decrease in RhoA-mediated signalling and leads to podosome formation [Schramp et al., 2008].

The isolated GAP region (652-798) alone does not have any functions, indicating that there are other regions on DLC1 that control the activity of this protein [Kim et al., 2008]. It has already been shown that binding of proteins such as tensin to the FAT region is essential for DLC1s function as a tumor suppressor [Liao et al., 2007]. A study conducted to identify mutations

present in this region in the genomic DNA of cancer patients' revealed mutations, namely, T301K and S308I. Though these mutations do not impair the focal adhesion targeting of DLC1, they affect GAP function of DLC1 as seen by the inability of these mutations to prevent colony formation [Liao et al., 2008]. DLC1s interaction with a pro-inflammatory protein S100A10 via 7 residues in the serine-rich region from amino acids 348-354 lead to degradation of S100A10, which had an inhibitory effect on the invasiveness, col ony forming ability and cell migration of A549 cells in a GAP-independent manner [Yang et al., 2011].

1.3.2 RhoGAP-independent functions of DLC1

DLC1, a multi-domain protein, has three known functional domains so far and an unstructured region, which has been implicated as being important for regulating DLC1s function. Out of the three domains, GAP is the most studied domain. Evidence suggests that RhoGAP activity of DLC1 is important but not exclusively responsible for the tumor suppression is shown by the ability of GAP-inactive mutant to inhibit to a great extent, the *in vitro* growth and invasive property of NSCLC [Healy et al., 2008]. One such finding was that SAM domain alone is seen to have an effect on cell migration by its specificity to bind to Elongation Factor1A1 (EF1A1) and localize to membrane periphery and ruffles [Zhong et al., 2009]. Other than protein synthesis, EF1A1 has been implicated in various aspects of cytoskeletal organization and cell growth. Also, GAP-inactive mutant of DLC1, which had tensin-binding residues still intact had the capability of inhibiting cell migration comparable to the DLC1 mutants that had lost the ability to bind tensins but retained its GAP activity [Qian et al., 2007], though not to the extent of wild-type protein. This observation implies that, unlike previous

belief, the tensin-binding ability and RhoGAP function of DLC1 might independently contribute to DLC1s role in tumor suppression. The most recent work showing DLC1s GAP-independent function was the binding of DLC1 to a cell surface plasminogen receptor, S100A10. Upon DLC1 binding, S100A10 becomes susceptible to degradation via the ubiquitin pathway, leading to the attenuation of the plasminogen activation as well as inhibiting cell migration and invasion. This effect was the same in both wild-type DLC1 as well as GAP-inactive DLC1 [Yang et al., 2011].

Given the ability of DLC1 to function in both GAP-dependent and –independent manner, it is no surprise that compared to the very many RhoGAPs, it is seen to be more frequently affected in cancers.

1.3.3 DLC family- Functions:

The family to which DLC1 belongs consists of two other members namely, DLC2 and DLC3. The genes encoding the three proteins arose by gene duplication and are paralogues of each other. DLC1 and DLC2 have 58% similarity, whereas DLC1 has only 44% similarity to DLC3. DLC2 and DLC3 are 52% similar.

DLC2 and DLC3 have been seen to localize to focal adhesions associated with vinculin, through possibly their interactions with tensin-family of proteins [Kawai et al., 2007; Kawai et al., 2009b]. Work done on the DLC family of proteins show that DLC2 displaces endogenous DLC1 from the focal adhesions, by competing with its focal adhesion targeting region, suggesting common mechanisms by which they target focal adhesions [Kawai et al., 2009b].

DLC2, like DLC1, causes rounding up of cells and disruption in the actin cytoskeletal structures by inhibiting RhoA activity, a function which is dependent on its GAP activity [Leung, 2005]. DLC2 has also been seen to localize with mitochondrial markers and were seen in lipid droplet-like

structures and the START domain was necessary for both these localizations [Ng et al., 2006]. Studies have shown that DLC2, unlike DLC1 is not embryonic lethal [Yau et al., 2009]. Also, DLC1s downstream effects include reduction in the phosphorylated states of paxillin and FAK [Kim et al., 2007], whereas as DLC2 has been seen to effect only the phosphorylation status of paxillin and not FAK. DLC2 regulates cell cycle via inhibition of Raf-1-ERK1/2-p70S6K pathway instead of modulating its effect via the CDK inhibitors [Leung et al., 2010].

The knowledge on DLC3 is much less as compared to DLC2. It has been seen that DLC3, like other DLCs, inhibits tumor growth [Durkin et al., 2007a]. DLC3 also binds to the SH2 and PTB domains of tensins [Qian et al., 2007].

DLC gene family are evolutionarily conserved. Initially identified in mouse (ARHGAP7 or STARD12) and rat (p122RhoGAP), they are now seen to have orthologues in other vertebrates namely *Canis lupus* (dog), *Gallus gallus* (chicken), *Xenopus tropicalis* (frog), *Lagocephalus lagocephalus* (puffer fish), *Pan troglodytes* (chimpanzee) and invertebrates such as *Drosophila* and *C. elegans* [Durkin et al., 2007b].

Initially discovered as a binding partner for phospholipase C-δ1 (PLC-δ1), rat p122RhoGAP protein is 93% identical to human DLC1. The expression of this rat orthologue is very similar to that of humans [Durkin et al., 2007b]. The GAP domain of this protein functions synergistically with PLC-δ1, inhibiting the activation of RhoA causing cell rounding via the loss of actin stress fibres as well as focal adhesions [Sekimata et al., 1999]. Unlike in humans, p122RhoGAP leads to the release of intracellular calcium and activates protein kinase C, by hydrolysing phosphatidylinositol (4, 5)-bisphosphate (PIP2) leading to the formation of second messengers inositol triphosphate and diacylglycerol [Healy et al., 2008; Homma and Emori, 1995].

The *crossveinless-c (cv-c)* gene in *Drosophila* encodes the protein homologous to the human DLC1. Its role was indispensible during

morphogenesis. It was seen to be necessary for regulation of RhoGTPase leading to cytoskeletal remodelling [Denholm et al., 2005] as well as for regulating tubulogenesis [Durkin et al., 2007b]. The ability of the human DLC1 to negatively regulate RhoA leading to the actin cytoskeleton reorganization is similar to that seen in both the rat and the *Drosophila* orthologues [Kawai et al., 2004; Sato et al., 2010].

1.4 Focal adhesion kinase

Focal adhesion kinase (FAK), in 1992, was independently identified by Steve Hanks, Jun-Lin Guan and Michael Schaller. It was initially identified as a tyrosine kinase which gets highly phosphorylated upon formation of intergrin-associated adhesion sites in cells and was seen to be a substrate of viral oncogene, Src [Guan and Shalloway, 1992; Hanks et al., 1992; Schaller et al., 1992]. FAK is also referred to as protein tyrosine kinase2 (PTK2) and is found in chromosome 8q24term [Mitra et al., 2005]. It is almost ubiquitously expressed and is a well conserved all the way from lower eukaryotes like *Drosophila* and zebrafish to mammals [Parsons, 2003]. FAK along with PYK2 are the two members of FAK family of non-receptor protein tyrosine kinases. They share similar structure and functions but also have other distinct functional properties. Both these proteins not only play an important role in the signalling cascade as kinases but also behave as scaffolds to allow assembly of the signalling complexes. Activation of FAK occurs through mainly integrin-mediated signalling and to some degree by growth factor receptor and cytokine receptor activation and mechanical stimulation. Focal adhesion Kinase (FAK) plays a role in three major cellular processes, namely, cell survival, cell proliferation and cell motility [Hall et al., 2011].

1.4.1 FAK: Structure

FAK is a 125kDa multi-domain protein with a kinase domain and two non-catalytic domains. The N-terminal domain of FAK shows sequence similarity to the family of proteins containing the FERM (erythrocyte band four.1-ezrin-radixin-moesin) domain. The C-terminal domain has focal adhesion targeting (FAT) sequences and the central domain is the kinase domain which is proline rich. In general, members of this family link transmembrane glycoproteins to the actin cytoskeleton [Mitra et al., 2005; Parsons, 2003].

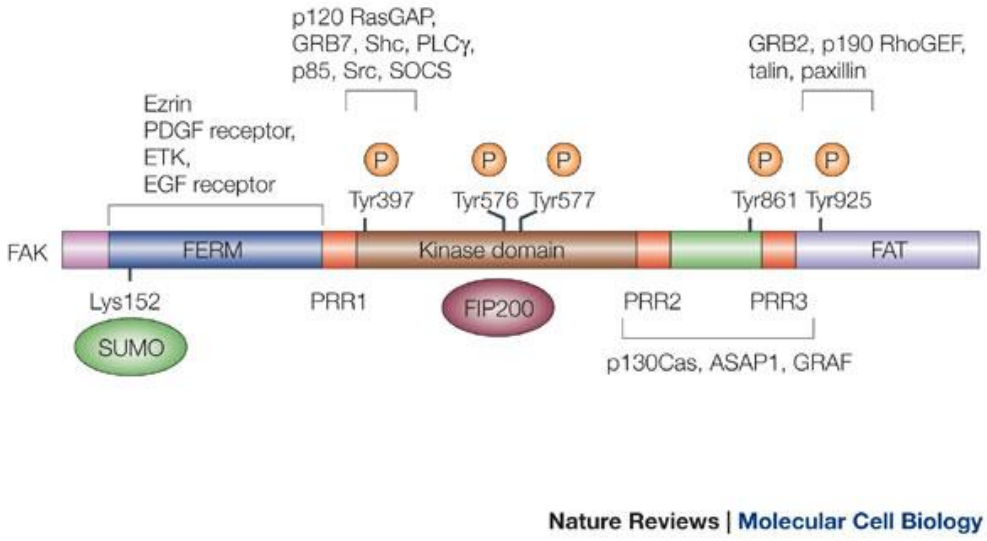

Fig 1.5: FAK domain structure and binding partners [Mitra et al., 2005]

1.4.1.1 FERM domain:

The function of the N-terminal domain of FAK is not completely understood. The FERM domain of FAK acts as the domain of interaction with

the receptor tyrosine kinases such as EGFR and PDGFR and allows for further activation of downstream signalling molecules [Sieg et al., 2000]. This domain mediates binding of FAK to β-integrin, which is necessary for activation of FAK itself as well as strengthening of integrin-associated adhesions [Mitra et al., 2005]. FAK is known to activate other non-receptor tyrosine kinases such as Etk which requires the binding of the FERM domain of FAK to the PH domain of Etk [Chen et al., 2001]. Furthermore adaptor proteins associated with actin like ezrin lead to an increased FAK activation by binding to the FERM domain, in an integrin-independent manner [Mitra et al., 2005]. This domain is also subjected to posttranslational modification, namely sumoylation (addition of a small ubiquitin-related modifier (SUMO)), which could be the possible stimulus for FAK accumulation in the nucleus, as is with other proteins. Upon sumoylation, FAK shows increased catalytic activity, indicating a potential signalling link between the focal complexes and the nucleus [Kadare et al., 2003]. So, the FERM domain may act to regulate the catalytic function of FAK by interaction with other proteins or by direct modification as well as link FAK to integrin- and growth factor receptor-linked signalling.

1.4.1.2 C-Terminal domain:

This domain is important for protein-protein interactions. It also contains the focal adhesion targeting (FAT) region that is about 100 amino acids long and is necessary for FAK localization to the focal adhesions. The FAT domain is similar to those in other focal adhesion localizing proteins such as Cas, vinculin etc. It consists of a four-helix bundle structure. The FAT domain also mediates the binding between FAK and paxillin [Hayashi et al., 2002]. There is a hydrophobic patch on either side of the domain which is

supposed to interact with the LD motif on paxillin. Paxillin acts as the docking partner for FAK at the focal adhesions as it binds to the cytoplasmic domain of the intergrin present in them [Hall et al., 2011; Hayashi et al., 2002]. Surprisingly though, mutants of FAK that fail to bind paxillin were still seen to target to the focal adhesions, suggesting that there are other possible mechanisms of FAK targeting to adhesion complexes. FAK is also seen to associate with the focal adhesions via its interaction with another focal adhesion protein, Talin [Cooley et al., 2000]. It was recently shown that FAK through its FAT region interacts with DLC1s LD-like motif. This interaction was important for DLC1 localization to the focal adhesions [Li et al., 2011].

This domain apart from the FAT region also consists of two proline-rich regions which allow interactions with SH3 domain containing proteins. One such example is the binding of p130Cas with FAK which is seen to be important for cell migration. This region is also necessary for FAK interaction with RhoGTPase regulators such as GRAF and ASAP1, hence providing a link between the assembly of focal adhesions and cytoskeletal dynamics [Mitra et al., 2005].

Also, a region called the FRNK (FAK-related-non-kinase) present in the C-terminal is expressed independently *in vivo* and functions possibly as a negative regulator of the catalytic activity of FAK. The expression of FRNK is controlled by transcriptional elements found between the last exon of the kinase domain and the first exon of the C-terminal domain. Over-expression of FRNK inhibits cell spreading and migration [Hall et al., 2011].

1.4.1.3 Kinase domain:

FAK kinase domain is highly similar to the kinase domains of other non-receptor tyrosine kinases as well as receptor tyrosine kinase. A feature unique to this kinase domain is the presence of a disulphide bond in the

terminal lobe of this domain observed via crystallographic studies. Presence of this bond could be possibly important for the kinase function [Nowakowski et al., 2002]. FAK gets phosphorylated at Tyr397 present in the kinase domain upon integrin clustering. It has been recently shown that FAK undergoes dimerization leading to intermolecular phosphorylation of Tyr397 [Katz et al., 2002]. This causes an increase in FAK's catalytic activity and subsequently leads to the phosphorylation of focal adhesion-associated proteins at the Tyr residue. Activation of this residue is also important for phosphorylation of Tyr576 and Tyr577, two highly conserved residues in the catalytic loop of the kinase domain, for the maximal FAK activity signalling the start of the downstream cascade [Mitra et al., 2005].

1.4.2 FAK activation and regulation:

The activation of FAK is started off by auto-phosphorylation of Tyr397 in *cis* or *trans*, which creates a binding motif for SH2 domain-containing proteins [Mitra et al., 2005]. This occurs once FAK localizes to the integrin-associated focal complexes via the FAT region at the C-terminal domain. Phosphorylation of this residue promotes binding of Src, causing a conformational activation of Src. Activation of Src leads to transphosphorylation of FAK at Tyr 576 and Tyr577, which lie in the activation loop of the kinase domain. This event is important for the maximal activation of FAK [Hanks et al., 2003].

FAK is regulated by conformational changes and protein-protein interactions. The kinase domain has a unique disulphide bond, which might regulate FAK activity through conformational changes [Parsons, 2003]. Binding of FAK-interaction protein, FIP200, to the kinase domain inactivates FAK [Abbi et al., 2002]. Intramolecular constrains as seen in alternatively

spliced isoforms of FAK which have additional residues around Tyr397 changes the activation kinetics of FAK from *trans*- to *cis*-phosphorylation [Toutant et al., 2002]. FERM domain binds to the kinase domain *in trans* and inhibits FAK activity as removal of FERM domain increases FAK activation. FERM domain also mediates binding of interacting partners like ezrin and TRIO, a GEF, which increases FAK's catalytic function [Mitra et al., 2005].

FAK plays an important role in cytoskeletal dynamics, which involves direct and indirect regulation of RhoGTPases. The activated Src in the Src-FAK complex leads to the phosphorylation of Tyr861 on FAK which mediates the binding of the proline-rich regions of FAK with SH3 domain-containing proteins, namely p130Cas. Tyr925 phosphorylation by activated Src is necessary for SH2-mediated binding site for Grb2, an adaptor protein, which leads to the activation of Ras and its downstream signalling. The following activation of MLCK downstream of Erk2 modulates focal adhesion dynamics, cell proliferation and survival signalling [Hanks et al., 2003; Mitra et al., 2005]. It has been shown that over-expression of DLC1 leads to dephosphorylation of FAK at Y397 and Y925 [Kim et al., 2007].

1.4.3 FAK: Regulation of RhoGTPases and their regulators

RhoGTPases are critical for cytoskeletal dynamics and directional cell movement. Coordinated functions of Rac and Rho drive the migration process, which requires a tight regulation by the GAPs and the GEFs [Jaffe and Hall, 2005]. Recent studies suggest that FAK functions so as to activate and inactivate RhoA in a cyclical manner. In the initial stages of cell spreading, there is an FAK mediated tyrosine phosphorylation and localization of p190RhoGAP to the focal adhesions. This interaction required the presence of p120RasGAP. This association led to the RhoA inactivation in early cell spreading and this promoted polarization of the cell [Tomar et al., 2009].

Whereas during the later stages of cell spreading, FAK associated with p190RhoGEF (Rgnef) along with paxillin and this complex localized to the focal adhesions as well. This also requires the tyrosine phosphorylation on Rgnef. By this temporally separated binding with a GAP and a GEF, FAK can control the process of cell spreading. The FAK-p190RhoGEF complex in neuronal cells during development controls axonal branching and synapse formation [Lim et al., 2008]. Apart from afore mentioned GAP and GEF, FAK also regulates the phosphorylation and localization of PDZ-GEF and LARG. FAK also indirectly controls Rac activity by the regulation of RhoA activity. This is due to the existing antagonism between RhoA and Rac. FAK also mediates Rac activation via phosphorylation of adaptor proteins such as p130Cas and PIX [Tomar and Schlaepfer, 2009]. Furthermore, FAK interacts with N-WASP which has been activated by Cdc42 prior to this association and this might work to couple the polymerization of actin with membrane protrusion during cell migration [Mitra et al., 2005].

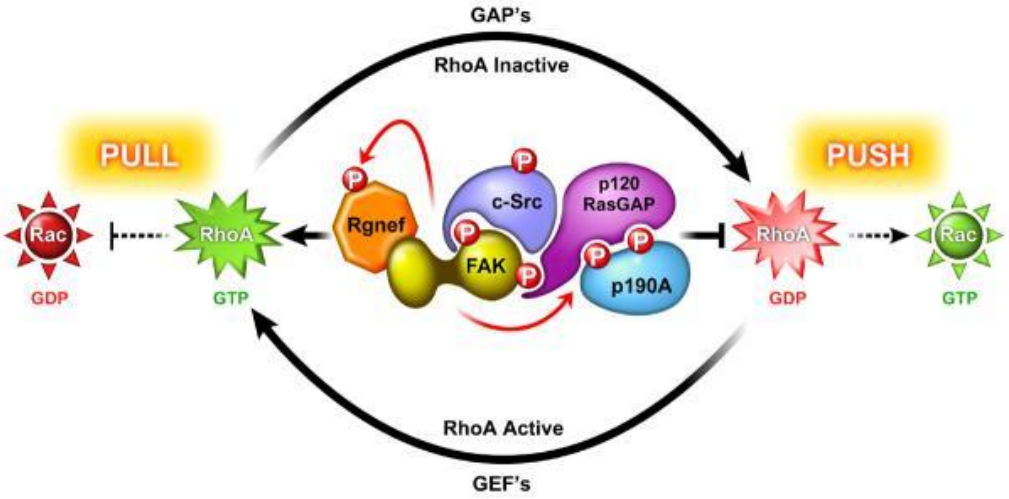

Fig 1.6: FAK-mediated regulation of GAPs and GEFs to control RhoA during cell migration [Tomar and Schlaepfer, 2009].

Lamellipodial extensions coupled with focal adhesion assembly and disassembly at the leading and the trailing edges controls directional migration. Recent studies show that FAK is a key regulator of this process as well [Tomar and Schlaepfer, 2009]. Contrary to previous believes that FAK is necessary for the formation of focal adhesions, accumulating evidence suggest that FAK association with FAs target them for disassembly or turnover. This is supported by studies in FAK-/- fibroblasts which show increased FAs at the periphery and decreased FA turnover at the leading and trailing edges of the cells. Activated FAK resides in the FAs for an increased period of time allowing the effectors needed for the disassembly to be recruited [Webb et al., 2004]. By controlling the RhoA and Rac activity, FAK controls the force generated during cell migration, which in turn decides the FA turnover.

1.5 Protein Phosphatase 2A

Phosphorylation is one of the major post-translational modifications which alter the protein function and localization and plays a major role in the process of cytoskeletal dynamics. The reversible event of phosphorylation is controlled by kinases and phosphatases. Kinases like FAK, LIMK, PAK etc have been extensively studied during this process. On the other hand involvement of phosphatases have not been delved into and the importance of the phosphatase family of proteins in cell migration is only recently being understood [Larsen et al., 2003]. Phosphorylation occurs on 3 amino acids, namely Serine (Ser), Threonine (Thr) and Tyrosine (Tyr) with phosphorylation of Ser accounting for 86.4%, Thr 11.8% and Tyr 1.8% of all phosphorylation [Olsen et al., 2006]. Hence Ser/Thr phosphatases are of great importance. Of

all the Ser/Thr phosphatases that are present, PP2A and PP1 together account for 90% of the Ser/Thr dephosphorylation [Eichhorn et al., 2009]. Use of Okadaic acid, a selective but not specific inhibitor of PP2A, was seen to inhibit cell migration and promote tumor growth in mice [Eichhorn et al., 2009; Wilson et al., 1991], indicating the importance of PP2A in physiological and pathophysiological conditions.

1.5.1 PP2A: Structure

PP2A belongs to a family of holoenzyme complexes which have distinct localizations, different substrate specificities and activities. PP2A are structurally complex existing in a dimeric ($PP2A_D$) or a trimeric form ($PP2A_T$). The dimer consists of the catalytic and the scaffold subunit, known as the core enzyme and the trimer consists of the core enzyme with the additional regulatory subunit and this is known as the holoenzyme and it is functionally active.

1.5.1.1 PP2A catalytic subunit ($PP2A_C$)

The catalytic domain is globular structure that gives rise to a bimetallic active site for hydrolysis. It shares 50% sequence identity with PP1. $PP2A_C$ has two isoforms, Cα and Cβ, expressed ubiquitously by two distinct genes and share 97% sequence identity and are 35 kDa in size. Cα, which has 10 times higher expression level due to a stronger promoter is mainly expressed in the plasma membrane compared to Cβ which is usually expressed in the cytoplasm and the nucleus [Eichhorn et al., 2009; Seshacharyulu et al., 2013].

As determined by the crystal structure, PP2Ac has a highly conserved C-terminal tail (^{304}TPDYEL^{309}) which binds the scaffold and regulatory subunits. Binding of the regulatory subunit depends entirely on methylation or phosphorylation of this region. Inadequacy of Cβ in substituting the function of Cα as observed in Cα knockout mice which are embryonic lethal suggests that the two isoforms of $PP2A_C$ are non-redundant in their functions despite being 97% identical in their sequences [Eichhorn et al., 2009; Seshacharyulu et al., 2013].

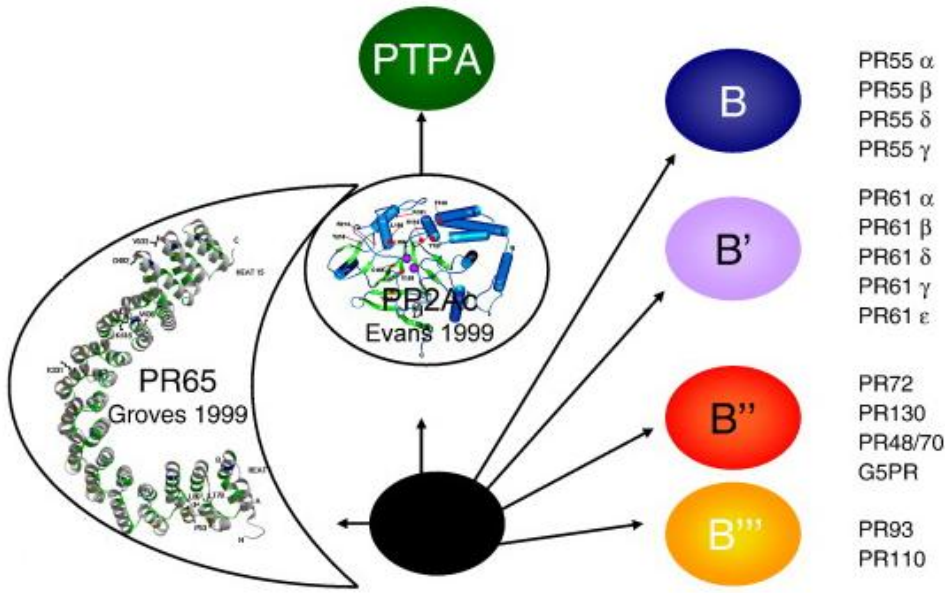

Fig 1.7: PP2A subunits

1.5.1.2 PP2A structural subunit (PR65 or PP2A-A)

PP2A structural or the scaffold subunit, like the catalytic subunit, is encoded by two ubiquitously expressed genes, giving rise to isoforms Aα and Aβ. They share 87% sequence identity, with Aα accounting for a higher

percentage in the holoenzyme in the adult. Aβ isoform is seen in abundance in oocytes and in early stages of development. Despite the high percentage of sequence identity, similar to the $PP2A_C$, Aβ cannot substitute for the loss of Aα in mice. At the C-terminal of this subunit there is Huntington/elongation/A-subunit/TOR (HEAT) sequence in 15 tandem repeats of 39 amino acids. Each repeat consists of two α-helices connected by intra- and inter- repeat loops. The first 10 repeats allow binding to the regulatory subunit and the rest mediate the binding with the catalytic subunit. In the dimeric form, the binding of structural subunit to the catalytic subunit changes its catalytic specificity. Once the core enzyme is formed, the scaffold subunit bends and forms a horseshoe shape-like structure. This allows the structural subunit to recruit the regulatory subunit to form the holoenzyme as well allows the catalytic subunit to interact with the substrate unimpeded.

The two isoforms bind to different regulatory subunits and even thought the Aβ is lower in abundance, many tumor-specific mutations which prevent it from forming the holoenzyme, have been identified in it [Eichhorn et al., 2009; Seshacharyulu et al., 2013].

1.5.1.3 PP2A regulatory subunit

This is the most diverse of the PP2A subunits with 15 genes that encode 26 different transcripts and splice variants. Subunit B is regulatory subunit and it determines the substrate specificity, controlling the activity of the holoenzyme in a temporal and spatial manner. Existence of multiple subunits gives rise to multiple functionally viable PP2A heterotrimeric complexes. With PP2A and PP1 controlling 90% of the Ser/Thr phosphatase activity, multiple PP2A holoenzymes can account for the diversity of the phosphoproteins and signalling pathways that get regulated by PP2A. The regulatory subunits do not share any sequence similarities except for the few

conserved amino acids which mediate the binding with the HEAT repeats in the scaffold subunit. This subunit has been divided into four families: B (B55/PR55), B' (B56/PR61), B'' (PR48/PR72/PR130), and B''' (PR93/PR110), with the numbers indicative of the size of the protein in kDa.
The regulatory subunits are unique in their expression patters in different tissues at various developmental stages [Eichhorn et al., 2009; Seshacharyulu et al., 2013]. It is interesting to note that the B56 subunit is responsible for targeting PP2A to the focal adhesions via its ability to interact with paxillin [Ito et al., 2000].

1.5.2 PP2A: tumorigenesis

PP2A's role in cancer was identified when Okadaic acid treatment was observed to induce tumor in mice. Aberrant expression or mutation of the regulatory and scaffold subunit of PP2A has seen in various cancers. Loss-of-function mutations in PR65 or the structural subunit was seen in lung, colon, breast, skin, cervical and ovarian cancers. Reduced expression of PP2A-A, observed in glioma as well as *in vivo* mice studies, led to decreased expression of the holoenzyme indicating a lower tumor suppressive function of PP2A. PP2A also, regulates GTPases like RalA which plays an important role in migration, cell proliferation, apoptosis, transcription and transport. Upon PP2A inhibition, RalA gets hyperphosphorylated [Eichhorn et al., 2009; Seshacharyulu et al., 2013].

Of the various regulatory subunits of PP2A, the B55 and B56 were the most commonly mutated ones in cancer. B55 suppression leads to sustained activation of Akt which enhanced cell proliferation [Manning and Cantley, 2007]. B56 subunit of PP2A is known to play an important role in controlling various signalling pathways. p53 gets directly dephosphorylated by B56-containing holoenzyme, which stabilizes and upon B56 inhibition, p53

mediated signalling pathway gets deregulated [Li et al., 2007]. This subunit also destabilizes c-myc, an oncogene, by dephosphorylating it and targeting it for ubiquitination [Arnold and Sears, 2006]. A truncation mutant of B56 increases the metastatic potential of B16 melanoma cells due to its inability to dephosphorylate paxillin [Ito et al., 2000]. Mitogen-activated protein kinase (MAPK) signalling and the WNT signalling is also directly affected by B56-associated PP2A. This holoenzyme directly dephosphorylates ERK thereby by acting as a negative regulator of the MAPK signalling cascade. Similarly in the WNT signalling PP2A destabilizes β-catenin, leading to its degradation. Deregulation of MAPK and WNT signalling are seen to play a part in tumorigenesis, hence fortifying the role of PP2A as a tumor suppressor [Eichhorn et al., 2009; Seshacharyulu et al., 2013].

1.5.3: PP2A: Cell adhesion and motility

Activation of Akt is critical to cell migration and it is tightly regulated and any disruption in this leads to loss of cell polarity. Regulation of Akt by PP2A makes it a key player in the process [Hunter, 1995]. PP2A also inhibited EGFR mediated chemotaxis in keratinocytes via its dephosphorylation of ERK [Pullar et al., 2003]. Dephosphorylation by PP2A maintains cell adhesion and cytoskeleton dynamics. Epithelial cell migration is important for the process of wound healing which was inhibited by PP2A, which was also seen to inhibit endothelial cell migration therefore affecting angiogenesis [Gabel et al., 1999]. PP2A is seen to localize with β1-integrin and hence regulates the FAK complex. It was seen that upon PP2A inhibition FAK/Src/paxillin complex gets hyperphosphorylated, which lead to disorganization of focal adhesion sites and increased endothelial cell migration [Young et al., 2002]. PP2A is also needed to maintain the cytoskeletal integrity, upon inhibition of which cell exhibit a rounded morphology as well as increased motility.

Conversely, PP2A is also seen to stimulate migration by dephosphorylation of actin depolymerising factor (ADF)/cofilin, which is required to be localized to the lamellipodium in a dephosphorylated state for directional migration [Ambach et al., 2000].

1.6 Hypothesis and Objectives

DLC1 is a focal adhesion localizing protein. It requires interaction with the tensin family of proteins and the residue Y442 was shown to be important for this function [Qian et al., 2007]. And more recently it was shown that it interacts with talin and FAK through an LD-like motif which is also seen to affect the ability of DLC1 to localize to the focal adhesions. In fact, the authors showed that DLC1 lacking the ability to interact with both the proteins was more impaired in its focal adhesion targeting than the one lacking binding capabilities to only one [Li et al., 2011]. It was also shown that over-expression of DLC1 leads to the dephosphorylation of FAK at Y397 and Y925 residues [Kim et al., 2008]. DLC1 being a tumor suppressor is deleted or mutated in a multitude of cancers. One such study done to identify potential mutational hotspots in DLC1 at the focal adhesion targeting region picked up two residues T301 and S308. Mutations in these residues affected DLC1s expression as well as the RhoGAP activity [Liao et al., 2008]. Why and how the mutations in these residues have such a profound effect on DLC1 is unknown. These residues being Ser/Thr could possibly be subject to posttranslational modifications such as phosphorylation which affects DLC1s function.

DLC1, due to the presence of a highly serine-rich region, is a potential target downstream of various kinases and phosphatases, which by controlling DLC1s phosphorylation status can control its regulation. A few phosphorylation sites on DLC1 have been delineated in the past decade which

have been seen affect its function as well as use of Okadaic acid, a PP2A inhibitor, has been shown to affect DLC1s phosphorylation status [Ko et al., 2013; Ko et al., 2010a; Scholz et al., 2008]. B56 subunit of PP2A is known to localize to focal adhesions as well [Ito et al., 2000]. What is lacking is a complete picture on how and by what mechanisms DLC1 gets regulated to switch between the active and inactive states. DLC1 being a GAP for RhoA, has to be under tight spatial and temporal control.

With the knowledge that we have on the localization of DLC1, FAK and PP2A and the role they play in cell migration, we hypothesize that interplay between these molecules might be responsible for DLC1-mediated regulation of cell dynamics.

The objectives of the project to delineate the finer details of this regulation are to:

1. Examine the interaction between PP2A and DLC1
2. Delineate the residues that might be potential target for PP2A
3. Identify a potential kinase which primes DLC1 as a PP2A target
4. Elucidate the interplay among the three molecules of interest
5. Determine the functional significance of this interplay

CHAPTER 2

Materials and Methods

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2 MATERIALS AND METHODS

2.1 Phosphoproteomic analysis

Flag-DLC1 construct was transfected in 293T cells as outlined in section 2.4.3 and starved in serum-free media for 18 hours. Following this, the cells were subjected to the following treatments: DMSO; DMSO+EGF; U0126+EGF; Okadaic acid; Okadaic acid + EGF. After which, the cells were lysed using RIPA buffer supplemented with fresh cocktail of protease inhibitor (Roche) and immunoprecipitated using M2 anti-Flag agarose beads (section 2.6.2) and washed thrice in ice cold RIPA buffer. The samples were then mixed with an SDS-buffer without β-mercaptoethanol (0.1 M Tris-HCl (pH 6.8), 3 % (v/v) sodium dodecyl sulfate (SDS) (*1st Base*), 15 % glycerol (*Merck*), 0.005 % (w/v) bromophenol blue (*BioRad*)). To this a final concentration of 10mM Dithiothreitol (DTT) was added and the samples were boiled for 30mins at 55-60 °C. This was then let to cool down to room temperature and to this 20mM final concentration of freshly prepared Iodoacetamide (IAA) was added and was allowed to stand at room temperature for 30mins. DTT acts as a reducing agent while IAA acts as an alkylating agent.

The sample was then ready to be loaded on to a 10% acrylamide gel and SDS-PAGE was performed. The gel was then fixed using a solution containing 10 % acetic acid and 25 % isopropanol. It was then washed a few times with deionized water with care being taken to prevent the gel from tearing and stained with PageBlue™ solution (*Fermentas*) overnight. The next day, the stain was removed and the gel was washed with deionized water multiple times for the purpose of destaining the gel. Once the bands become clearer on the gel, they were excised using a sterile scalpel and put into an eppendorf tube containing 100 µl of deionized water. The bands were then

analysed using AB SCIEX TripleTOF® *5600+* mass spectrometry system to analyse the phosphorylated residues on DLC1. Only phospho-residues containing peptides with a confidence level score above 95% was chosen. The residues were compared across the different treatments and only those that were unique to the treatment of Okadaic acid+EGF were used for further studies.

2.2 Generating DLC1 and $PP2A_C$ constructs

The full-length DLC1 constructs, the GAP-inactive DLC1 (DLC1-R677E) and the truncation mutants of DLC1, namely, Flag-SR1, -SR2 and SR3 were previously generated by Wong Ming Zhi, Denise, Dr. Zhou Yi Ting and Dr Zhong Dandan. Using Polymerase Chain Reaction (PCR), gel extraction, restriction digestion, ligation, transformation and plasmid extraction, the internal deletion mutant of DLC1 (DLC1-ΔS), point mutants of DLC1 (T301/S308A and T301/S308D) point mutant of $PP2A_C$ were generated The protocols of the afore mentioned experiments are discussed in detail in this section.

The previously generated DLC1 constructs were all cloned into the pXJ40 vector (Flag and GFP-tagged), kindly provided by Dr. E. Manser (Institute of Molecular and Cell Biology, Singapore). This vector is a mammalian expression vector containing the CMV promoter with the tag attached to its 5'end. The template used for construction of PP2A mutants was myc-tagged $PP2A_C$, a kind gift from Prof Lin Sheng-Cai (Xiamen University).

2.2.1 Polymerase Chain Reaction (PCR)

The existing plasmid constructs were used for the purpose of carrying out PCR to create point mutations and internal deletions in the case of DLC1, sub-coning of FAK and point mutations in the case of PP2A. Templates used for DLC1 point mutants and internal deletions were pXJ40-Flag-DLC1 and pXJ40-GFP-DLC1, for FAK sub-cloning was mouse FAK cloned into pCDNA and for $PP2A_C$-CS mutant was pXJ40-myc-$PP2A_C$. The primers used for the purpose are given in table (2.1).

For the PCR, the set up was as follows: For a 50µl reaction- 2.5 U of *Pfu Turbo* DNA Polymerase, corresponding 1 X *Pfu Turbo* DNA Polymerase reaction buffer (Stratagene, USA), 10pmol of the specific forward and reverse primer oligonucleotides each, 0.2mM dNTP mix, 100ng of the template and the reaction mix was topped upto 50µl with deionized autoclaved water. Pfu Turbo was used because the templates to be amplified were greater that 1 kb in length.

The PCR reaction was carried out in a thermal cycler (BioRAD icycler). The parameters of the PCR cycles were set as follows: Initial denaturation step at 95 °C for 2mins followed by 20 cycles consisting on the following steps: Denaturation at 95 °C for 2 mins; Annealing at 55-65 °C for 30secs (Temperature determine by the Tm of the primer pair); Extension at 72 °C for 1min/kb of DNA template. This was followed by a final extension step at 72 °C for 12-15mins. For the point mutation PCR products, 10 µl of the reaction mix was subjected to DpnI digestion at 37°C for 30-45mins post-PCR.

For the purpose of creating the internal deletion mutant of DLC1, nested PCR was done. For this 2 pairs of primers (ΔS_F1 and ΔS_R1; ΔS_F2 and ΔS_R2) were designed, with the forward of the first pair (ΔS_F1)

containing the BamHI restriction site and the reverse primer of the second pair (ΔS_R2) carrying the XmaI restriction site. The reverse primer of the first pair (ΔS_R1) and forward primer of the second pair (ΔS_F2) were designed for the region surrounding but excluding the amino acids to be deleted. For the first round of PCR, two set ups were made with each primer pair. For the second round of PCR, the products from the previous rounds were used with the ΔS_F1 and ΔS_R2 as the primer pair. These were then subjected to restriction digestion at 37°C for 4hours.

Table 2.1: Primer sequences used for cloning of DLC1 and $PP2A_C$ mutants

Construct Name	Primer Name	Primer sequence
T301/S308A	AA_F	5’ – CAGCAGCAGCAGCGCACAGTCGGAG ACCAGC – 3’
	AA_R	5’ – GCTGGTCTCCGACTGTGCGCTGCTGC TGCTG – 3’
T301/S308D	DD_F	5’ –CAGCAGCAGCAGCGACCAGTCGGAG ACCAGC – 3’
	DD_R	5’ – GCTGGTCTCCGACTGGTCGCTGCTG CTGCTG - 3’
DLC1_ΔS	ΔS_F1	5'- CGCGATATCGACAAAGAAGATGATAT TACTGAGCT -3'
	ΔS_R1	5' –CGCTGCTGGTCTCCGACTGGGTCTGCGT GGAGTTGGAAACG - 3'

DLC1_ΔS	ΔS_F2	5'-CGTTTCCAACTCCACGCAGACCCAGTCGG AGACCAGCAGCG -3'
	ΔS_R2	5'- CGCGATATCGTGGTTATTTGATGGGATC CAAGAGA -3'
$PP2A_C$-CS	CS_F	5’ – GCTCCAAACTATAGTTATCGTAGTGGT AACCAAG – 3’
	CS_R	5’ – CTTGGTTACCACTACGATAACTATAGTT TGGAGC – 3’
FAK	FAK_F	5’ – ATGCGGATCCATGGCAGCTGCTTATCTT – 3’
	FAK_R	5’– ATGCGGTACCTCAGTGTGGCCGTGTCTG – 3’

2.2.2 Agarose gel electrophoresis

Only in the case of DLC1 internal deletions, once the PCR products were obtained (after restriction digestion the products were subjected to the same), agarose gel electrophoresis was carried out to separate the DNA fragments of the required size from the rest. 1% (w/v) agarose gel was made by dissolving the required weight of Agarose (1^{st} Base) in 1 X TAE buffer (40 mM Tris base, pH 8.0 with glacial acetic acid, 10 mM EDTA). For visualization of DNA under ultraviolet light, SYBR®Safe DNA gel stain (Invitrogen) at a dilution of 1:50,000 was mixed with the gel prior to setting. This gel was run using 1 X TAE as the buffer. Alongside the products, 1 kb DNA ladder (Fermentas) was run to assist in size determination.

2.2.3 Gel extraction

Following the agarose gel electrophoresis, the separated DNA bands were visualized under UV light in the Molecular Imager Gel Doc XR system (BioRad) and the required bands were excised out using a sterile scalpel. These bands were then extracted from the gel using the QIAquick Gel Extraction Kit (QIAGEN) using the instruction manual provided by the manufacturer. The bands were eluted out using deionized autoclaved water and were used for further processing as required.

2.2.4 Restriction enzyme digestion

PCR products after gel extraction along with the vectors/constructs were digested with corresponding enzymes as designed into the primers. 1.5 µg of each of the DLC1 plasmid construct and the PCR product was digested using BamHI (New England Biolabs) and XmaI (New England Biolabs) in a total volume of 50 µl consisting of 1X reaction buffer 4 (as determined by the manufacturer), 1X bovine serum albumin (BSA), 4 U of the enzyme for every µg of DNA to be digested in the case of vector digestion and for the PCR products a total of 25 U was used. This set up was kept for 4 hours at 37 °C. The digested products were subjected to agarose gel electrophoresis and gel extraction as described previously.

For the purpose of FAK sub-cloning, BamHI and KpnI (New England Biolabs) were used to digest the PCR product as well as pXJ40-Flag and pXJ40-GFP vectors. The protocol used was similar to that used for DLC1.

2.2.5 Ligation

For internal deletion, fragment of DLC1 lacking the 5 amino acids (15bps) was inserted back into pXJ40-Flag/GFP DLC1 constructs after digestion of both the constructs and the PCR product. Ligation carried out overnight at 16 °C. The PCR product to the digested construct concentration was about 2:1, for a total volume of 20 µl , with 1X ligase buffer (New England Biolabs) and 1µl of 200 U T4 DNA ligase (New England Biolabs).

The digested vector and PCR product were ligated using the same protocol for FAK sub-cloning.

2.2.6 Preparation of competent cells

E. coli DH5α was used as the preferred choice for cloning because of a mutation in its *endA* gene that reduces its synthesis of endonuclease enzyme, it allows for production of plasmid DNA of high purity as well as yields a high concentration of DNA.

These bacterial cells were made competent using the calcium/manganese based (CCMB: 80 mM $CaCl_2.2H_2O$, 20 mM $MnCl_2.4H_2O$, 10 mM $MgCl_2.6H_2O$, 10 mM KOAc pH 7, 10 % (v/v) Glycerol; pH 6.4, filtered) method. The preferred growth media and agar for *E. coli* DH5α cells are Luria Bertani (LB) broth and agar (BD Difco). The bacterial cells were first streaked onto an LB Agar plate without antibiotics from glycerol stock and incubated overnight at 37 °C. Inoculating a colony from this plate the next day into 5ml of LB broth, a starter culture was prepared and incubated overnight at 37 °C in a bacterial shaker incubator at 250 rpm. The next day, 50 ml of LB broth was inoculated with the starter culture and the

culture was grown at 37 °C with shaking till an OD measured at 600 nm reached 0.6. The culture was then chilled on ice for 10 minutes before it was pelleted at 2,500 rpm for 10-15 minutes at 4 °C. The pellet obtained in this step was resuspended by gentle vortexing with 1/3rd the original volume of the culture (17ml) of ice cold CCMB. This was then incubated on ice for 20 mins. This is pelleted again by centrifuging at 3,000 rpm for 10 minutes at 4 °C, following with the pellet was resuspended again in ice cold CCMB which is 1/12th the original volume of culture (4ml). The cells are then aliquoted (100µl each), snap frozen in liquid nitrogen and stored at -80 °C for future use.

2.2.7 Transformation of ligated products into competent bacterial cells

Transformation of the DNA into competent cells can be done using two different protocols. For the purpose of transforming ligated products the heat-shock method is used which has high efficiency of transformation. The other method is the KCM method, which is mainly used for the purpose of retransformation of plasmid DNA.

In the heat-shock method, the *E. coli* DH5α of competent cells were first thawed out on ice. To this about 10 µl of the ligation product was added, mixed and incubated on ice for 30 mins and then was subjected to heat shock by keeping it at 42 °C for 90 seconds followed by 5 mins incubation on ice again. To this mixture, 600 µl of LB broth (without antibiotics) was then added and the sample was incubated in 37 °C at 250 rpm for 1 hour for recovery. The sample was then concentrated by pelleting and plated onto LB agar plates with ampicillin (100µg/ml) and incubated overnight at 37 °C.

In the KCM method, to about 20 µl of KCM (100 mM KCl, 30 mM $CaCl_2$, 50 mM $MgCl_2$) 1µl of the plasmid DNA was added and incubated on ice for 5mins. Simultaneously *E. coli* DH5α cells were thawed out on ice. 20 µl of the competent cells were added to the nixture and incubated on ice for 20-30mins and plated onto LB agar plates with ampicillin (100µg/ml). The plates were incubated overnight at 37°C.

2.2.8 Plasmid DNA extraction

Colonies were picked from the LB agar plates, inoculated in 5ml LB broth with ampicillin (100µl/ml) and incubated overnight at 37°C at 250rpm. These cultures were pelleted down and plasmid DNA was extracted using the AxyGen minprep kit, following the manufacturer's instruction. The plasmid DNA was eluted using deionized autoclaved water and its concentration checked using the NanoDrop 2000. OD_{260}:OD_{280} ratio was checked to ensure the purity of plasmid DNA obtained (ratio of 1.8-2 is indicative of highly pure DNA devoid of protein contaminants). This plasmid DNA can be used for the purpose of DNA sequencing and checking for plasmid expression.

For the purpose of higher concentration and purity 50ml of inoculated culture can be used for plasmid DNA extraction using AxyGen Midiprep kit as per the manufacturer's instructions.

2.2.9 Sequencing of DNA constructs

The purified plasmid was processed for the purpose of sequencing so as to ensure that the insertion was within frame and there were no mutations in the

case of the DLC1 internal deletion and that the correct substitutions were made for the point mutants generated for DLC1 and $PP2A_C$. For this purpose T7 forward primer as well as DLC1 and $PP2A_C$ internal primers were used. The reaction mix for this PCR contained 100ng of plasmid DNA, 0.8µl of the relevant primer, 1Xsequencing buffer, 1µl of BigDye. The PCR set up was as follows: : Denaturation for 30 seconds at 96°C; Annealing for 15 seconds at 55°C and Extension for 4 minutes at 60°C. This was repeated for 25 cycles.

The PCR product obtained is ethanol precipitated to purify. The reaction mix for this procedure contains 62.5µl of 95% Ethanol, 3µl of 3M sodium acetate, with 5µl of PCR product. The total volume was maed upto 80µl and incubated at room temperature for 15-20mins. The DNA was pelleted by centrifuging the reaction mix at maximum speed (14,000 rpm) for 10 mins. The supernatant was decanted and 500µl of 75% ethanol was added and this was centrifuged at 14,000 rpm for 5mins. Ethanol was removed and the remaining ethanol in the tube was air-dried or dried at 85°C. The samples were then processed by HiDi and sequenced in the the ABI PRISM 3130 Genetic Analyzer (*Applied Biosystems*) by the Department of Biological Sciences. The obtained sequences were analysed using the BLAST ((Basic Local Alignment Search Tool) program on the NCBI (National Center for Biotechnological Information) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.2.10 Checking expression of cloned constructs

The different mutants of DLC1 and $PP2A_C$ were first checked for expression. This was done by transfecting the plasmid DNA into 293T cells, followed by lysis of the cells by RIPA buffer. The lysate was then separated by SDS-PAGE and analysed by Western blotting using antibodies specific to

the tag attached to the protein or the protein itself. The protocols for these are outlined in the coming sections.

2.3 Expression and purification of GST-fusion proteins in bacteria

pGEX-4T-1 vector containing GST-tagged RBD was transformed into *E.coli.* DH5α cells. Inoculating a colony from this plate the next day into 5ml of LB broth, a starter culture was prepared and incubated overnight at 37 °C in a bacterial shaker incubator at 250 rpm. The next day, 50 ml of LB broth was inoculated with the starter culture and the culture was grown at 37 °C with shaking till an OD measured at 600 nm reached 0.6. Once the culture reaches the correct OD, 0.1 mM IPTG was added in to induce the expression of GST-tagged proteins with over-night shaking at 25 °C. The next day, the cell pellets were collected by centrifugation at, followed by freezing in -80 °C for 1 hour. The pellets were then thawed on ice, resuspended in chilled RBD lysis buffer (1XPBS, 1% Triton-X-100). To this 100mM DTT (w/v) and a mixture of protease inhibitors (Roche Applied Science) were freshly added. The resuspended pellet was lysed by sonication using MISONIX Sonicator XL 2020, set to a program of 3.0 sec pulses with a 6.0 sec lag period for a period of 3 mins at an amplitude of 20%. The cell lysate were centrifuged at 5000 rpm for 20 mins at 4°C. The supernatant was incubated with required amount of Glutathione Sepharose 4B beads (Amersham Sciences) at 4°C over night. Beads with GST-tagged proteins were washed 3 times with chilled RBD lysis buffer followed by 2-times washing with chilled 1XPBS and finally the beads were resuspended in equal volume of 1XPBS.

To check for the expression, 10 µl of the beads were boiled with the loading buffer and together with BSA standards were run on acrylamide gel.

The gel was stained with Coomassie Brilliant Blue stain for 1 hour and destained using water till the bands were clearly visible.

2.4 Mammalian cell culture and Transfection

For the purpose of the experiments in this thesis, the mammalian cells lines used were 293T and HeLa JW.

2.4.1 293T

293Ts are human embryonic kidney epithelial cells (American Type Cell Culture) with stable expression of SV40 T antigen. These cells were cultured as a monolayer on tissue culture dishes (*Nunc*) in Roswell Park Memorial Institute (RPMI) 1640 medium containing glutamine (10.4 g of HyQ RPMI - 1640 powder in 1 L of deionized autoclaved water) supplemented with 2 g/L sodium bicarbonate, 10 mM HEPES free acid, 100 U/ml penicillin-streptomucin (all from Hyclone Laboratories) and 10 % (v/v) defined fetal bovine serum (FBS) (LifeTech) and maintained in an incubator at 37 °C with 5 % carbon dioxide.

2.4.2 HeLa JW

These cells were a kind gift ftom Dr Alexander Bershadsky's Lab. These cells are a modification of HeLa cells, which are immortalized cervical cancer cell line. They were grown in Dulbecco's Modified Eagle's Medium containing glutamine (13.2g of HyQ DMEM-High Glucose powder in 1 L of

deionized autoclaved water) supplemented with 5g/L sodium bicarbonate, 100 U/ml penicillin-streptomycin solution (Hyclone Laboratories) and 10% (v/v) FBS (LifeTech) as a monolayer in tissue culture dishes (Nunc) and maintained in an incubator at 37 °C with 5 % carbon dioxide.

2.4.3 Transfection of 293T cells

24 hours prior to transfection the cells were subjected 0.25 % trypsin-EDTA (*LifeTech*) treatment and plated in 10% RPMI-1640 onto required size of tissue culture dishes so as to achieve 80% confluency for the purpose of transfection. Before seeding the cells, the culture dishes were coated with poly-D-lysine for 15mins at 37°C and then washed thrice with PBS. For transfection, Mirus TransIT®- Transfection reagent (*Mirus Bio Corporation*) was used (For every µg of plasmid DNA 3µl of transfection reagent was used). The required volume of transfection reagent was incubated in 250-500 µl of serum-free RPMI - 1640 for 5 mins at room temperature prior to addition of plasmid DNA, followed by 20 min incubation at room temperature. This mix was added drop-wise to the culture plates, which were swirled to ensure even distribution of the complex. These plates were incubated at 37 °C for 24 hours with 5 % carbon dioxide. Before transfection, the media in the tissue culture dishes were replaced with fresh 10% RPMI-1640. 4 hours post-transfection, this medium was replaced with 0% RPMI-1640, subjecting the cells to 18 hours of starvation for experimental purposes.

2.4.4 Transfection of HeLa JW cells

24 hours prior to transfection the cells were subjected 0.25 % trypsin-EDTA (*LifeTech*) treatment and plated in 10% DMEM onto required size of

tissue culture dishes so as to achieve 80% confluency for the purpose of transfection. For transfection Lipofectamine™ 2000 transfection reagent (*Life Technologies*) was used (For every 2µg of plasmid DNA 4.5 µl of transfection reagent was used.). The required volume of transfection reagent was incubated in OPTI-MEM® reduced serum medium (*Gibco*) for 5 minutes at room temperature (For every 2µg of plasmid DNA 150 µl of OPTI-MEM® was used.). The plasmid DNA was similarly diluted in OPTI-MEM® reduced serum medium and this was then added to the transfection reagent complex and incubated for 20 mins at room temperature. This mix was added drop-wise to the culture plates, which were swirled to ensure even distribution of the complex. These plates were incubated at 37 °C for 24 hours with 5 % carbon dioxide. Before transfection, the media in the tissue culture dishes were replaced with fresh 10% DMEM. 4 hours post-transfection, this medium was replaced with 0% DMEM, subjecting the cells to 18 hours of starvation for experimental purposes.

2.5 EGF stimulation, U0126/Okadaic Acid/FAK inhibitor Treatment:

For doing a time-dependent study on the effect of EGF stimulation on the binding of DLC1 and $PP2A_C$ as well as other effects, 4 hours post-transfection, the cells were kept in a serum-free media for 18-24 hours. These cells were then treated with EGF (Sigma) at a concentration of 100ng/ml. The cells were then lysed at set time intervals using RIPA lysis buffer and analysed using SDS-PAGE and western blotting.

To confirm if the effect seen in the experiments were indeed downstream of MEK/ERK1/2, the cells were subjected to MEK/ERK inhibitor U0126 treatment. The concentration of U0126 (Promega) used is 5µM. It was added 1 hour prior to stimulation with EGF. U0126 is stably dissolved in

DMSO hence the control cells were treated with DMSO of the same volume as U0126, to negate the effect of DMSO from the experiment.

FAK inhibitor used was PF-573228 (Sigma) at a concentration of 300ng/ml. The treatment protocol followed is same as that of U0126, with the treatment starting 1 hour prior to EGF stimulation/lysis. In the case of Okadaic Acid (Enzo Life Sciences), it was used as at a concentration of 100nM and the cells were treated 2 hours prior to EGF stimulation/lysis. In both the cases the control cells were treated with DMSO as both the compounds were dissolved in DMSO. Following the treatments, the cells were lysed in RIPA buffer and analysed with SDS-PAGE and western blotting.

2.6 Co-immunoprecipitation

2.6.1 Preparation of mammalian whole cell lysates

The mamamlian cells were lysed after the respective treatments using RIPA buffer (150 mM sodium chloride, 50 mM Tris, pH 7.3, 0.25 mM EDTA, 1% (w/v) sodium deoxycholate, 1% (v/v) Trition X-100, 50 mM sodium fluoride). To this a mixture of protease inhibitors (Roche Applied Science) was freshly added before every experiment. The cells were centrifuged at maximum speed (14,000 rpm) for 10 mins and the supernatant collected for further studies.

2.6.2 Co-immunoprecipitation

For co-immunoprecipitation, cells were transfected with the required constructs, proteins of which were being tested for binding. For this, atleast one of the transfected constructs was Flag-tagged. After lysing the cells, some amount of the whole cell lysate was set aside for checking for various proteins. The rest of the whole cell lysate as incubated with 7-10 µl of M2 anti-Flag agarose beads (Sigma-Aldrich) at 4 °C for 4 hours. The agarose beads were then washed with RIPA buffer thrice and the bound complex analysed by SDS-PAGE and western blotting.

2.7 RBD assay

The endogenous RhoA activity was examined with pull-down assays with GST-tagged RBD domain of rhotekin which can specifically bind to GTP-bound RhoA (Ren *et al.,* 1999; Wheeler and Ridley, 2004; Shang *et al.,* 2003). The transfected cells were subjected to respective treatments and lysed with RIPA buffer. To 20 µl of lysates, 180 µl of RIPA buffer was added and incubated with 20 µl of GST-tagged RBD bound to glutathione sepharose beads for 45mins at 4 °C. This complex was then washed thrice with RIPA buffer. This was processed using SDS-PAGE. Immunoblotting was done for rulled-down active RhoA, endogenous RhoA and the over-expressed proteins in the whole cell lysate.

2.8 SDS-PAGE gel eletrophoresis and western blot analysis

The proteins were analyzed on 4-12.5% SDS-polyacrylamide gels (SDS-PAGE) with a Mini Protean II electrophoresis apparatus (BioRad Laboratories). The gels were cast with glass plates containing 1.5 mm spacers and 15-well combs (BioRad Laboratories). 12.5% gels were used for resolving smaller molecular weight proteins around 20-25 kDa, 4% gels were used for the purpose of resolving phosphorylated DLC1 and 10% gels were used for the rest of the experiments. The resolving or the separating gel contained 4-12.5% (w/v) acrylamide, 0.48% (w/v) N-N'-methylbisacrylamide, 0.375 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.0075% (w/v) APS and 0.05% (v/v) TEMED (N, N, N', N'-tetramethylethylenediamine). The stacking gels contained 4% (w/v) acrylamide, 0.133% (w/v) N-N'-methylbisacrylamide, 0.125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.0075% (w/v) APS and 0.08% (v/v) TEMED. To the protein samples of 6X loading dye (3% (w/v) SDS, 15% (v/v) glycerol, 7.5% (v/v) b-mercaptoethanol, 0.1 M Tris-HCl pH 6.8, 0.005% (w/v) bromophenol blue) was added and boiled at 85 °C for 3mins. The samples together with protein markers (Bio-Rad Laboratories) were loaded into the gels. Electrophoresis was performed at 25mA/gel for 1.5-4 hours (depending on the gel percentage) at room temperature in SDS-running buffer (25 mM Tris, 192 mM glycine and 0.75% (w/v) SDS).

After eletrophoresis, proteins separated on the gels were transferred onto PVDF membrane (Millipore) in transfer buffer (33.7 mM Tris, 256 mM glycine, 20% (v/v) methanol and 0.01% (w/v) SDS) at 100 Volt for 45mins-1hour 10min at 4oC with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories).

The PVDF membrane onto which the proteins were transferred, they were incubated in 1% blocking buffer (1 X PBS, 0.1 % Tween-20, 1 % BSA) for 1 hour at room temperature, after which the blots were incubated with

primary antibody diluted in 1% blocking buffer for 2 hours at room temperature or overnight at 4 °C. The blots were washed 3 times for a duration of 7-10 minutes each with wash buffer (1 X PBS, 0.1 % Tween-20). This was followed by incubation with secondary antibody diluted in wash buffer or blocking buffer for 2 hours with a repeat of the washes. To detect the proteins, the membranes were then treated for chemiluminescence detection using the ECL kit (Pierce).

The antibodies used were polyclonal anti-Flag (Sigma) [1:10000], polyclonal anti-c-myc (Zymed) [1:2500], monoclonal anti-dualphospho-threonine-tyrosine (anti-phospho ERK1/2) (Sigma) [1:1000], monoclonal anti-ERK2 (anti-pan ERK) (Transduction Laboratories) [1:1000], anti-DLC1 (BD Biosciences) [1:1000], monoclonal pan FAK(AbCam) [1:1000], anti-FAK pY397(BD Biosciences) [1:1000], anti-FAK pS910 (AbCam) [1:1000] and PP2A-C(α/β) (Santa Cruz) [1:1000].

2.9 Cell Spreading

For cell spreading studies, HeLa JW cells were transfected with pXJ40-GFP-DLC1, pXJ40-GFP-DLC1-R677E and pXJ40-GFP and starved as described in section 2.4.4. Serum contains fibronectin and other matrix protein which assist in cell attachment to the surface of the plates. So as to maintain similar conditions as those used for binding studies as well as RBD assay, 6-well plates (Nunc) was functionalized using 10% DMEM media and kept overnight in the 37°C incubator. After, 18-24 hours of starvation, the cells were subjected FAK inhibitor treatment or DMSO treatment for 1 hour. Following which, the cells were trypsinized, centrifuged in a falcon tube to remove the trypin. The cells were then re-suspended in serum free-media with or without FAK inhibitor, depending on the prior treatment and left for

recovery at 37°C with 5% CO2 for 45 mins in the incubator. 35mins into the recovery, to some of the falcon tubes EGF was added and left for another 10mins. After recovery, the cells were aliquoted into the pre-functionalized 6-well dishes. These were then left at 37°C with 5% CO2 in the incubator and fixed at various time points (30mins, 45mins, 60mins and 90mins). For the purpose of fixing, 4% Paraformaldehyde (PFA) was used. At each time point, the 6-well dishes were removed and washed thrice with 1XPBS. To then plates following the wash 1ml of PFA was added and the plates were incubated at 37°C for 15mins. The wash step with 1XPBS was repeated to remove residual PFA. 1ml of 1XPBS was added to each well to prevent drying up of the cells and the dishes were stored at 4°C till time of imaging. The cells were imaged in Perking Elmer Spinning Disk with a Olympus IX Inverted Microscope and a UPlansSApo 10X objective using the 488 DPSS laser and the bright-field.

2.10 Wound Healing

For the wound healing experiment, PDMS strips were used to create a gap or a wound in the cultured cells. This was chosen over the traditional scratching of a wound so as to avoid injuring the cells, which might affect the final outcome of the experiment. PDMS substrates were prepared using a Sylgard 184 silicone elastomer kit (Dow Corning). The silicone elastomer component was mixed with the curing agent in a ration of 10:1 and degassed for 30mins after which it was poured into a 35mm plate to a required depth. Subsequently, crosslinking of the elastomer was carried out at 70 °C for 2 hours [Prager-Khoutorsky et al., 2011]. This gives the PDMS block just the right amount of stiffness to be easily cut. The PDMS block was allowed to

cool down to room temperature. Following this, using a clean scalpel, the PDMS block was cut into thin strips of the required thickness.

Before using the PDMS strips for the experiment, they were thoroughly washed in 100% ethanol and rinsed with deionized autoclaved water. These along with the tweezers were disinfected using UV light. The strips were then carefully placed into the wells of a 12-well dish (Nunc) and pressed down to ensure they stick to the surface of the dish. The HeLa JW cells were trypsinized and seeded at high confluency into the wells, making sure not to dislodge the PDMS strips.

24 hours later, the HeLa JW were transfected with pXJ40-GFP-DLC1, pXJ40-GFP-DLC1-R677E and pXJ40-GFP as described in section 2.4.4 and 4 hours later starved in serum-free media. 18-24 hours later, the cells were subjected to FAK inhibitor treatment and EGF stimulation as per requirement. The PDMS strips were carefully pulled out without disturbing the cells. This forms a gap/wound in the monolayer of cells, which was then imaged to study the ability of the cells to migrate and close the gap/wound. The imaging was done using Olympus Live EZ with a Olympus IX Inverted Microscope and a UPLFLN 10X objective at 37°C, with 5% CO2. Warm White LED was used for bright field imaging and XCite Series 120Q Fluorescence light source was used for fluorescence imaging with a FITC (U-MNIB) filter over a period of 48 hours.

CHAPTER 3

RESULTS

3 RESULTS

3.1 RhoGAP function of DLC1 can be modulated by EGF stimulation

The RhoGAP function of DLC1 is known to contribute towards its ability to regulate various cellular processes. Its regulation of key cellular processes also implicates it in tumor suppression. It has been shown that the GAP domain of DLC1 alone is not sufficient for its role as a tumor suppressor and that the regions surrounding the GAP domain are vital for its activity [Kim et al., 2008]. Furthermore, there are multiple factors contributing to this function and we have shown that the pathway downstream of the EGF receptor is one such factor. EGF stimulation activates EGF receptor (EGFR), which in turn is responsible for a multitude of downstream signalling cascades which include the Ras-MAPK pathway, JAK/STAT pathway and PI3K/Akt pathway. It is thus important to elucidate the signalling cascade important for further downstream functions.

We showed that DLC1 requires stimulation by EGF to exert its GAP function on the endogenous RhoA (Fig 3.1). This possible effect of EGF could be either a direct one via phosphorylation of DLC1 or an indirect effect involving other potential regulators of DLC1. We explored the possibility that this effect was due to direct phosphorylation of DLC1 by using a lower percentage of acrylamide gel, to better resolve higher molecular weight proteins. DLC1 showed a lower electrophoretic mobility shift as seen in the lane 2 of the top panel of Fig 3.2. This recovers upon treating the cells with the Mek/Erk inhibitor U0126 (lane 3 of the top panel of Fig 3.2), indicating that Ras-MAPK pathway is involved in phosphorylation of DLC1. It has been previously shown that DLC1 gets regulated by the event of phosphorylation as

seen in the case of Akt phosphorylation at S567 which leads to the inactivation of DLC1 RhoGAP activity [Ko et al., 2010a]. However this is the first time that the Ras-MAPK pathway has been shown to regulate the GAP function of DLC1.

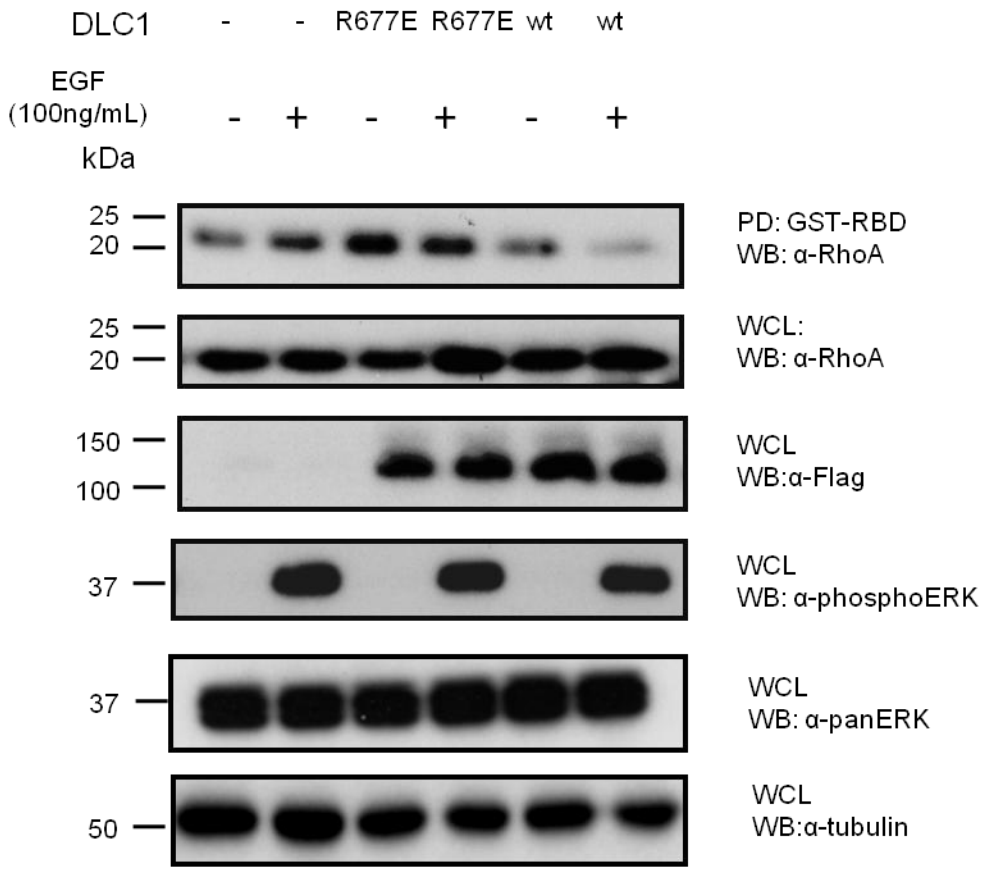


Figure 3.1: EGF stimulation triggers DLC1 GAP activity towards RhoA. Hela JW cells were transfected with Flag-tagged DLC1 full length (FL), Flag-R677E (GAP negative mutant), and Flag-vector. The cells were starved for 18-24 hours in serum-free media and then stimulated for 10mins with EGF (100ng/ml). The cells were lysed and the whole cell lysates (WCL) were used for pull-down assay (PD) with glutathione-sepharose beads bound to GST-tagged RBD. Proteins in the WCL and the PD were processed by SDS-PAGE and immunoblotting and probed with the appropriate antibodies. Upon EGF stimulation, DLC1 FL shows reduced amounts of active RhoA (top panel, lane

6) whereas the GAP mutant shows no difference in active RhoA levels with or without EGF stimulation (top panel, lanes 3 and 4).

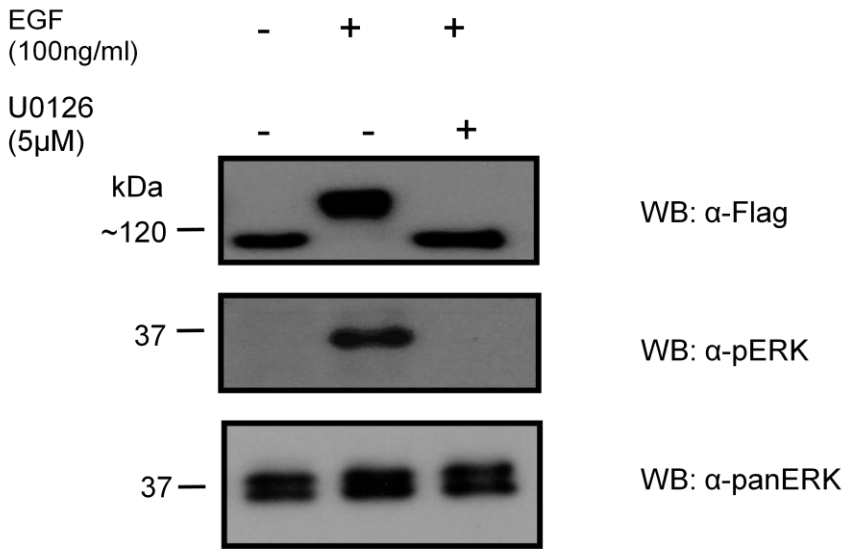


Figure 3.2: DLC1 shows an electrophoretic mobility shift upon EGF stimulation. 293T cells transfected with Flag-tagged DLC1 were starved for 18-24 hours in serum-free media and then stimulated for 10mins with EGF (100ng/ml). The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the IP were separated using 4% gel and immunoblotted with anti-Flag, anti-phosphoERK and anti-panERK antibodies. Upon EGF stimulation, there is an apparent increase in the DLC1 molecular weight (top panel, lane 2), which is lost upon treatment with U0126 (top panel, lane 3) indicative of possible phosphorylation of DLC1, involving the Ras-MAPK pathway.

3.2 Identifying PP2A as a potential interacting partner of DLC1

Studies have also shown that, upon inhibition by Okadaic acid, the phosphorylation status of DLC1 increases, indicating that PP2A might be playing a significant role in regulation of DLC1. This increase in phosphorylation increased the association of DLC1 with a scaffold protein 14-3-3 and blocks its GAP activity [Scholz et al., 2008]. Okadaic acid was shown to specifically inhibit PP2A and to a much lesser extent PP4 and PP5 [Favre et al., 1997]. It also targets PP1, but at a concentration 100 times more than that required for PP2A. It has been shown that upon Okadaic acid treatment, the cells become more motile [Wilson et al., 1991] and the process of cell migration encompasses multiple steps. With the event of phosphorylation playing a key role, phosphatases have a part throughout this process. PP2A via its interaction with paxillin through its B56 (B') subunit [Ito et al., 2000] localized to focal adhesions, which have seen to be an important site for DLC1s GAP function.

3.2.1 Confirmation of OA mediated regulation of DLC1 phosphorylation downstream of EGF stimulation and identification of potential target sites

With the knowledge that OA treatment causes an electrophoretic mobility shift in DLC1, we wanted to see whether this effect is downstream of EGF stimulation or independent of it. We used 100nM of OA, a concentration at which it specifically inhibits PP2A and observed that this electrophoretic mobility shift is maintained only upon EGF stimulation as seen in lane 4 of Fig 3.3 compared to lane 2 where there was no EGF treatment. This indicates that PP2A might be a potential regulator of DLC1 downstream of EGF stimulation.

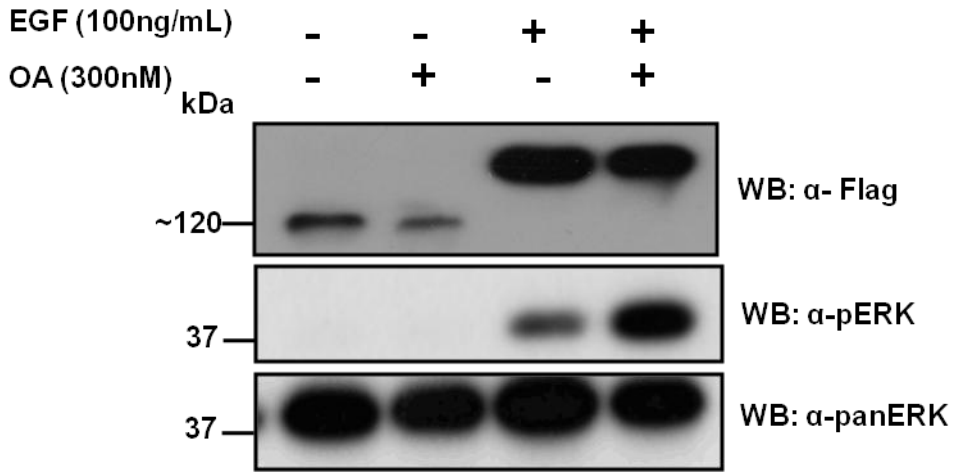


Figure 3.3: Okadaic acid treatment maintains the observed DLC1 electrophoretic mobility shift downstream of EGF stimulation. 293T cells transfected with Flag-tagged DLC1 were starved for 18-24 hours in serum-free media. The cells were treated with Okadaic acid (OA) (100nM) for 2hours and then stimulated for 10mins with EGF (100ng/ml). The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the IP were separated using 4% gel and immunoblotted with anti-Flag, anti-phosphoERK and anti-panERK antibodies. Upon treatment with EGF and OA, the observed DLC1 electrophoretic mobility shift is maintained.

To delineate which residues on DLC1 might be potential targets downstream of EGF stimulation and OA treatment, phosphoproteomic analysis was carried out on DLC1 with various treatments. After comparing the phosphorylation sites between the different treatments, various sites were identified in the serine-rich region of DLC1 (Table 3.1).

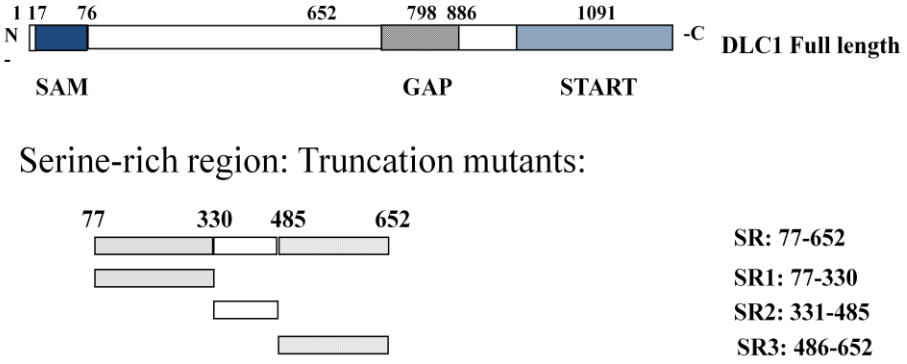
Table 3.1: Identification of potential phosphorylation sites on DLC1 by phosphoproteomics. 293T cells transfected with Flag-tagged DLC1 were starved for 18-24 hours in serum-free media. The cells were then treated with Okadaic acid (100nM) for 2hours and then stimulated for 10mins with EGF

(100ng/ml). The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation with M2 anti-Flag agarose beads. The samples were boiled, and then subjected to reduction and alkylation. Proteins were then separated using SDS-PAGE and the gel was stained with PageBlue™ solution and de-stained using water. The bands were excised and used for mass spectrometry analysis and the sites S305-307 were identified as potential phosphorylation sites on DLC1.

Conf	Sequence	Modification
99	296-SVSNSTQTSSSSSQSETSSAVSTPSPVTRTR	Phospho(S)@10; Phospho(S)@11
99	296-SVSNSTQTSSSSSQSETSSAVSTPSPVTRTR	Phospho(S)@3; Phospho(S)@13
99	296-SVSNSTQTSSSSSQSETSSAVSTPSPVTRTR	Phospho(S)@12

As a confirmation we also used various truncation mutants of the serine rich region of DLC1, namely, SR1 (77-330), SR2 (331-485) and SR3 (486-652). Figure 3.4 shows that only SR1 showed mobility shift in SDS-PAGE whereas SR2 and SR3 did not. The sites identified with phosphoproteomics lie within the SR1 region, thus providing further proof of the potential sites under the EGF/OA regulation.

A



B

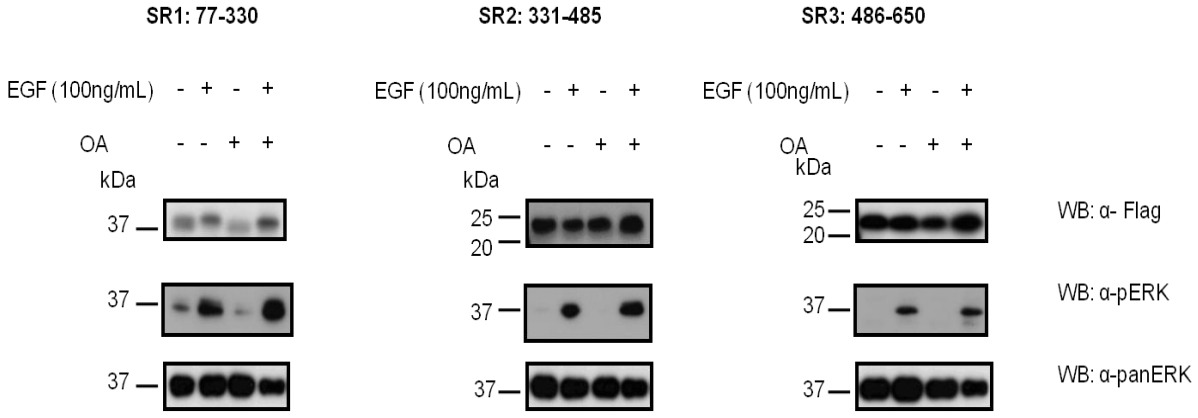


Figure 3.4: Electrophoretic mobility shift in DLC1 truncation mutant upon Okadaic acid treatment and EGF stimulation. A) Schematic representation of DLC1 serine-rich truncation mutants. B) 293T cells were transfected with Flag-tagged DLC1 truncation mutants. The cells were starved for 18-24 hours in serum-free media. The cells were treated with Okadaic acid for 2hours and then stimulated for 10mins with EGF (100ng/ml). The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the IP were separated using 12.5% gel and immunoblotted with anti-Flag, anti-phosphoERK and anti-panERK.

3.2.2 PP2A interaction with DLC1: EGF-dependent process

As shown by the Okadaic acid treatment, PP2A is a potential regulator of DLC1. Furthermore, this regulation seems to be downstream of EGF stimulation. Keeping this in mind all experiments were performed using EGF treatment. The cells were transfected with DLC1 and $PP2A_C$-CS and stimulated with EGF. The cells were lysed at various time points post-EGF stimulation. It was observed that the maximal interaction between DLC1 and PP2A takes place at 10mins post-stimulation (Fig 3.5, top panel, lane 3), which also seems to be the peak in the phosphoERK cycle, hence reiterating that this regulation is indeed downstream of EGF stimulation and is probably controlled by the Ras-MAPK pathway.

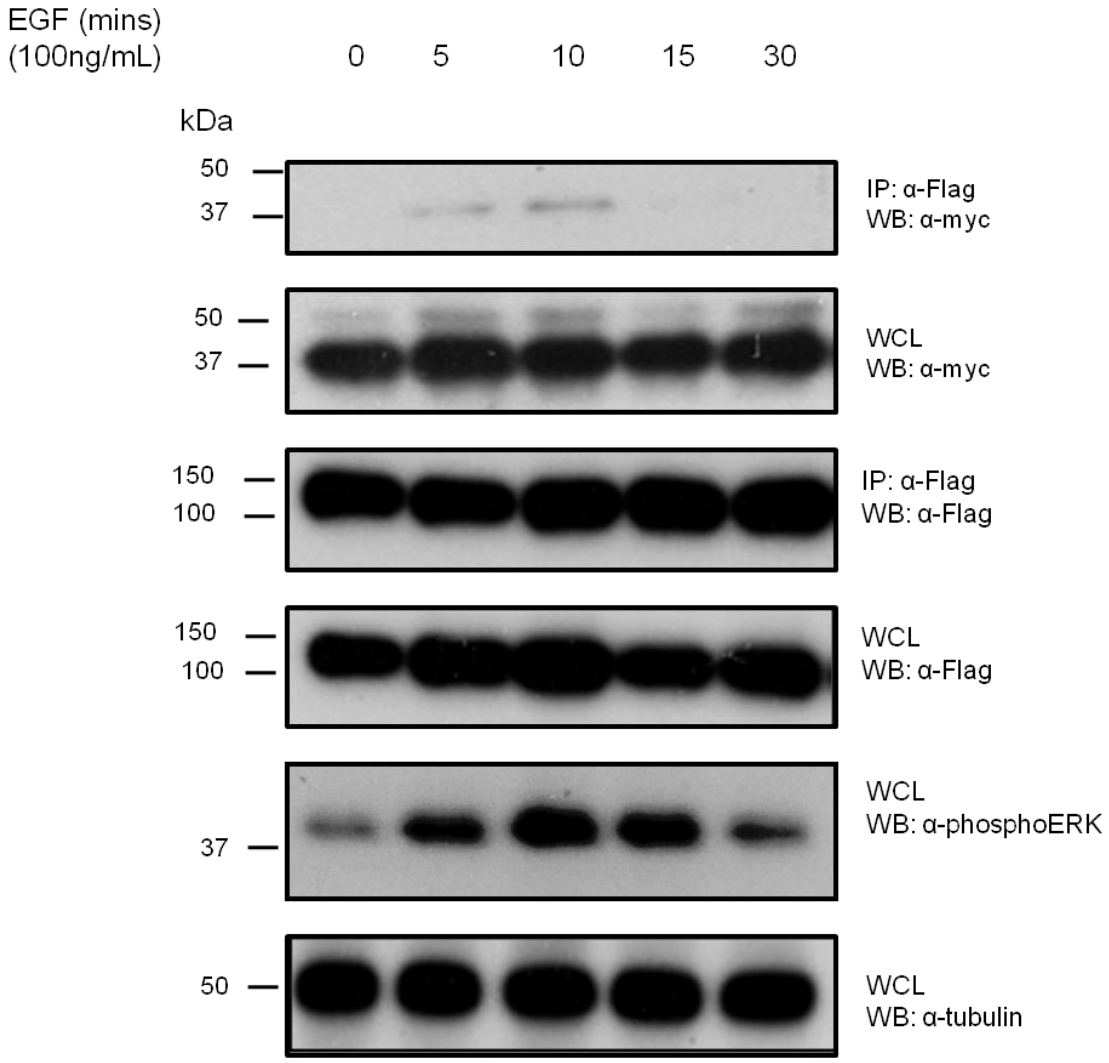


Figure 3.5: The DLC1-PP2A-C-CS binding in HeLa JW cells is dependent on EGF stimulation. HeLa JW cells transfected with Flag-tagged DLC1 and myc-tagged PP2A-C-CS were starved for 18-24 hours in serum-free media and then stimulated with EGF (100ng/ml). The cells were lysed 0, 5, 10, 15 and 30mins post stimulation and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the WCL and IP were separated by SDS-PAGE and immunoblotted with respective antibodies. The binding between DLC1 and PP2A-C-CS is dependent on EGF stimulation (top panel) with the binding profile mimicking that of the ERK1/2 phosphorylation profile (5th panel from top) with a peak at 10min post stimulation.

3.2.3 Confirmation of site-specific binding between DLC1-PP2A

Different sets of mutants were created for the potential target site on DLC1 to elucidate their binding capabilities with PP2A. A deletion mutant encompassing the 5 serines in the region identified by phosphoproteomics was created, namely, DLC1-ΔS (ΔS304-S308). Fig 3.6 shows that DLC1-ΔS does not interact with PP2A even upon EGF stimulation, thereby confirming that these might indeed be the target for PP2A-mediated dephosphorylation of DLC1.

Residues T301 and S308 have previously been identified as loci that are prevalently mutated in DLC1 in cancer patients [Liao et al., 2008]. S308 falls under the serine island identified by phosphoproteomics. To determine whether these residues might be targets of PP2A, they were mutated to Alanine and Aspartate creating the double mutants T301/S308A and T301/S308D. T301/S308A mutant acts as the phospho-dead mutant whereas the T301/S308D mutant acts as the phospho-mimetic mutant. Immunoprecipitation studies of these mutants with PP2Ac- CS revealed, as expected, that phospho-mimetic mutant of DLC1 binds much stronger than the phospho-dead mutant of DLC1, which even upon EGF stimulation did not show any interaction (Fig 3.7, top panel, lane 2-6). The phospho-mimetic mutant showed binding, but to a much lesser degree, even without stimulation, whereas the wild-type DLC1 binding occurred, as previously shown, only upon stimulation (Fig 3.7, top panel, lane 1-4). The preference of $PP2A_C$ towards the phospho-mimetic mutant of DLC1 indicates that T301/S308 is probably the site of dephosphorylation for PP2A. Also, to confirm that the binding is due to the activation of the Ras-MAPK pathway downstream of EGF stimulation, we carried out the binding between DLC1 mutants and PP2A in the presence of constitutively active Mek2 (Mek2-SD) and dominant negative Mek2 (Mek2-K). We observed the binding only in the presence of Mek2-SD and not Mek2-K for both wtDLC1 and DLC1-T301/308D.

Whereas, as with the previous experiment, DLC1-T301/S308A did not bind to PP2A (Fig 3.8). These results are similar to that observed with the binding done in the presence and absence of EGF stimulation. We can conclude that EGF stimulation activates the Ras-MAPK pathway which regulates the interaction.

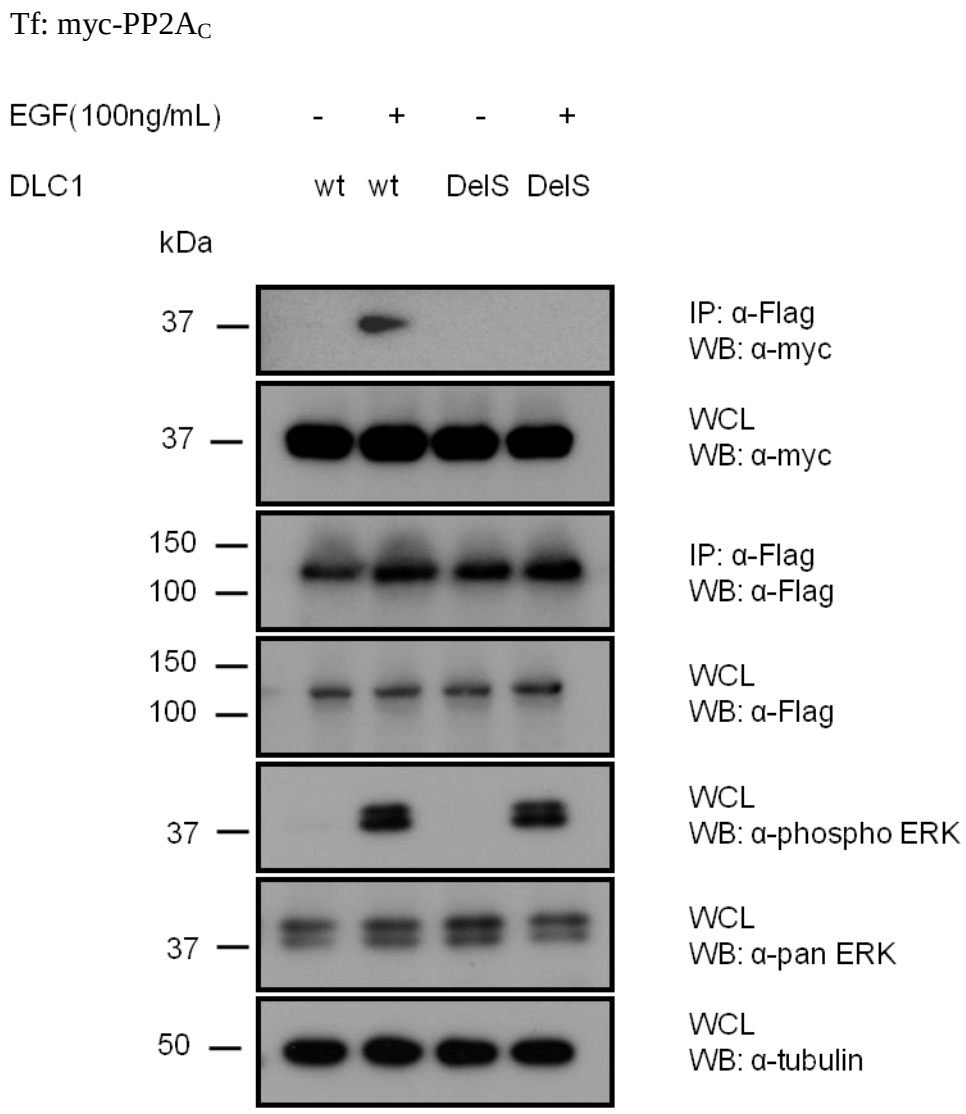


Figure 3.6: DLC1-ΔS binding with PP2A-C-CS upon EGF stimulation. HeLa JW cells were transfected with Flag-tagged DLC1 or Flag-tagged DLC1ΔS and myc-tagged PP2A-C-CS. The cells were starved for 18-24 hours in serum-free media and then stimulated for 0 or 10mins with EGF (100ng/ml) as indicated. The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the

WCL and IP were separated by SDS-PAGE and immunoblotted with the respective antibodies. The binding between DLC1 and PP2A-C-CS (top panel, lane 2 is lost upon deletion of S304-S308 of DLC1 (DLC1-ΔS) (top panel lane 4).

Tf: myc-PP2A$_C$

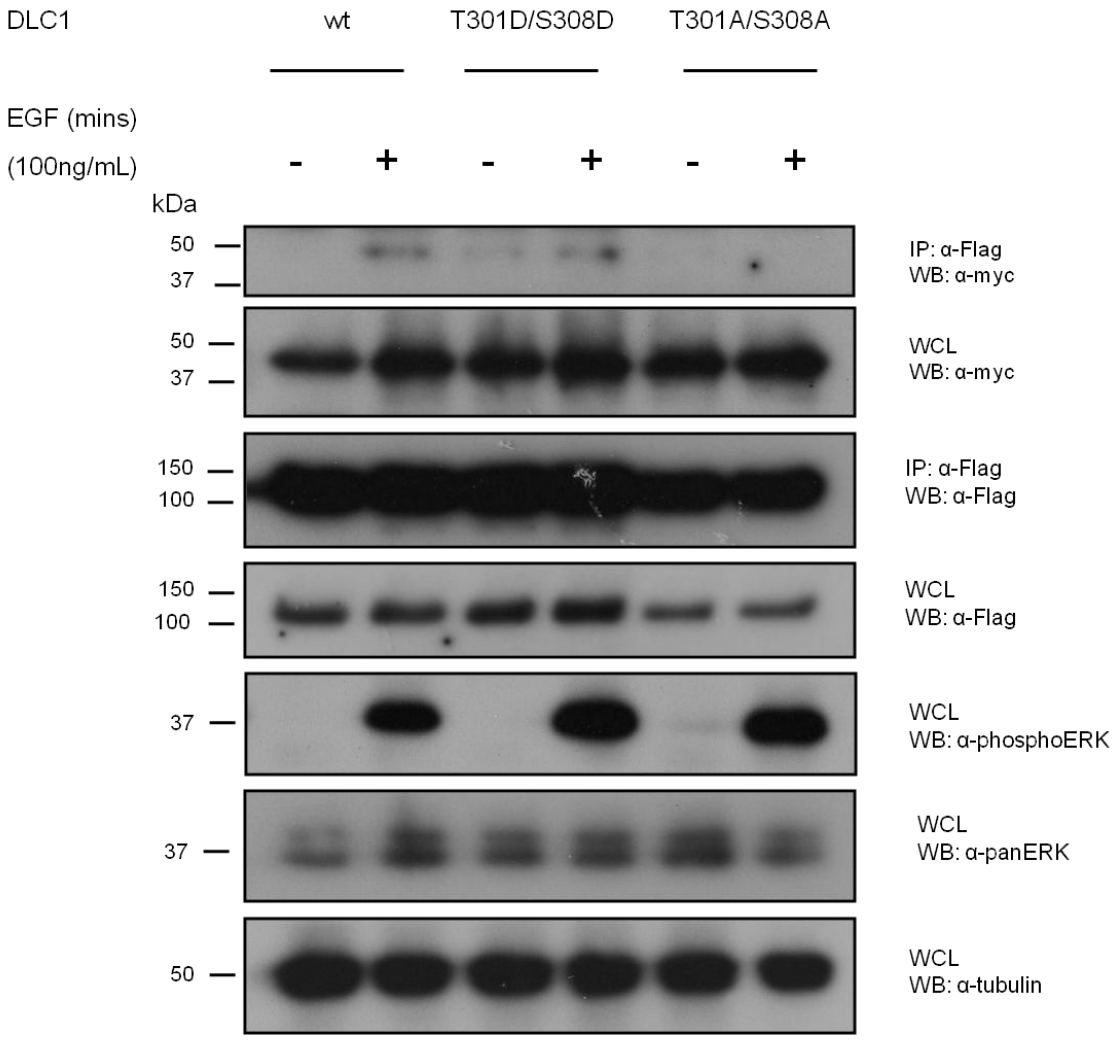


Figure 3.7: PP2A-C-CS binding with DLC1 phospho-mimetic and phospho-defective mutants. HeLa JW cells were transfected with Flag-tagged DLC1, Flag-tagged DLC1 (T301/S308D) and Flag-tagged DLC1 (T301/S308A). The cells were starved for 18-24 hours in serum-free media

and then stimulated for 0 or 10mins with EGF (100ng/ml). The cells were then lysed and the whole cell lysate (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the WCL and IP were separated by SDS-PAGE and immunoblotted with respective antibodies. PP2A-C-CS binds to the phosphor-mimetic DLC1 T301/S308D and not to the phosphor-defective DLC1 T301/S308A suggesting that T301/S308 is a site of dephosphorylation for PP2A on DLC1.

Tf:myc-PP2A$_C$-CS

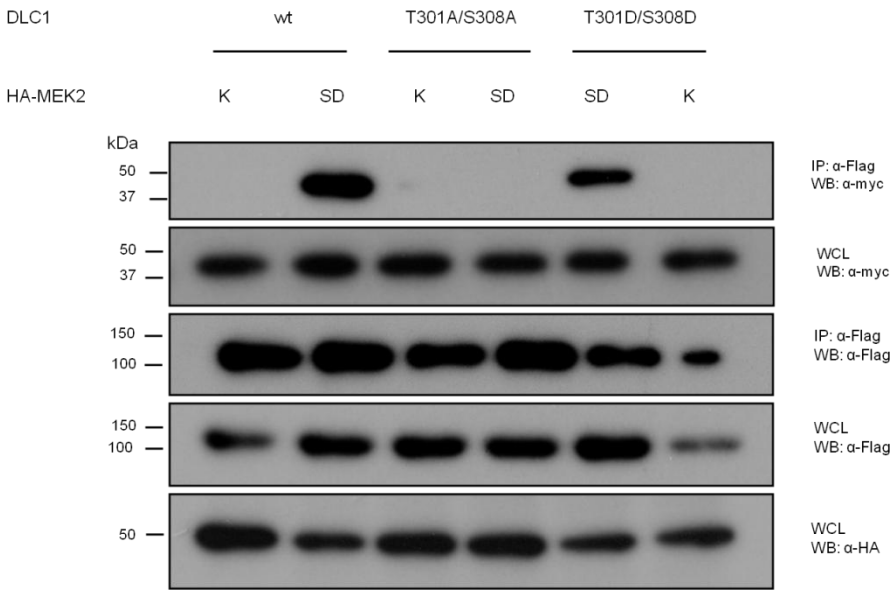


Figure 3.8: DLC1 interaction with PP2A is regulated by Ras-MAPK pathway downstream of EGF stimulation. HeLa JW cells were transfected with Flag-tagged DLC1, Flag-tagged DLC1 (T301/S308D) and Flag-tagged DLC1 (T301/S308A) as well as HA-Mek2-SD and HA-Mek2-K as indicated. Myc-PP2A$_C$-CS was transfected into all wells. The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the WCL and IP were separated by SDS-PAGE and immunoblotted with respective antibodies. wtDLC1 and the phospho-mimetic DLC1 (T301/S308D) bind to PP2A$_C$-CS in the presence of Mek2-SD (top panel, lanes 2 and 5 respectively) and not in the presence of Mek2-K (top panel, lanes 1 and 6). The phosphor-defective mutant DLC1 (T301/S308A) does not bind to PP2A$_C$-CS even with Mek2-SD indicating that the Ras-MAPK pathway activated downstream of EGF regulates DLC1-PP2A$_C$-CS interaction.

3.3 Effect of PP2A regulation on DLC1 GAP activity

As seen previously, EGF stimulation is required for DLC1 GAP activity (Fig 3.1) as well as for PP2A interaction with DLC1 (Fig 3.5). We wanted to confirm whether these two events were correlated or independent of each other. In addition, we wanted to check whether PP2A-mediated regulation of DLC1 by dephosphorylation had any direct effect on the RhoGAP function of DLC1 as it has been shown in previous studies that DLC1 can also be regulated to perform GAP-independent functions [Healy et al., 2008]

3.3.1 Dephosphorylation mediated by PP2A regulates DLC1 GAP activity

To further explore the role of phospho-mimetic and phospho-defective mutants of DLC1 on its GAP activity, we performed the RhoGAP assay by transfecting HeLa JW cells with vector, wtDLC1, DLC1-R677E, DLC1-T301/S308A and DLC1-T301/S308D. The sample set was subjected to EGF stimulation prior to lysis. Interestingly, the dephosphorylated-like form of DLC1, namely the phospho-dead mutant, and not the phosphorylated-like form (phospho-mimetic mutant) of DLC1 showed RhoGAP activity upon stimulation, which was similar to that of the wtDLC1 (Fig 3.9, top panel). This indicates that DLC1 has to undergo dephosphorylation by PP2A for it to be active.

We created a deletion mutant for the serine-rich island identified via phosphoproteomics, namely, DLC1-ΔS. With the DLC1-ΔS mutant by-passing the need for dephosphorylation, we hypothesized that DLC1 might get activated earlier. Hence, we proceeded to do a time-dependent EGF stimulation and compared the active RhoA levels between vector, wtDLC1

and DLC1-ΔS in HeLa JW cells. Fig 3.10a shows that wtDLC1 has maximal activity at 10mins post-stimulation (Top panel, centre section), whereas DLC1-ΔS seems to attain maximal activity earlier than wtDLC1 (Top panel, right section). It is also noteworthy to see that despite the deletion of the PP2A interacting sites, DLC1 still requires EGF stimulation for its activity. This attests the need for EGF stimulation and presence of another phosphorylation that is important for DLC1s GAP activity.

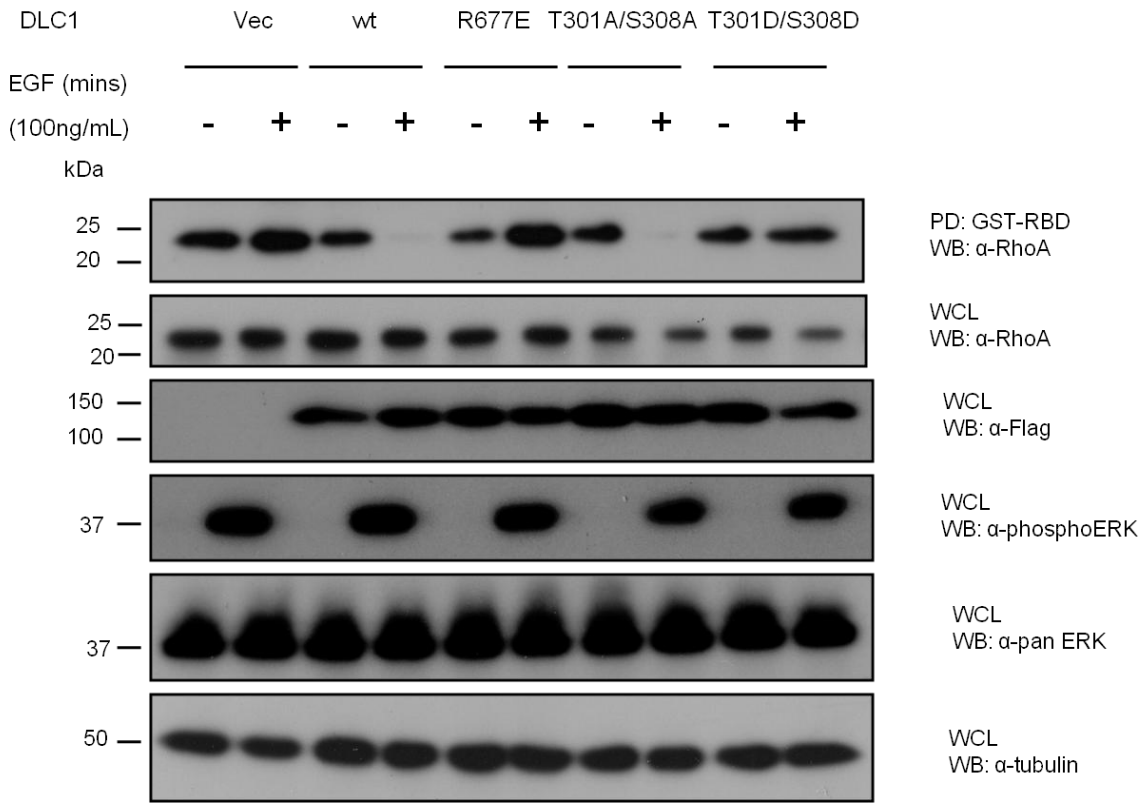
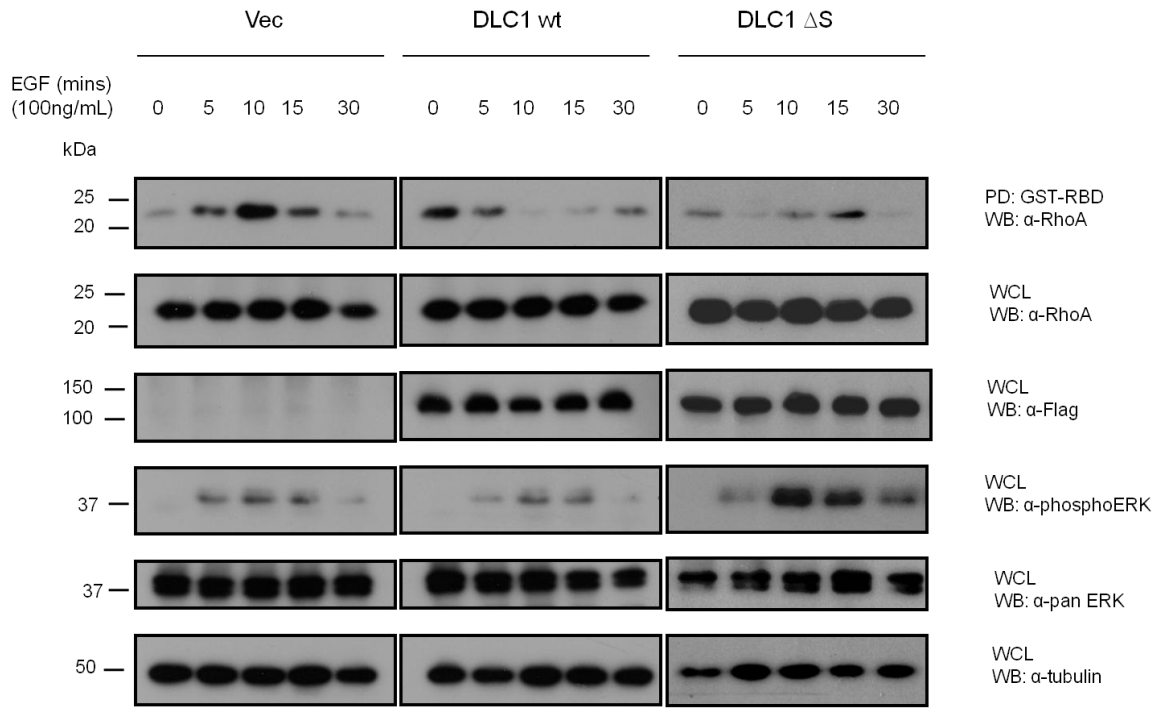


Figure 3.9: In vitro GAP activity of DLC1 phospho-defective and phospho-mimetic mutants. HeLa JW cells were transfected with Flag-tagged DLC1 and its mutants. The cells were starved for 18-24 hours in serum-free media and then stimulated for 0 or 10mins with EGF (100ng/ml). The cells were lysed and the whole cell lysate (WCL) were used for pull-down (PD) with glutathione sepharose 4B beads attached to GST-tagged RBD. Proteins in the WCL and PD were separated by SDS-PAGE and immunoblotted with

respective antibodies. EGF stimulation activates GAP activity of wtDLC1. The phospho-defective mutant DLC1 (T301/S308A) is also activated upon EGF stimulation whereas the phospho-mimetic mutant DLC1 (T301/S308D) remains inactive even with EGF stimulation. This indicates that DLC1 is active only upon dephosphorylation of T301/S308 residues.

a.



b.

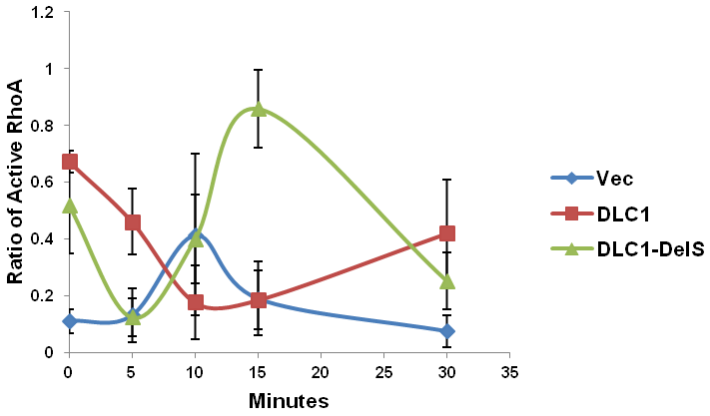


Figure 3.10: Time-dependent effect of EGF stimulation on the in vitro GAP activity of DLC1 and mutant. a) HeLa JW cells were transfected with

Flag-tagged DLC1, Flag-tagged DLC1-ΔS and Flag-tagged vector. The cells were starved for 18-24 hours in serum-free media and then stimulated with EGF (100ng/ml) for time intervals of 0, 5, 10, 15 and 30mins. The cells were lysed and the whole cell lysates (WCL) were used for pull-down (PD) with glutathione sepharose 4B beads attached to GST-tagged RBD. Proteins in the WCL and PD were separated by SDS-PAGE and immunoblotted with respective antibodies. b) shows the analysis done for RBD assay indicating the active levels of RhoA over time; n=3, $p<0.05$.

3.4 DLC1-PP2A interaction: Is there another regulator?

To confirm the binding between DLC1 and PP2A, immunoprecipitation was carried out in 293T cell line. Surprisingly, unlike the interaction in HeLa JW cells the binding between DLC1 and PP2A did not show dependence on EGF stimulation in 293T cells (Fig 3.11).

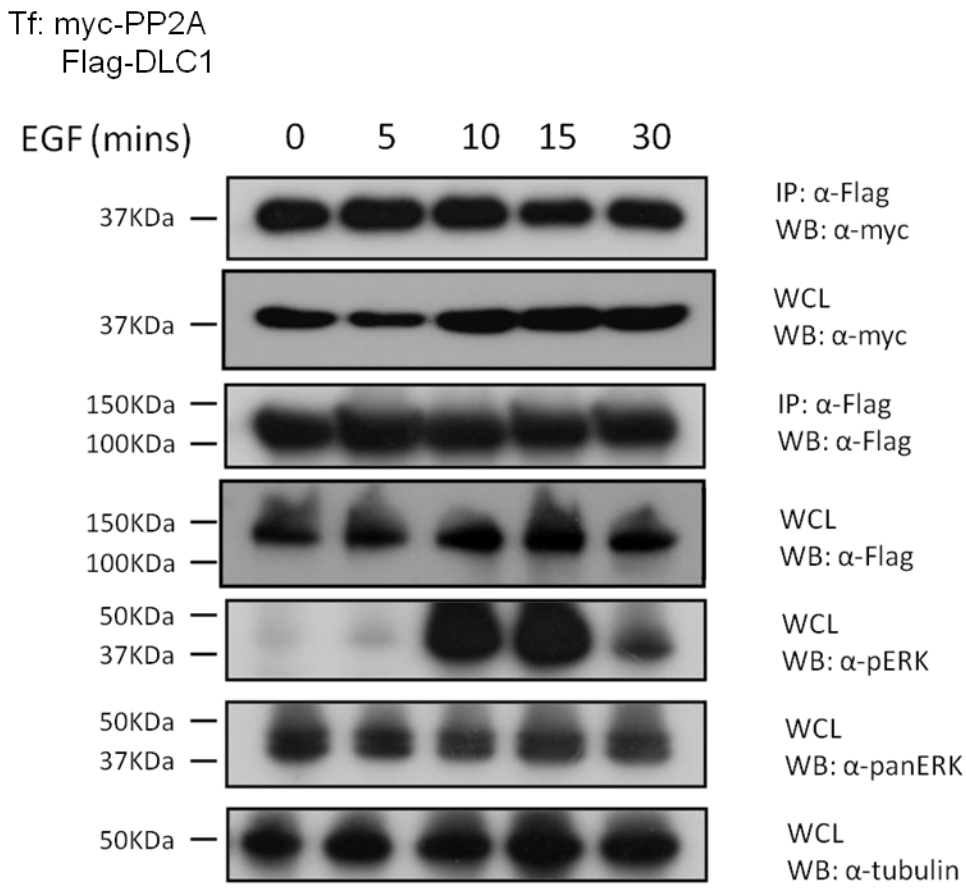


Figure 3.11: DLC1-PP2A-C-CS binding in 293T cells. 293T cells were transfected with Flag-tagged DLC1 and myc-tagged PP2A-C-CS. The cells were starved for 18-24 hours in serum-free media and then stimulated with EGF (100ng/ml) for duration of 0, 5, 10, 15, 30mins. The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the WCL and IP were separated by SDS-PAGE and immunoblotted with respective antibodies. The interaction between DLC1 and $PP2A_C$-CS occurs independent of EGF stimulation in 293T cells.

3.4.1 Focal Adhesion Kinase (FAK) check on DLC1-PP2A interaction

Since HeLa JW and 293T cells showed different binding pattern upon EGF stimulation, we wanted to check whether this effect was simply a cell-

line dependent one or there was another molecule involved. We compared the lysates of HeLa JW and 293T and probed for various proteins which might potentially regulate this interaction. The proteins initially probed for were based on the prior knowledge of their interaction with DLC1. Interestingly, 293T cells lacked total FAK and the active form of FAK pY397 but HeLa JW was found have both the proteins in abundant quantity (Fig 3.12, 3rd and 4th panel from the top).

FAK has been previously shown to be a DLC1 interacting partner and is important for DLC1s tumor suppressive functions ([Li et al., 2011]. To confirm that the differential binding seen in HeLa JW and 293T was indeed because of FAK, we used two cell lines, namely, wild-type mouse embryonic fibroblasts (MEFs) and FAK-/- MEFs. The two cell lines were transfected with DLC1 and $PP2A_C$ and EGF stimulation was carried out before cell lysis. The immunoprecipitation study showed DLC1-PP2A interaction in wtMEFs is EGF stimulation dependent whereas it is not in the case of FAK-/- MEFs (Fig 3.13). This proves that the interaction between the two proteins is not a cell line-dependent effect but dependent on FAK as a regulator.

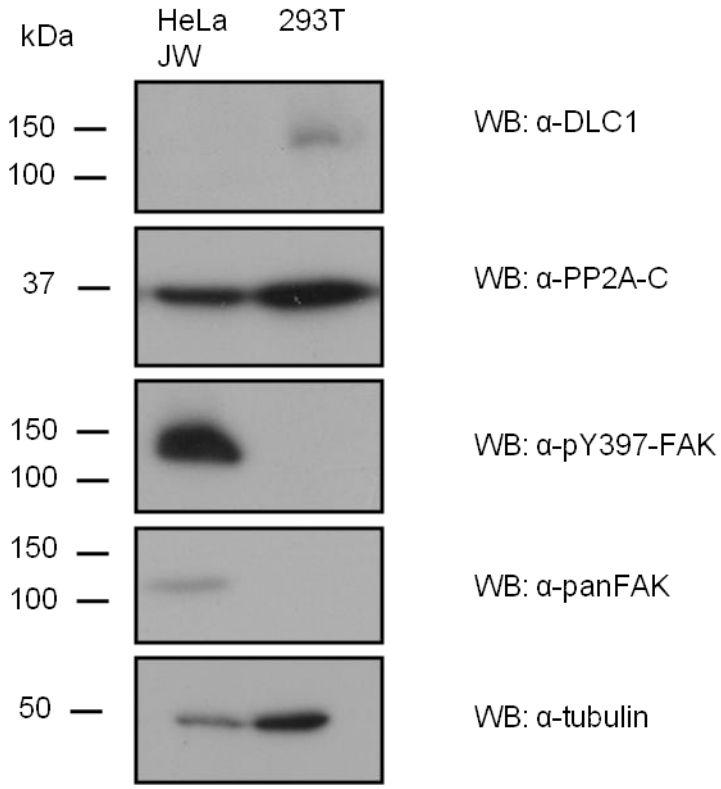


Figure 3.12: FAK expression profile in HeLa JW and 293T cells. HeLa JW and 293T cells were seeded in serum-containing media. The cells were lysed and the whole cell lysate (WCL) proteins were separated by SDS-PAGE and immunoblotted with respective antibodies. DLC1 is present in 293T cells and absent in HeLa JW cells whereas FAK is present in HeLa JW and absent in 293T cells.

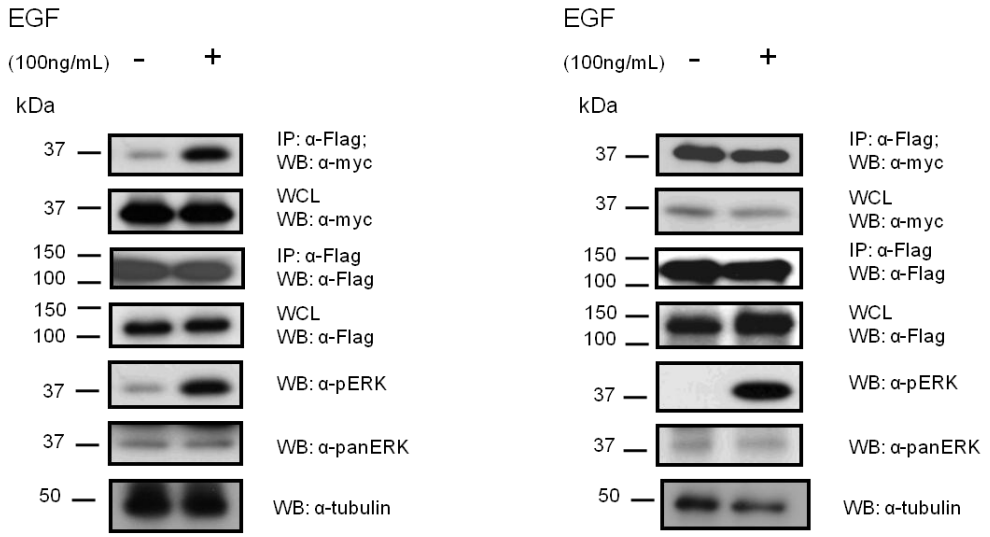


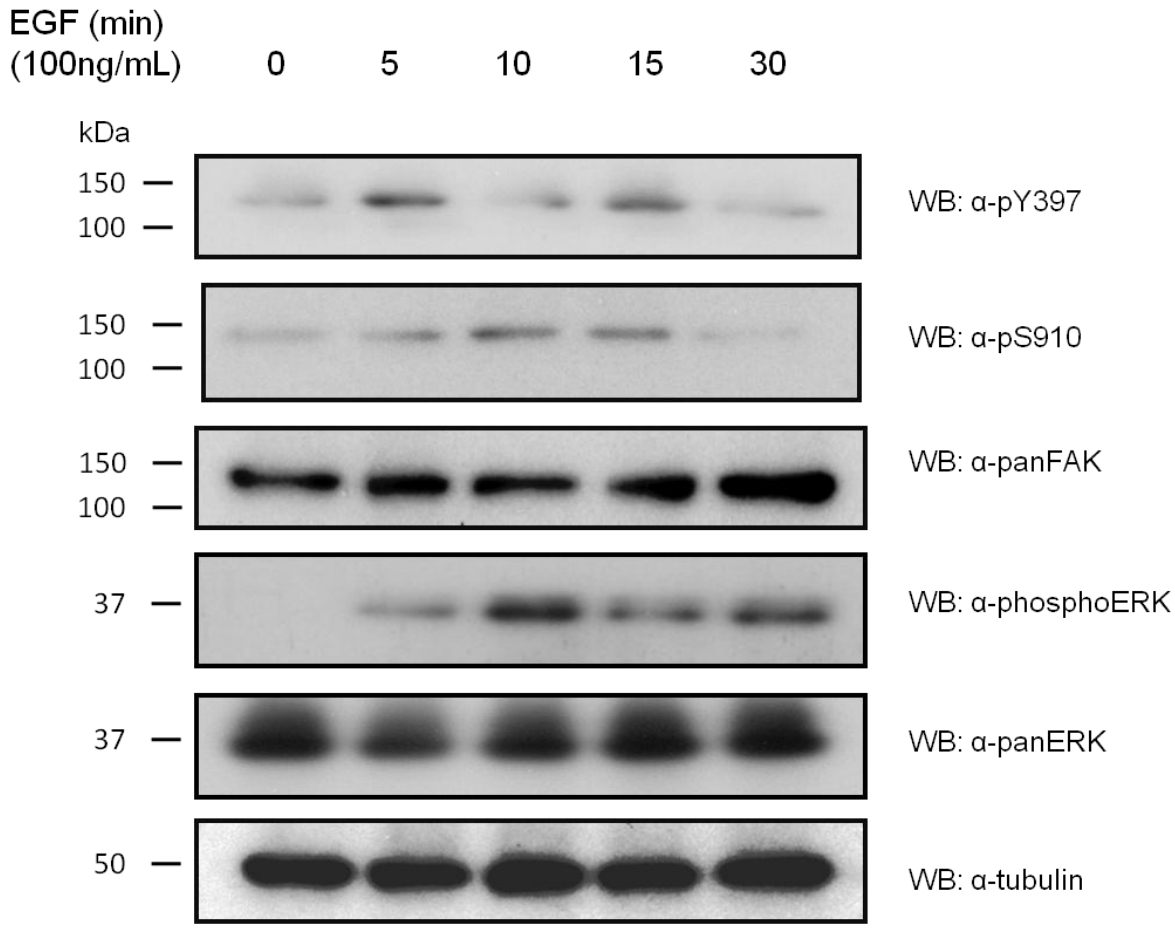
Figure 3.13: DLC1-PP2A-C-CS binding in wtMEFs and FAK-/- MEFs. wtMEFs (left) and FAK-/- MEFs (right) were transfected with Flag-tagged DLC1 and myc-tagged PP2A-C-CS. The cells were starved for 18-24 hours in serum-free media and then stimulated for 10mins with EGF (100ng/ml). The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the WCL and IP were separated by SDS-PAGE and immunoblotted with respective antibodies. DLC1-PP2A$_C$-CS interaction is dependent on EGF stimulation in wtMEF whereas in FAK-/- MEFs this interaction is independent of EGF stimulation.

3.4.2 Inactivation of FAK by Ras-MAPK pathway allows for PP2A interaction with DLC1

The next question we asked was- In what manner does EGF stimulation regulate FAK to mediate the binding between DLC1 and PP2A? The answer to this might lie in the inactivation of FAK by EGF. EGF stimulation- dependent phosphorylation of the S910 residue on FAK recruits Pin1 and PTP-PEST, a tyrosine phosphatase to FAK, leading to the dephosphorylation of Y397, which is important for FAKs active status [Zheng et al., 2009].

To determine the phosphorylation status of FAK upon EGF treatment, HeLa JW cells were subjected to EGF stimulation and were lysed at varying pre-fixed time points and the lysates were blotted for pS910, pY397 and panFAK. Fig 3.14a shows that at maximal activation of the Ras-MAPK pathway (10mins post stimulation), the pS910 levels was also maximum. In contrast the Y397 phosphorylation levels were the lowest at this time point, indicating that FAK was getting inactivated at this stage of stimulation. This is in concurrence with the EGF regulation of FAK phosphorylation that has been previously observed by others [Zheng et al., 2009]

a.



b.

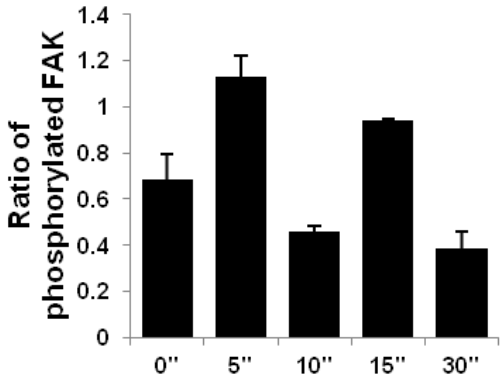


Figure 3.14: EGF stimulation dependent change in FAK S910 and Y397 phosphorylation. a) HeLa JW cells were seeded in 6-well plates. The cells were starved for 18-24 hours in serum-free media and then stimulated with EGF (100ng/ml) for 0, 5, 10, 15 and 30mins. The cells were lysed and the whole cell lysate (WCL) proteins were separated by SDS-PAGE and immunoblotted with respective antibodies. b) shows statistical analysis for the ratio of pY397 to total FAK present in the cells; n=3, $p<0.05$.

To ensure that the FAK phosphorylation on S910 residue and the subsequent dephosphorylation on Y397 residue is downstream of the Ras-MAPK pathway, we used U0126, a Mek/Erk inhibitor, followed by EGF stimulation and blotted for the different phosphorylated states of FAK. As shown in Fig 3.15, U0126 treatment reduces the S910 phosphorylation and consequently inactivation of FAK is inhibited.

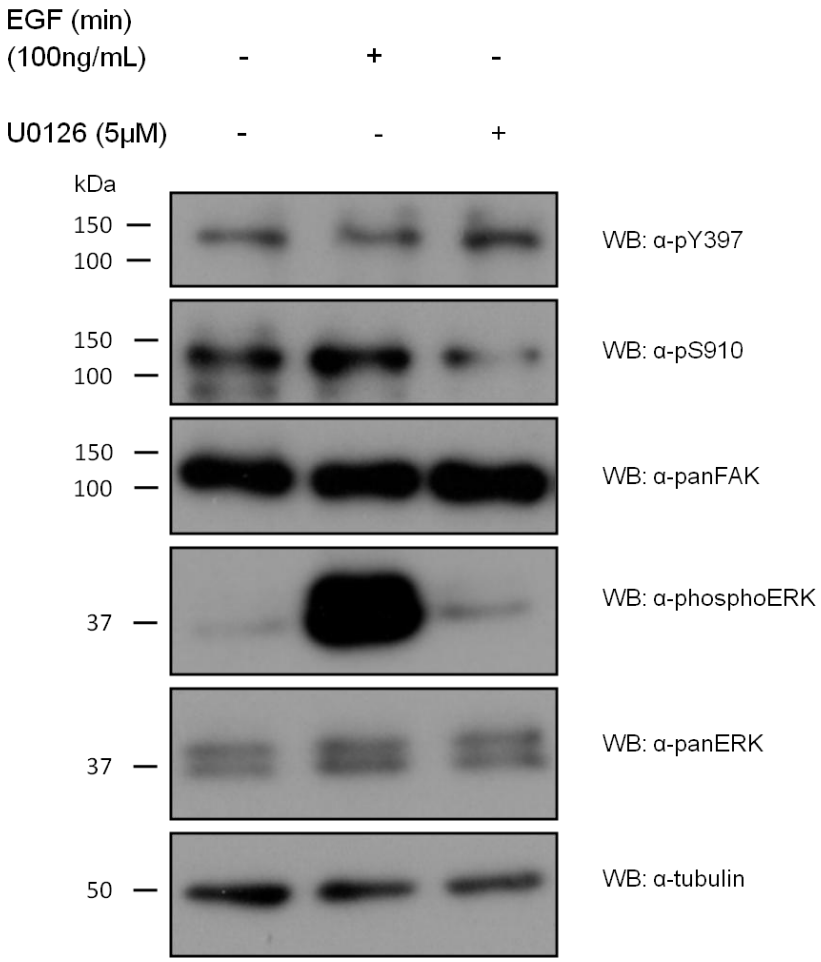


Figure 3.15: U0126 treatment inhibits EGF-mediated change in phosphorylation of FAK S910 and Y397. HeLa JW cells were seeded in 6-well plates. The cells were starved for 18-24 hours in serum-free media. This was followed by U0126 treatment for 1hr and EGF stimulation (100ng/ml) for 0 and 10mins as indicated. The cells were lysed and the whole cell lysate (WCL) proteins were separated by SDS-PAGE and immunoblotted with respective antibodies. Upon EGF stimulation, the phosphorylation of FAK on S910 increases (2nd panel, lane 2) and upon U0126 treatment, the levels of S910 phosphorylation decreases (2nd panel, lane 3). A consequent increase in phosphorylation of Y394 on FAK is observed (top panel, lane 3) upon decrease of S910 phosphorylation.

To confirm the regulation of DLC1-PP2A$_C$-CS by FAK, we repeated the DLC1-PP2A$_C$ interaction in HeLa JW and 293T cells with FAK inhibitor treatment and over-expression of FAK respectively. We observed that the

binding profile of HeLa JW with FAK inhibitor treatment now matches that of 293T without FAK over-expression and that of 293T with FAK over-expression matches HeLa JW without the inhibitor treatment (Fig 3.16 and Fig 3.17). We now conclude that FAK in its active form inhibits the DLC1-PP2A binding and upon FAK inhibition, this constraint is lifted, allowing the interaction to occur.

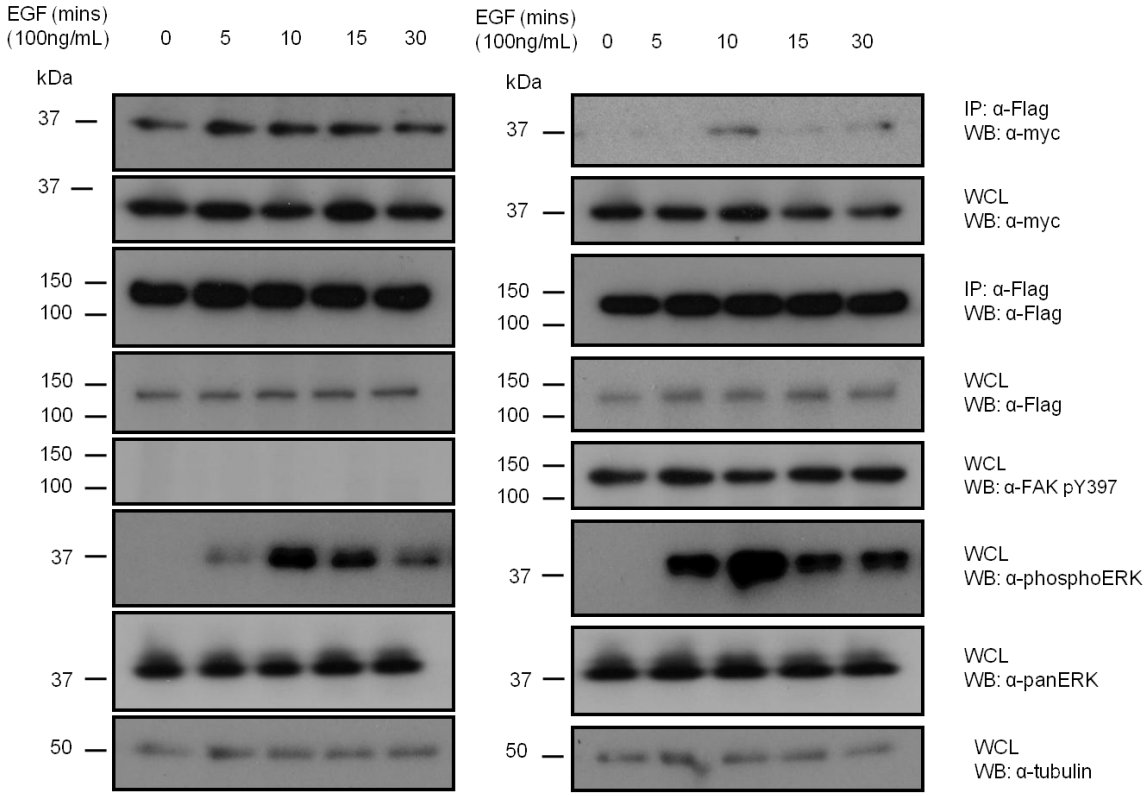


Figure 3.16: DLC1-PP2A-C-CS binding in HeLa JW cells with and without FAK inhibitor treatment. HeLa JW cells were transfected with Flag-tagged DLC1 and myc-PP2A-C-CS. The cells were starved for 18-24 hours in serum-free media, treated with FAK inhibitor (left) or DMSO (right) and then stimulated with EGF (100ng/ml) for 0, 5, 10, 15, 30mins. The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the WCL and IP were separated by SDS-PAGE and immunoblotted with respective antibodies. Upon inhibition of FAK in HeLa JW cells, the DLC1-$PP2A_C$-CS interaction is independent of EGF stimulation (left) whereas under normal conditions the interaction is dependent on EGF stimulation (right).

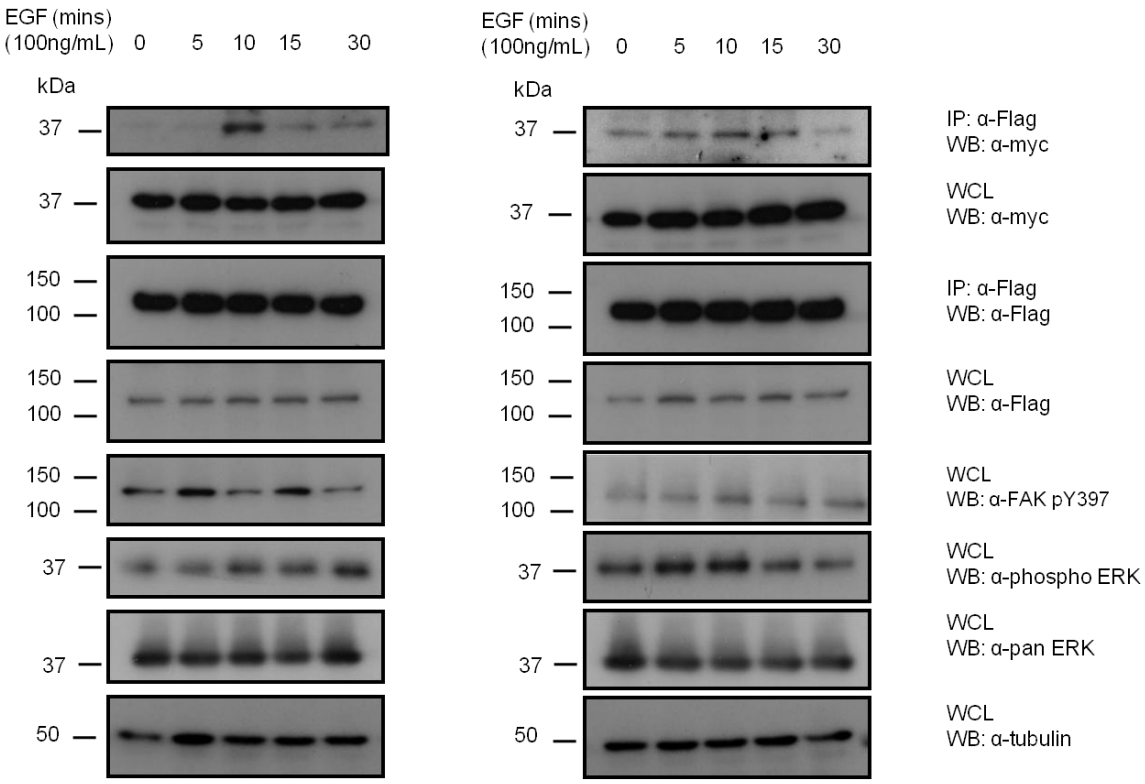


Figure 3.17: DLC1-PP2A-C-CS binding in 293T cells with and without FAK overexpression. 293T cells were transfected with Flag- DLC1, myc-PP2A-C-CS and either mcherry-FAK (left) or mcherry-vector (right). The cells were starved for 18-24 hours in serum-free media and then stimulated with EGF (100ng/ml) for 0, 5, 10, 15, 30mins. The cells were lysed and the whole cell lysates (WCL) were used for immunoprecipitation (IP) with M2 anti-Flag agarose beads. Proteins in the WCL and IP were separated by SDS-PAGE and immunoblotted with respective antibodies. In 293T cells, upon transfection with FAK, the previously EGF independent binding of DLC1-$PP2A_C$-CS becomes dependent on EGF stimulation with a peak interaction at 10min post stimulation.

3.4.3 EGF stimulation controls DLC1 activity in a two-pronged manner

FAK plays a major role in regulating the active RhoA levels. In FAK-/- cells the intrinsic active RhoA levels are elevated. Also, FAK seems to be involved in inactivating RhoA [Orr et al., 2004]. We wanted to see how the interplay of DLC1, PP2A and FAK affected the GAP activity of DLC1. For this purpose we used inhibitors of FAK and PP2A, PF-573228 and Okadaic acid respectively and carried out the RhoGAP assay post-stimulation. In Fig 3.18 we observed that DLC1 lacks GAP activity when treated with Okadaic acid (top right panel, lane 4) whereas upon FAK inhibitor treatment the GAP function of DLC1 is still retained (top right panel, lane 6). With or without treatment of either of the inhibitors, DLC1 gets activated only upon stimulation with EGF. This suggests that EGF stimulation is necessary but not sufficient to activate DLC1 RhoGAP function and it requires dephosphorylation by PP2A for full activity. This dephosphorylation event is control indirectly by EGF as well, because of its control on the active state of FAK, which in the absence of stimulation prevents PP2A from interacting with DLC1.

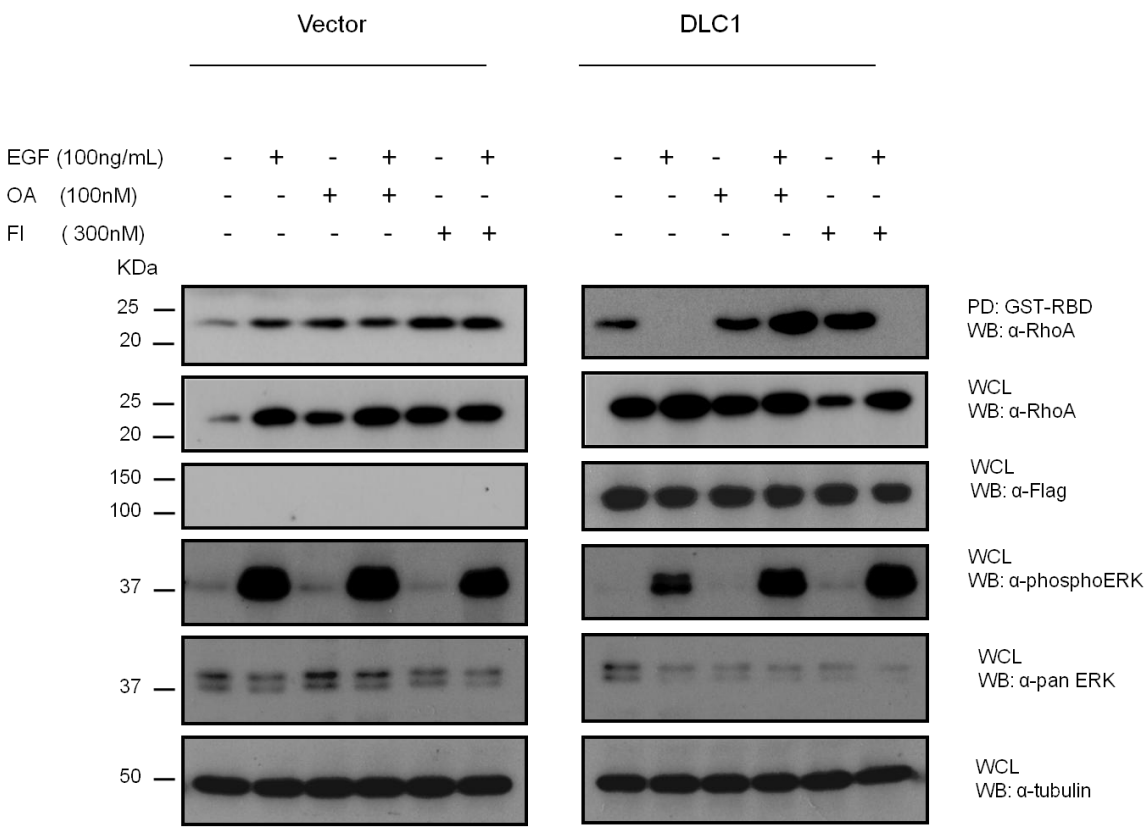


Figure 3.18: In vitro GAP activity of DLC1 on endogenous RhoA upon Okadaic acid and FAK inhibitor treatment. HeLa JW cells were transfected with Flag-tagged DLC1 (right) and Flag-vector (left). The cells were starved for 18-24 hours in serum-free media and then treated with Okadaic acid (OA), FAK inhibitor (FI) and/or EGF (100ng/ml) as indicated. The cells were lysed and the whole cell lysates (WCL) were used for pull-down (PD) with glutathione sepharose 4B beads attached to GST-tagged RBD. Proteins in the WCL and PD were separated by SDS-PAGE and immunoblotted with respective antibodies. Treatment with OA overrides the activation of DLC1's GAP activity brought about by EGF stimulation whereas treatment with FI has no effect on the activation of DLC1 upon EGF stimulation.

3.5 DLC1 mediated change in cell spreading and motility

DLC1 is a known tumor-suppressor and it is important to study the role of this protein in the process of cancer initiation and progression. It has been

seen that various cancers carry mutations in the *DLC1* gene or it is entirely deleted from the genome [Durkin et al., 2007b]. For cancer progression, the affected tissues have to migrate from the region of origin to a different location and this is called metastasis. Cell migration is the first step in metastasis. Cell migration itself can be broken down to multiple steps, with the attachment and spreading of the cells on the extracellular matrix initiating the process. These processes are spatially and temporally controlled by RhoGTPases. DLC1 expression in cells has been attributed to change in cell morphology as well as inhibition of directional cell migration [Kim et al., 2008].

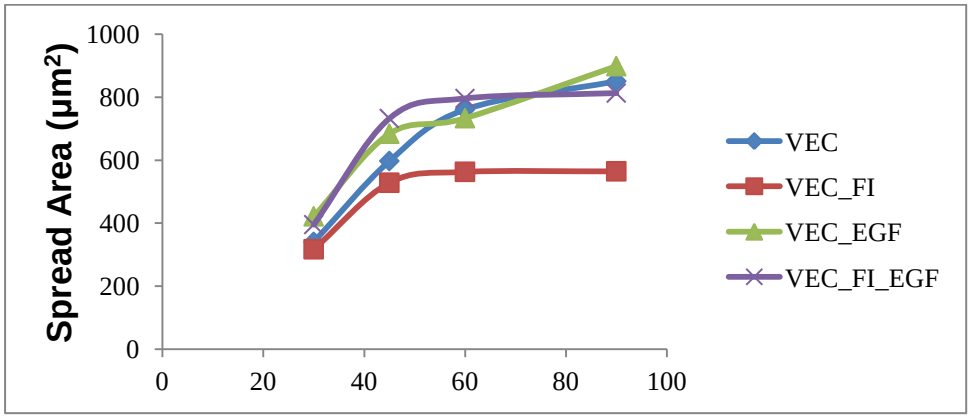
3.5.1 DLC1 enhances cell spreading in a GAP-dependent manner

We have elucidated a novel mechanism by which DLC1s activation is controlled in a temporal manner. Our next step was to extrapolate our findings to physiological functions and see the effect of DLC1-GAP activity on different yet connected cellular processes. We wanted to see if DLC1 has an effect on cell spreading as well and whether this was a GAP-dependent process. Cell spreading is a result of cyclic activation of Rac and RhoA. Decrease in cell spreading is a result of inhibition of membrane protrusion, mediated by Rho-dependent increase in cellular contractility. It has previously been shown that p190RhoGAP controls cell spreading in a RhoA-dependent manner [Arthur and Burridge, 2001]. We expected DLC1 to behave in a similar manner.

HeLa JW cells transfected with vector-GFP, DLC1-GFP and DLC1-R677E-GFP we plated as described in materials and methods and fixed at given time-points. The spreading trend for each sample was plotted over the course of 90minutes. We observed that in all cases, the spread area increased

over time Fig 3.19. To make the process of analysis simpler, we picked one constant time point across the sample sets for comparison (60 mins).

Even without stimulation, DLC1-transfected cells spread much better than vector- and DLC1-R677E- transfected cells. With FAK inhibitor treatment, there is a drastic decrease in spread area in all the sample sets (Fig 3.20). It has been previously shown that in FAK-/- cells, the levels of active RhoA are high. Increase in RhoA levels translates to higher contractility in the cells and as seen in the spreading experiment this decreases the spread area of the cells. Stimulating the cells with EGF reverses the effect of FAK inhibitor treatment alone, increasing the spread area of the cells transfected with DLC1 (Fig 3.21). This corresponds to DLC1-mediated suppression of active RhoA levels in the cell. This effect is a GAP-dependent function because cells transfected DLC1-R677E, the GAP-dead mutant do not show change in spread area even upon EGF treatment and are comparable to the vector transfected cells (Fig 3.22).



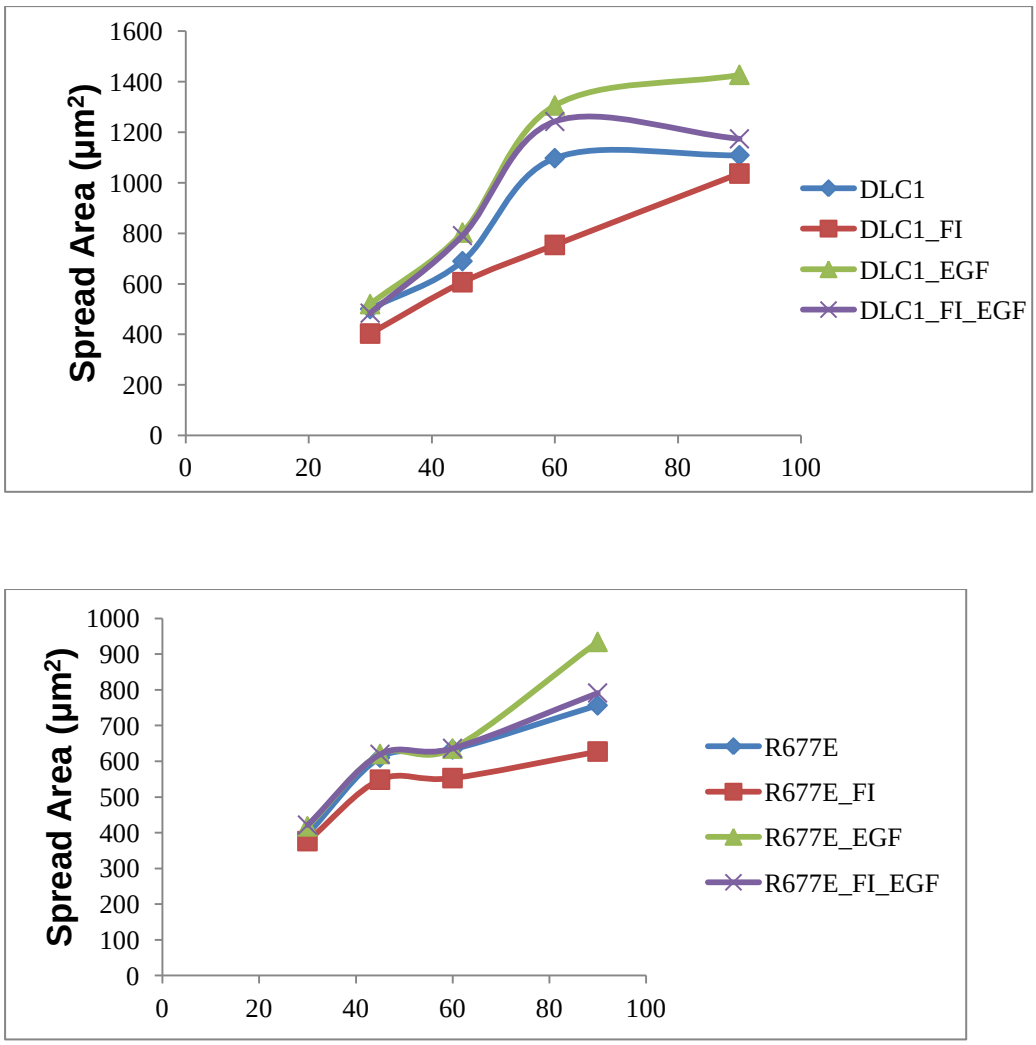
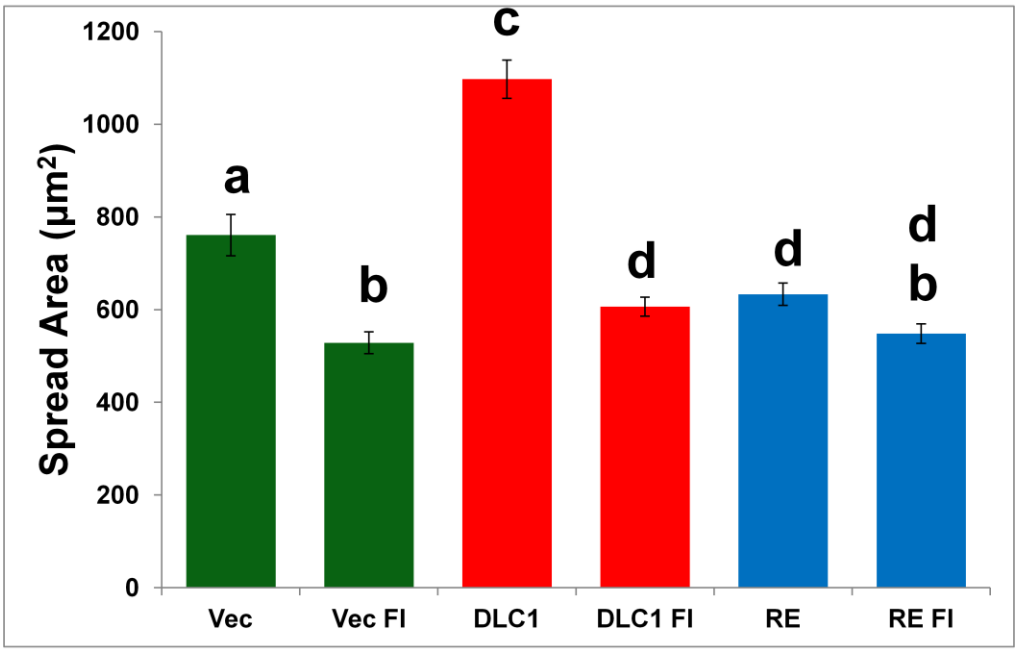
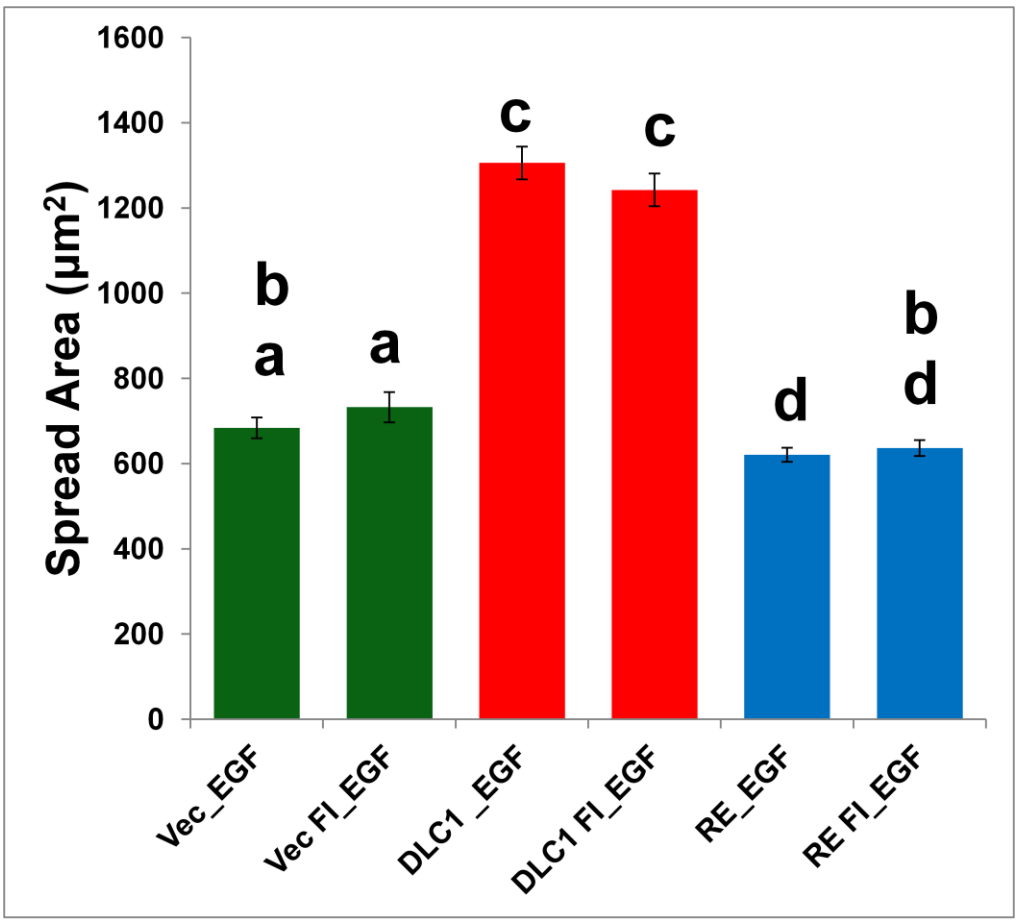


Figure 3.19: Spreading trend of cells over a period of 90mins: HeLa JW cells were transfected with GFP-vector, -DLC1 and –DLC1-R677E. Cells were subjected to different treatments as indicated. FI indicates FAK inhibitor treatment and EGF indicates EGF stimulation. The cells were plated and fixed at 0, 30, 45, 60 and 90mins. Spread area of the cells was calculated by drawing an outline of the cells using ImageJ software and measuring the area enclosed in the outline. The graphs were plotted as spread area over time.



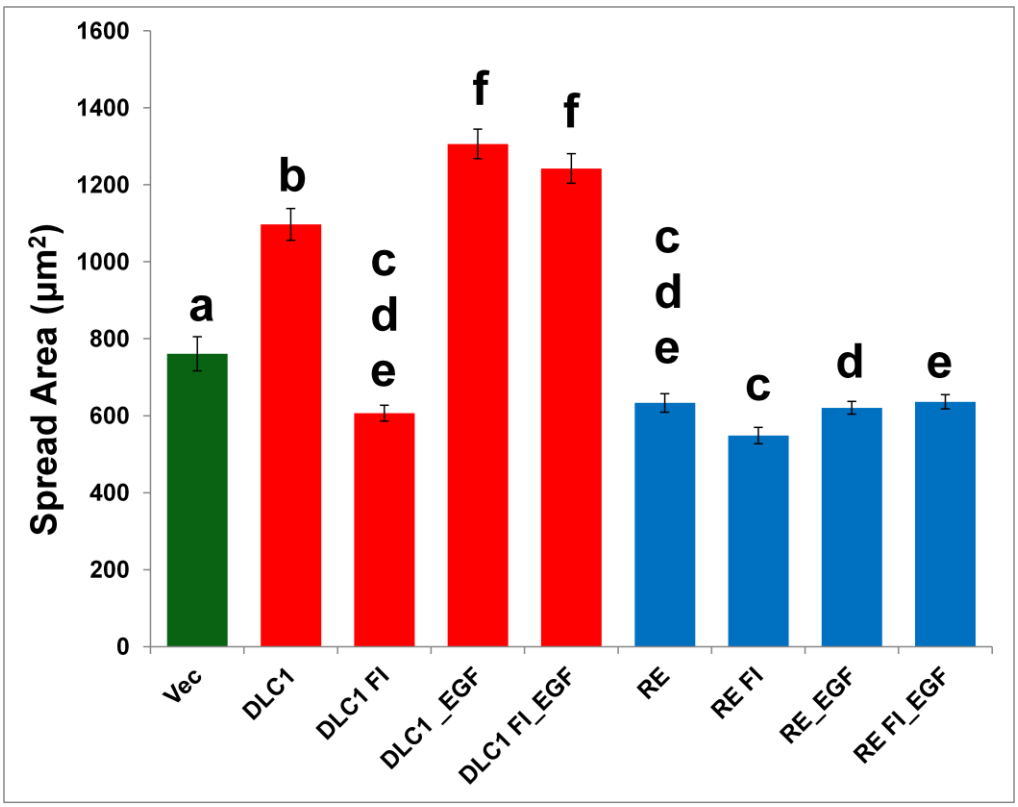
FI: FAK inhibitor treatment
RE: R677E

Figure 3.20: DLC1-transfected cells spread better, an effect that is reversed by FAK inhibitor treatment: HeLa JW cells were transfected with GFP-vector, -DLC1 and –DLC1-R677E. The cells were starved for 18-24 hours in serum-free media and then subjected to different treatments as indicated. FI indicates FAK inhibitor treatment. Spread area of the cells was calculated by drawing an outline of the cells using ImageJ software and measuring the area enclosed in the outline. Annova was used for statistical analysis, n=160, $p<0.05$. The analysed data shown is for cells fixed 60mins post-plating. The different alphabets are indicative of statistical significance with respect to each other and the error bars denote standard error.



FI: FAK inhibitor treatment
EGF: EGF stimulation
RE: R677E

Figure 3.21: EGF stimulation reverses the effect of FAK inhibitor treatment: HeLa JW cells were transfected with GFP-vector, -DLC1 and –DLC1-R677E. The cells were starved for 18-24 hours in serum-free media and then subjected to different treatments as indicated. FI indicates FAK inhibitor treatment and EGF indicates EGF stimulation. Spread area of the cells was calculated by drawing an outline of the cells using ImageJ software and measuring the area enclosed in the outline. Annova was used for statistical analysis, n=160, $p<0.05$. The analysed data shown is for cells fixed 60mins post-plating. The different alphabets are indicative of statistical significance with respect to each other and the error bars denote standard error.

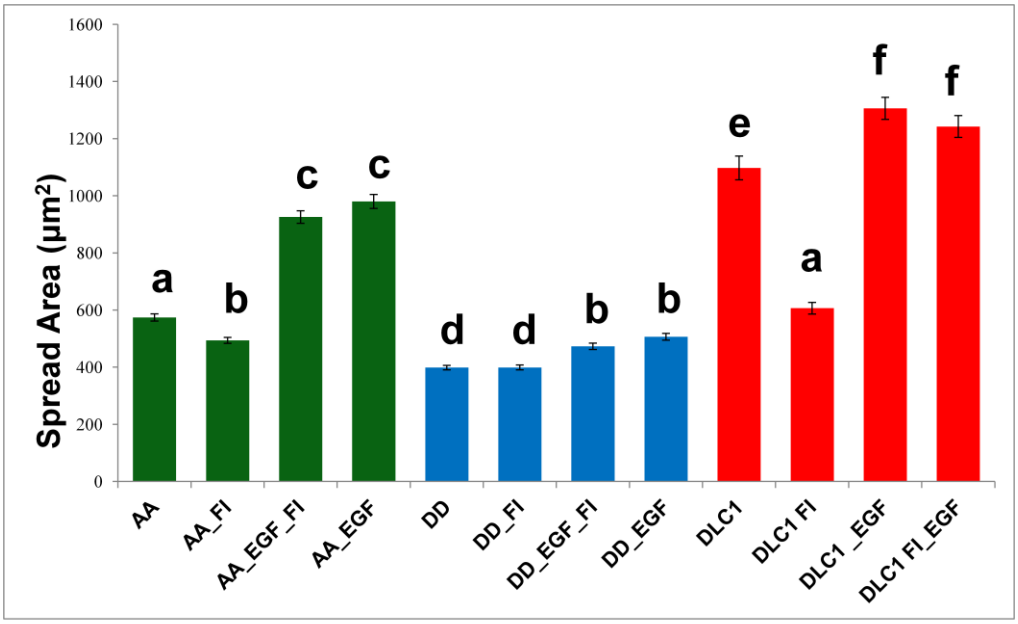


FI: FAK inhibitor treatment
EGF: EGF stimulation
RE: R677E

Figure 3.22: Cell spreading is a GAP-dependent function of DLC1: HeLa JW cells were transfected with GFP-vector, -DLC1 and –DLC1-R677E. The cells were starved for 18-24 hours in serum-free media and then subjected to different treatments as indicated. FI indicates FAK inhibitor treatment and EGF indicates EGF stimulation. Spread area of the cells was calculated by drawing an outline of the cells using ImageJ software and measuring the area enclosed in the outline. Annova was used for statistical analysis, n=160, $p<0.05$. The analysed data shown is for cells fixed 60mins post-plating. The different alphabets are indicative of statistical significance with respect to each other and the error bars denote standard error.

To confirm the GAP-dependence of the spreading capabilities of the cells, they were transfected with the phospho-mutants of DLC1, namely, DLC1-T301/S308A and DLC1-T301/S308D. These two mutants showed

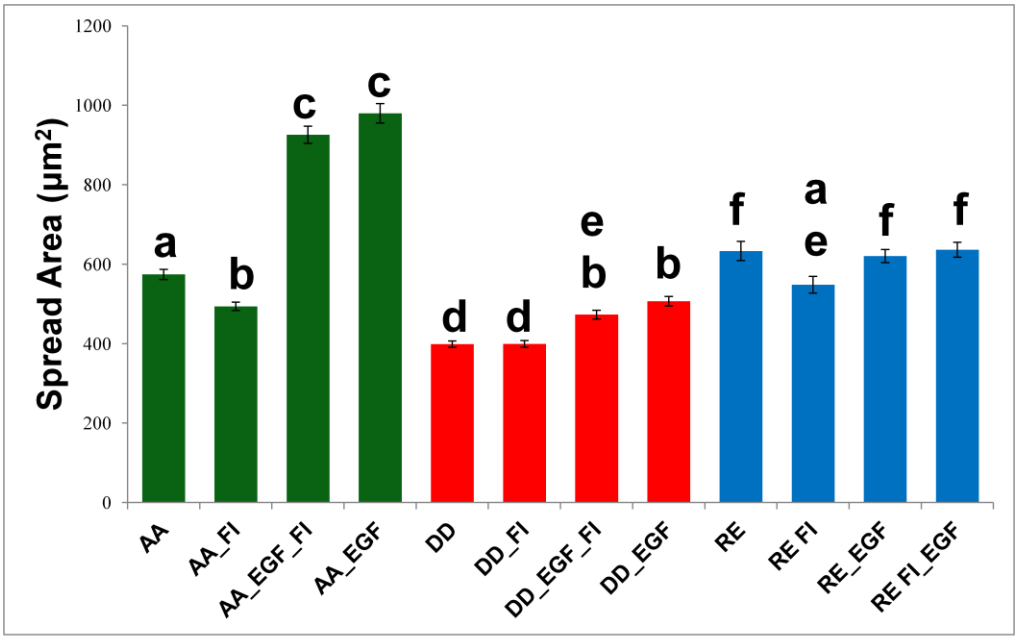
differential RhoGAP activity, with phospho-dead mutant GAP activity mimetic the wtDLC1 and the phospho-mimetic mutant activity being similar to DLC1-R677E. The cell spreading experiment indeed confirms that the alanine mutant spreads better than the aspartate mutant (Fig 3.23 and Fig 3.24). This shows that the novel mechanism of regulation of DLC1 comes into play as early as spreading.



FI: FAK inhibitor treatment
EGF: EGF stimulation
RE: R677E

Figure 3.23: Phospho-defective mutant cell spreading pattern is similar to that of wtDLC1: HeLa JW cells were transfected with GFP-DLC1, DLC1-T301/S308A and –DLC1-T301/308D. Cells were subjected to different treatments as indicated. The cells were starved for 18-24 hours in serum-free media and then subjected to different treatments as indicated. FI indicates FAK inhibitor treatment and EGF indicates EGF stimulation. Spread area of the cells was calculated by drawing an outline of the cells using ImageJ software and measuring the area enclosed in the outline. Annova was used for statistical analysis, n=160, $p<0.05$. The analysed data shown is for cells fixed 60mins post-plating. The different alphabets are indicative of statistical

significance with respect to each other and the error bars denote standard error.

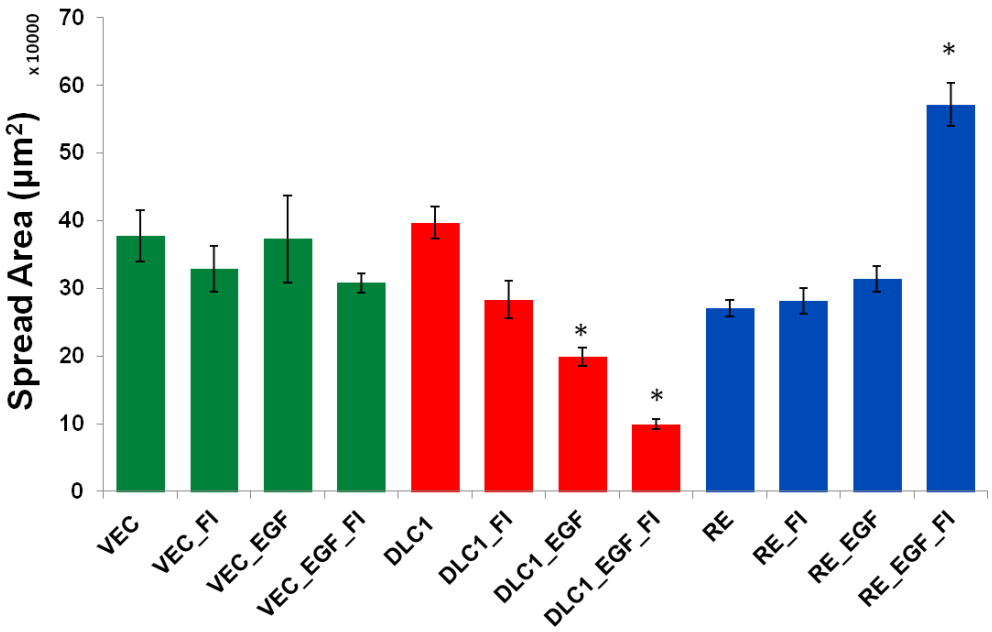


FI: FAK inhibitor treatment
EGF: EGF stimulation
RE: R677E

Figure 3.24: Phospho-mimetic mutant cell spreading pattern is similar to that of DLC1-R677E: HeLa JW cells were transfected with GFP-DLC1-R677E, DLC1-T301/S308A and –DLC1-T301/308D. The cells were starved for 18-24 hours in serum-free media and then subjected to different treatments as indicated. FI indicates FAK inhibitor treatment and EGF indicates EGF stimulation. Spread area of the cells was calculated by drawing an outline of the cells using ImageJ software and measuring the area enclosed in the outline. Annova was used for statistical analysis, n=160, $p<0.05$. The analysed data shown is for cells fixed 60mins post-plating. The different alphabets are indicative of statistical significance with respect to each other and the error bars denote standard error.

3.5.2 DLC1 inhibits cell migration only upon EGF stimulation

Deregulation of the RhoGTPases, decreases cell polarity leading to the loss of directional cell migration. It has been previously shown that DLC1 impairs wound healing. This is mainly due to the reduction in the directionality and not due to the decreased velocity. In the light of the steps that might be leading to activation of DLC1, we wanted to see how the aforementioned treatments affect the migration of the cell. When cells were transfected with DLC1, vector and DLC1-R677E, DLC1 seemed to impair the closure of the wound only upon EGF or EGF/FAK inhibitor treatment (Fig 3.25a and b).

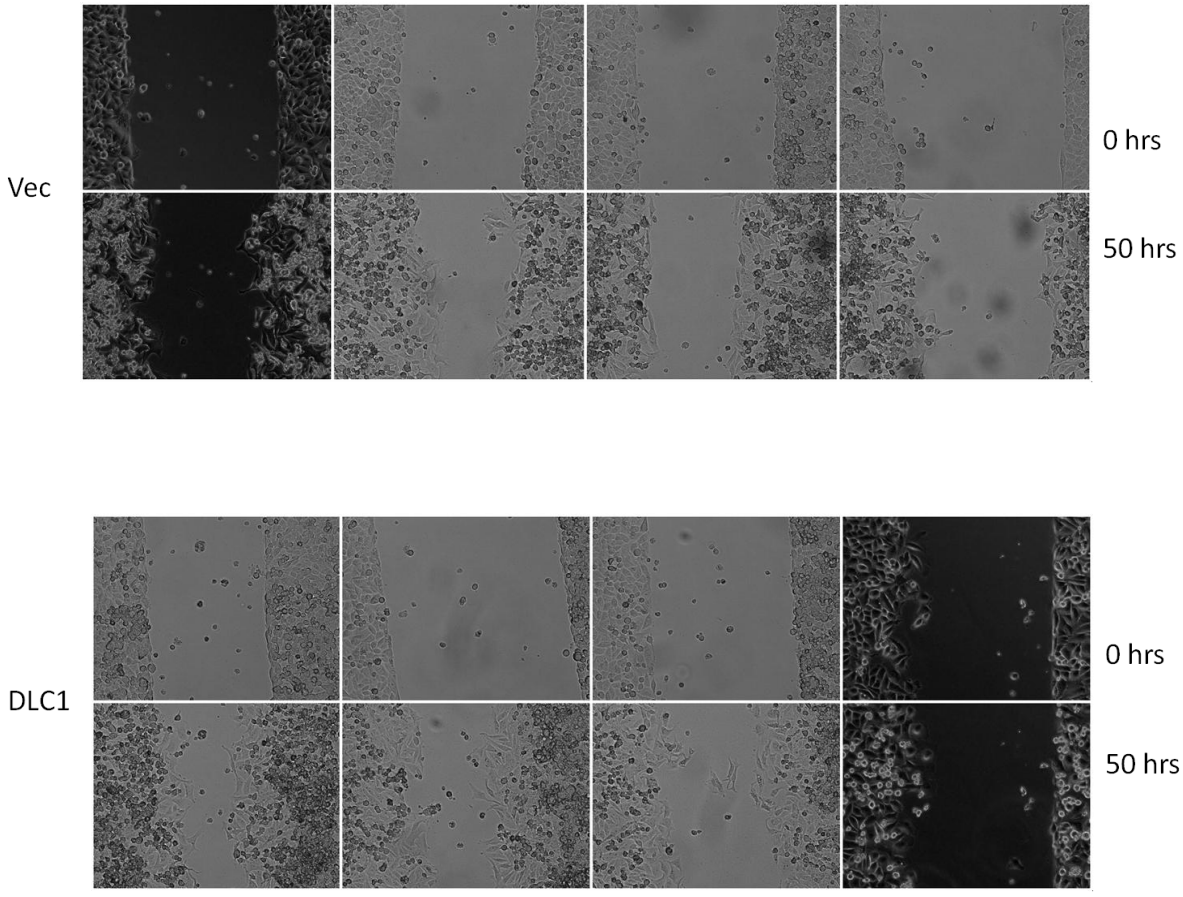


FI: FAK inhibitor treatment
EGF: EGF stimulation
RE: R677E

Figure 3.25 a: DLC1 requires EGF stimulation to be able to inhibit cell migration: HeLa JW cells were transfected with GFP-vector, -DLC1 and –

DLC1-R677E. The cells were starved for 18-24 hours in serum-free media and then subjected to different treatments as indicated. FI indicates FAK inhibitor treatment and EGF indicates EGF stimulation. The area covered by the cells during migration was calculated using ImageJ software. The first and the last time point of the migration were used to outline the edges of the cell monolayer. The area between the edges were calculated and area of last time point was deducted from the starting time point to give the area migrated by the cells. Annova was used for statistical analysis, n=4, $p<0.05$. The * represents treatments that are statistically significant with respect to other treatments and the error bars denote standard error.

EGF (100ng/ml)	-	+	-	+
FI (300nM)	-	-	+	+



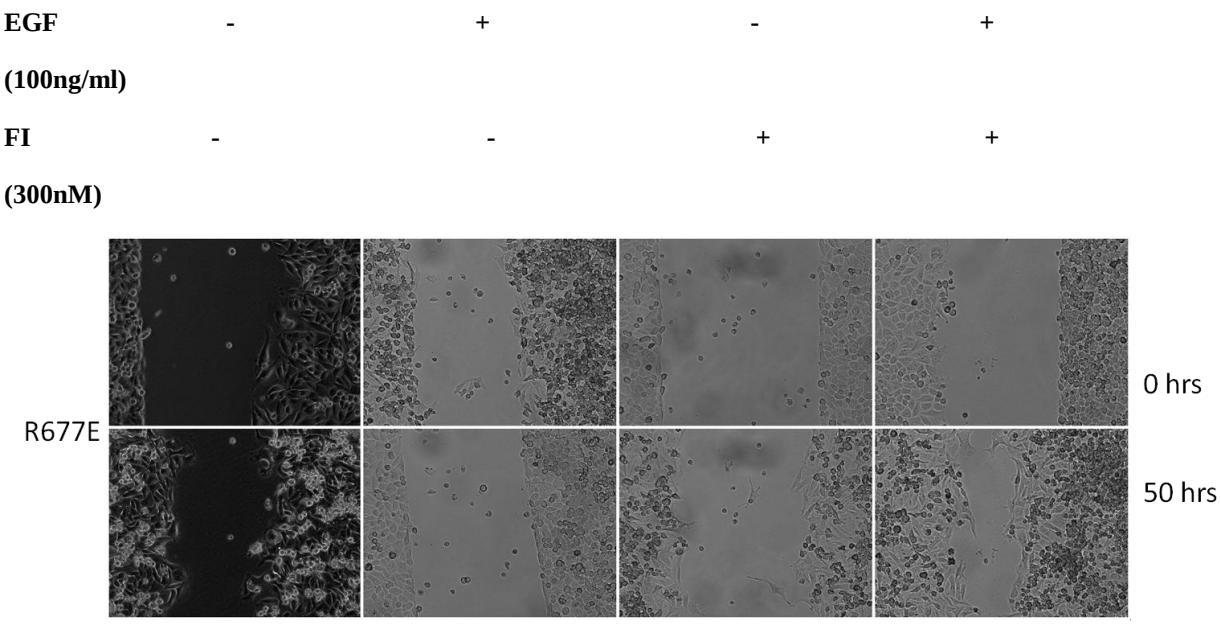


Figure 3.25b: Cell migration: This is representative data of the images obtained for the cell migration experiment done. For Vec, DLC1 and R677E the top panel is the image of the wound at 0hrs and the bottom panel is the image of the wound at 50hrs.

CHAPTER 4

DISCUSSION

4 DISCUSSION

4.1 EGF-mediated MEK-ERK activation acts as a master key to unlock DLC1 GAP activity

Information on DLC1s GAP function has always asserted that GAP domain of DLC1 alone is not an active domain and requires regions around it to function as a GAP [Kim et al., 2008]. Previously done work has shown that various posttranslational modifications regulate RhoGAP activity of DLC1 either directly or by mediating protein-protein interactions. Akt phosphorylation of DLC1 at S567 inhibits the GAP activity [Ko et al., 2010b], whereas S440 and Y442 phosphorylation mediates DLC1 interaction with the tensins which in turn is important for its RhoGAP function [Chan et al., 2009; Yam et al., 2006]. Recent work also shows that phosphorylation of DLC1 by PKA at S549 is important for its dimerization and this regulates DLC1 function as well [Ko et al., 2013].

4.1.1 Activation of DLC1-GAP function: A two-step process

We recently found that EGF-mediated activation of the Ras-MAPK pathway is involved in the activation of DLC1. This activation is carried out in a two-step process, which involves two other proteins, namely, FAK and PP2A. The first step in this process is the phosphorylation of DLC1. We found that DLC1 get phosphorylated (Fig 3.2) upon EGF treatment and this mediated through the Ras-MAPK pathway as demonstrated by the U0126 treatment. But this phosphorylation event does not activate DLC1. This was shown by Okadaic acid, a PP2A inhibitor, treatment. It was seen that upon stimulation,

in the event that PP2A is inhibited, DLC1 is still inactive (Fig 3.18). This shows that DLC1 also has to undergo PP2A mediated dephosphorylation to be activated. The sites identified by phosphoproteomics show that, EGF primes DLC1 for interaction and eventual dephosphorylation by PP2A (Table 3.1). But interaction between DLC1 and PP2A is not a simple event of priming mediated by the Ras-MAPK pathway. It also requires the inactivation of FAK, which interferes with DLC1-PP2A interaction. This is the second step in the EGF-mediated activation of DLC1. FAK inactivation is carried out by the ERK-mediated phosphorylation of FAK at S910, making it a target for PTP-PEST, which dephosphorylates Y397 thus inactivating FAK [Zheng et al., 2009]. This inactivation allows for DLC1-PP2A interaction leading to the dephosphorylation of DLC1 and hence its activation.

4.1.2 Phosphorylation of DLC1 at site(s) other than those targeted by PP2A is essential for active GAP function

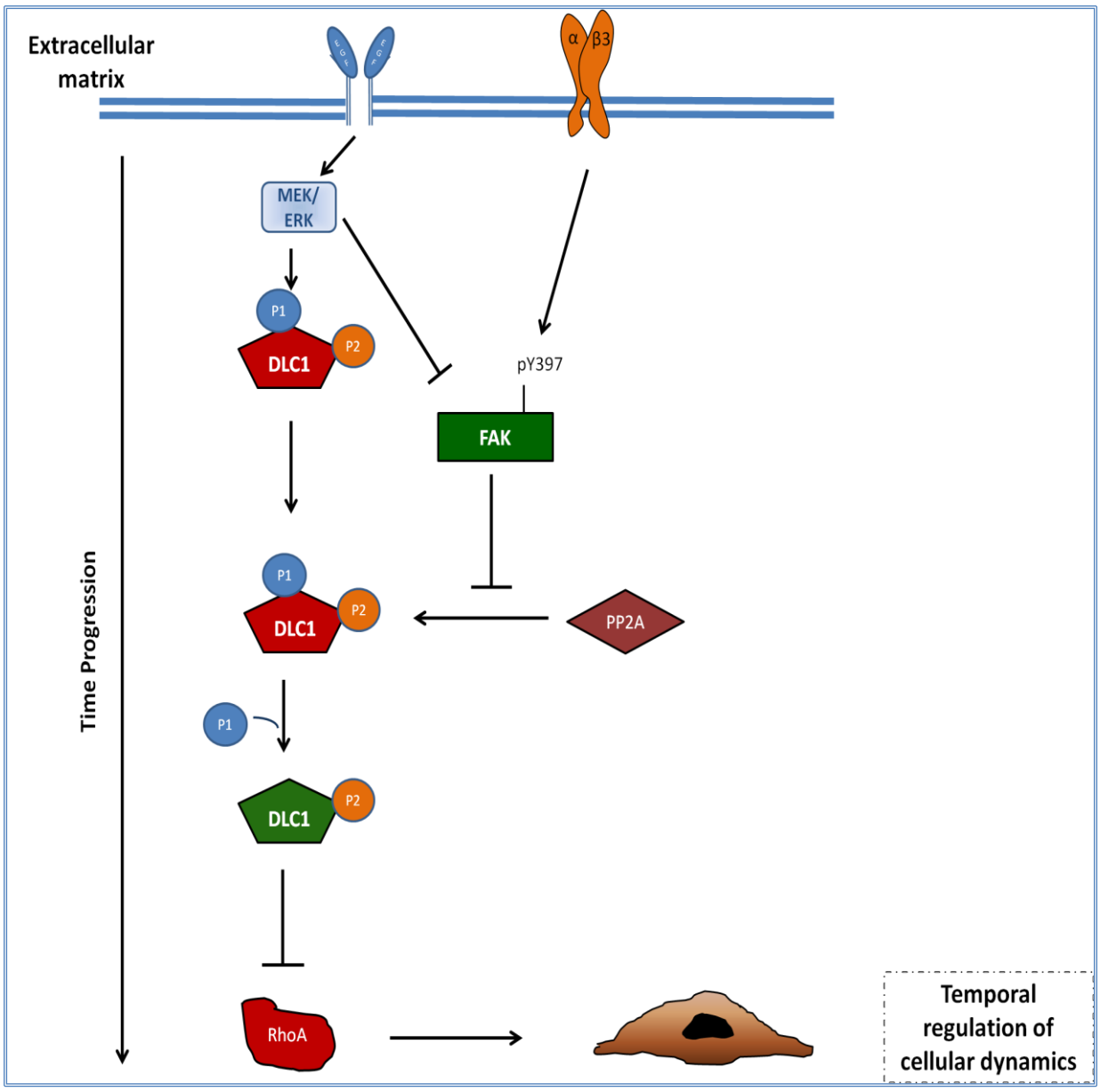
To ensure that the target sites found for PP2A were indeed the ones necessary for DLC1 function by dephosphorylation; we substituted Ser for Ala to create a phospho-dead mutant. Unexpectedly this mutant is inactive unless the cells were stimulated with EGF (Fig 3.9). To rule out the functionality of these sites in the activation process, we created phospho-mimicking mutant which contains Ser to Asp substitution. Surprisingly, this mutant was inactive even upon EGF stimulation (Fig 3.9), indicating that the dephosphorylation of the residues was an important step in the activation of DLC1-GAP function. This led us to the conclusion, that the event of EGF stimulation triggering the downstream Ras-MAPK pathway itself is important. This could possibly be due to presence of sites other than those targeted by PP2A which are phosphorylated by EGF that are necessary for the RhoGAP activity, answering

our question of how important EGF really is to this regulation. The phosphorylation of these other sites might be directly responsible or might be priming DLC1 for interaction with other proteins which are responsible for the GAP activity.

4.1.3 EGF stimulation allows for temporal activation of DLC1

Why is it necessary for DLC1 to contain multiple sites downstream of EGF? What regulatory advantage do multiple phosphorylation sites, which need to be dephosphorylated for DLC1s GAP activity, give DLC1? For directional cell migration, it is important that RhoGTPases are regulated in a spatial and temporal manner. With GAPs and GEFs controlling the activation status of RhoGTPases, it is necessary that these proteins are also under a tight regulation to ensure a cyclical activity of Rho, Rac and Cdc42. Activation of DLC1 by EGF is possibly one such temporal control. ERK activation by EGF is a cyclical process. Phosphorylation of ERK is seen within a couple of minutes of EGF stimulation. The levels reach a maximum and then drop before recovering again. With DLC1 phosphorylation and the dephosphorylation mediated by PP2A being dependent on pERK, it is possible that DLC1 gets phosphorylated at an earlier stage of ERK activation, which is followed by interaction with PP2A and dephosphorylation at a later stage when pERK levels reach a maximum. Hence, this cyclical ERK activation could in turn be controlling DLC1 activation in a time-dependent manner.

With the knowledge we have about the steps leading to the activation of GAP activity of DLC1, we have proposed the following model:


Extracellular matrix
α
β3
MEK/ ERK
P1
DLC1
P2
pY397
FAK
P1
DLC1
P2
PP2A
P1
DLC1
P2
RhoA
Time Progression
Temporal regulation of cellular dynamics

4.2 DLC1-PP2A interaction: What is the role of activated FAK?

We have shown that DLC1-PP2A interaction occurs only upon inactivation of FAK (Fig 3.16 and Fig 3.17). The question arises as to how the inactivation of FAK mediates this interaction? The two possibilities are that FAK interaction with DLC1 prevents the interaction of PP2A with DLC1 or FAK being a tyrosine kinase mediates inactivation of PP2A.

FAK, being a tyrosine kinase could be directly affecting PP2A by phosphorylation. It has been shown that phosphorylation of Y307 in the catalytic subunit of PP2A ($PP2A_C$) transiently inactivates PP2A. This phosphorylation is carried out by Src [Chen et al., 1992], which in turn requires FAK for α5β1 integrin mediated activation, although Src in some cases can be activated independent of FAK [Wu et al., 2008]. We have shown that in Hela JW cells that upon FAK inhibitor treatment the binding profile of DLC1-$PP2A_C$ is altered (Fig 3.17). It is possible that inhibition of FAK prevents the activation of Src, thereby preventing further phosphorylation of Y307, allowing PP2A to be active. PP2A could also be activated directly downstream of Ras/MAPK pathway. As seen in Fig 3.5, PP2A interaction occurs only at the stage where the EGF pathway is maximally activated at which point FAK gets inactivated. So is it necessary for PP2A to be activated to be able to bind to its substrates? If not, then what prevents DLC1-PP2A interaction?

FAK is known to interact with DLC1 with its FAT domain to the LD-like motif on DLC1. This LD-like motif lies between the residues 469-476 amino acids [Li et al., 2011]. The site of interaction for PP2A lies just upstream of these residues (Table 3.1). Both FAK and PP2A are large proteins, as well as, the serine-rich region is highly unstructured and with the binding of FAK, it might sterically hinder the binding of PP2A to DLC1. Information on as to whether DLC1-FAK interaction is dependent on the

active state of FAK is unavailable. The first question that needs to be answered is if Y397 phosphorylation of FAK is important for its interaction with DLC1. If the answer to this question is yes, it might shed some light on the possibility of steric inference. It would also explain the inactivation of FAK by Ras-MAPK, leading to the binding of PP2A, as this would remove any physical constraints for the interaction to take place.

4.3 Ras/MAPK-mediated DLC1 activation: A possible feedback loop

The Ras/MAPK pathway is one of the signalling cascades that are activated downstream of the EGF-receptor and is one of the best characterized pathways. Physiological functions regulated by this pathway include cell growth, division and differentiation; cell cycle regulation, integrin-mediated signalling and cell migration. Dysregulation of this signalling cascade is therefore very frequently observed in tumorigenesis [Mor and Philips, 2006]. This pathway begins with the binding of epidermal growth factor (EGF) to the EGFR activating the tyrosine kinase activity of the cytoplasmic domain of the receptor leading to auto-phosphorylation. This allows for docking proteins containing SH2 domain like GRB2 to bind to the phosphotyrosine residues of the receptor. Via its two SH3 domains GRB2 binds to SOS, a GEF, activating it. This in turn activates the Ras/MAPK cascade [Zarich et al., 2006]. GRB2-mediated activation can also be downstream of PDGFR and FGFR.

Activation of FAK leads to its binding with Src, forming a complex which is essential for Src activation and for complete activation of FAK. In this activated complex, Src phosphorylates the Tyr925 residue on FAK, creating a GRB2 binding site. This binding is a mechanism by which integrin engagement leads to activation of the Ras/MAPK pathway via FAK through its binding to GRB2 [Schlaepfer et al., 2004]. The phosphorylation of ERK

leads to the activation of the myosin light chain kinase. This is important for the modulation of the focal adhesion dynamics in migrating cells [Mitra et al., 2005].

We have shown that MEK/ERK mediated phosphorylation of DLC1 (Fig 3.2) is important for DLC1s RhoGAP function (Fig 3.1). Previous studies have shown that upon over-expression of DLC1, FAK undergoes dephosphorylation at two residues, namely, Tyr397 and Tyr925 [Kim et al., 2008]. With the dephosphorylation of Tyr925 the downstream ERK activation is inhibited preventing further or continuous activation of DLC1, completing the feedback loop.

4.4 Mechanical cues to biochemical signalling

All organisms ranging from bacteria to higher eukaryotes respond to mechanical stimuli, which are important for various physiological as well as pathophysiological processes. This event of conversion of physical stimuli to a biochemical response is known as mechanotransduction. Change in physiological characteristics due to physical forces is seen in the morphology of the heart, shape of the bone structure to the sensory responses like touch and hearing. Coordinated tissue growth and cell migration are a result of this event as well. And at a more molecular level, these physical forces also lead to conformational changes in proteins, which can act as a trigger in signalling events. The mechanical stimuli can be in the form of stress, pressure, tension etc and are translated into biochemical signals through specialized molecules called mechanotransducers. These are capable of sensing the change in the physical environment and undergo a conformational change to start the biochemical signalling. Cancer can be also a result of failure of a system to respond to these mechanical signals [Orr et al., 2006].

In processes like embryonic development, the stiffness of the surrounding tissue environment and the contractile forces exerted by the cells in response to the environment are the two major physical stresses seen. These two factors are important for mechanotransduction. The interplay between these two mechanical stressed is important for the process of embryogenesis [Wozniak and Chen, 2009].

4.4.1 Role of integrins in mechanotransduction

Focussing at a more cellular level, the external environment consists of a diverse collection of connective tissue components which together form the extracellular matrix (ECM). The cells react to both forces that are generated via the interaction with ECM (externally) or internally [Bershadsky et al., 2003; Chen, 2008]. These responses are bring about changes either locally, which affects the adhesion sites or globally that lead to a signalling cascade bringing about changes in the different physiological processes [Geiger et al., 2009]. For the purpose of detecting the signals in a coherent manner there has to be sensing machinery in the cell. Integrins, a family of transmembrane receptors found at adhesion sites are one such example which helps in recognition of different substrate properties ranging from biochemical to physical attributes. Integrin engagement induces the formation of focal adhesions. Focal adhesions are points where the cell physically adheres to the ECM as well as within the cell is the link with the cytoskeletal network, thus acting as a scaffold for further downstream cascades. At the adhesion sites, integrins associate with a diverse range of proteins which are part of biochemical signalling pathways [Geiger and Bershadsky, 2002; Geiger et al., 2001; Riveline et al., 2001].

Upon a physical cue, integrins respond by a structural change, allowing the adhesion-associated proteins to bind to it and triggering the signalling cascade that activate G-protein or phosphorylation-mediated pathways. mDia1 and ROCK activation downstream stream of Rho activation

leads to actin polymerization and increase in contractility of the cell, respectively, which promote the assembly of focal adhesions. This can also be carried out by activation of Src kinases [Riveline et al., 2001; von Wichert et al., 2003]. This change in cytoskeletal dynamics brings about changes in cell shape and motility and eventually affecting cell processes such as proliferation, differentiation and cell survival. The activated adhesions themselves generate forces via the actomyosin network. This process is a feedback loop, with integrins being responsible for both sensing and response modules [Geiger et al., 2009].

4.4.2 FAK-mediated regulation of RhoGTPases

Small GTPases play a crucial role in the process of relaying biochemical information from the mechanical stresses that translate into changes in the cell, making their regulation even more important. Hence the factors regulating the regulators, namely, GEFs and GAPs in response to integrins-mediated signalling become key players in the process of mechanotransduction [Geiger et al., 2009]. One such example is FAK. FAK associates with integrins clusters through its ability to bind with focal adhesion associated proteins such as paxillin, talin and p130Cas. This interaction leads to activation of FAK and Src Kinases leading to downstream signalling [Mitra et al., 2005]. It has been shown that there is FAK-mediated change in cell behaviour upon mechanical stimulus. This could be due to a conformational change causing the Y397 site to be exposed [Wang et al., 2001]. Phosphorylation of this site leads to FAK activation which is necessary for its various functions. FAK has been seen to act on p190RhoGEF and p190RhoGAP, by phosphorylating and activating both the molecules in a temporal as well as spatial manner [Tomar and Schlaepfer, 2009]. Thus FAK is responsible for transducing the physical forces as read by the integrins into biochemical signals by regulating the small GTPases through GEFs and GAPs.

4.4.3 Does DLC1 regulation have a role to play in mechanotransduction?

Recent study showed that FAK binds to DLC1 and has been shown to be important for its tumor suppressive functions [Li et al., 2011]. We have shown that FAK is also involved in regulating the RhoGAP function of DLC1 by inhibiting PP2A interaction with DLC1. ERK phosphorylation, that is essential to the activation of the RhoGAP activity of DLC1, can be stimulated downstream of FAK. ERK itself is seen to respond to mechanical cues leading to activation of its effectors without the involvement of FAK. With ERK responding to mechanical cues, either directly or indirectly through FAK [Martineau and Gardiner, 2001; Weyts et al., 2002], it is possible that DLC1 will play an important role in regulating the conversion of physical forces to biochemical process, eventually leading to the control of cellular dynamics.

4.5 Conclusions and future perspectives

In this study we have identified a novel EGF stimulation-mediated regulation of DLC1 RhoGAP function through FAK and PP2A. We have shown PP2A to be a novel binding partner for DLC1. The potential target sites for PP2A identified on DLC1 downstream of EGF stimulation and OA treatment were T301 and S308, which have been previously identified as residues that were seen to be highly mutated in cancer samples as compared to normal human tissue samples. These residues have been shown to be indispensable to the DLC1-PP2A interaction. Our study also shows that FAK, a previously identified binding partner of DLC1, needs to be inactivated in order for this binding to occur.

This lead us to delineate a temporally control DLC1 activation in which activated MEK/ERK acts as a “master key” by not only

phosphorylating DLC1 and hence priming it for activation but also inactivating FAK thereby allowing DLC1 to interact with PP2A. This interaction leads to dephosphorylation of DLC1, leading to its complete activation. The phosphorylation of DLC1 occurs in the initial stages of MEK/ERK activation which is followed by FAK inactivation once the phosphoERK levels reach a maximum.

With multiple key players in this regulation, there were many questions raised during the process of the study.

Is PP2A activation dependent on phosphorylation upon EGF stimulation or dephosphorylation upon FAK inhibition?

PP2A-mediated interaction followed by dephosphorylation occurs only upon maximal activation of the EGF pathway as seen by the pERK levels. This observation leads us to the question if PP2A activity and its ability to interact with its substrates correlated. It has been shown that crude lysates can be used to detect the catalytic activity of the enzyme using colorimetric assay [McAvoy and Nairn, 2010]. Lysates of cells transfected with $PP2A_C$ lysed at various time points downstream of EGF stimulation can be used to check for the active catalytic function of PP2A. Comparison of colorimetric data with the existent immunoprecipitation data will divulge the correlation between the PP2A's ability to interact and its active status.

Also, it is important to study how exactly EGF stimulation brings about activation of PP2A. As discussed, PP2A can be inactivated by Src-mediated Y307 phosphorylation. It will be paramount to see if this inactivation by Src is an FAK controlled event. For this purpose, FAK inhibitor treatment followed by SDS-PAGE and immunoblotting can be used to check for the

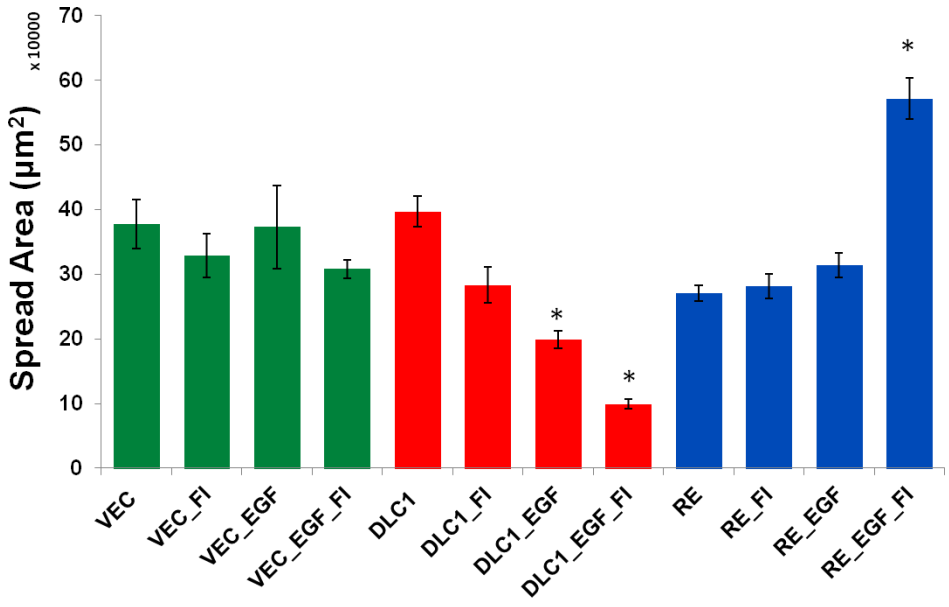
pSrc levels and pY307-PP2A levels. A change in pY307 levels upon FAK inhibitor treatment will show the effect of FAK on PP2A activation/inactivation. Also, quantifying pSrc levels will also tell us if FAK being a tyrosine kinase, can directly phosphorylate PP2A or whether its effects are mediated downstream of Src inactivation.

Does FAK play a physical role in preventing the DLC1-PP2A interaction?

A previous study has shown FAK-DLC1 interaction and the importance of the LD-like motif present on DLC1 which is necessary for the interaction [Li et al., 2011]. Also, we have, in our study shown DLC1-PP2A interaction and delineated the residues which are required for this interaction. As discussed in our first question, FAK could be leading to the inhibition or EGF stimulation could be responsible for the PP2A activation. Though the interacting sites are known, the requirements for the various interactions are unknown. Using immunoprecipitation, SDS-PAGE and western blotting, we can characterize binding between DLC1-FAK in a time-dependent manner post EGF stimulation. Blotting for pY397 and total FAK in the immunoprecipitates will answer the question as to whether the binding between DLC1-FAK is dependent on the activated state of FAK or not. Also, we have shown the binding between DLC1-PP2A is a stimulation-dependent characteristic (Fig 3.5). Comparing this profile to the DLC1-FAK binding will give us clues to whether FAK and PP2A can bind DLC1 simultaneously or if PP2A interaction requires FAK to detach itself from DLC1. This will also explain if there is competition and/or any form steric hindrance from FAK to prevent PP2A interaction.

Cell migration: Is it a GAP-dependent or independent function?

It has been previously shown that DLC1s role in cell migration is a GAP-independent process as the GAP-inactive mutant of DLC1, R677E also prevents the wound from healing. Its ability in wound closure is more similar to DLC1 than vector as expected [Kim et al., 2008]. The cell migration experiment performed in this study seems to contradict it.



FI: FAK inhibitor treatment
EGF: EGF stimulation
RE: R677E

Comparing the various treatments, we can see that DLC1s ability to inhibit wound closure is seen only upon EGF stimulation or EGF stimulation and FAK inhibitor treatment. These two treatment conditions were previously seen to be associated with DLC1 RhoGAP activity. Comparing the DLC1 and vector data with various treatments, this process seems to be a GAP-dependent

function. This is in contradiction with the previously published data which all show that transfection of DLC1 is alone sufficient to inhibit migration [Kim et al., 2007]. This can be explained by the fact that during the course of the wound healing experiment performed in our lab, cells were subjected to serum-free media, whereas the previously conducted experiments were carried out in serum-containing media. Hence there is some amount of activated MEK/ERK in media which might be sufficient for DLC1 activation and hence the observed results.

DLC1-R677E does not seem to have any effect on the process of wound closure as seen in the first three cases which are similar to effect seen in vector-transfected cells. But upon EGF stimulation and FAK inhibitor treatment, the ability of the R677E cells seems to increase. Even though this does not indicate towards a GAP-independent effect on cell migration, it does raise many questions regarding the probable causes for the seen effect. DLC1 is a protein with multiple domains, which have been shown to play a role in various GAP-independent tumor suppressive functions. It is possible that the effect on cell migration could be a concerted action of the various domains and not completely GAP-independent. Also, the role of FAK inhibitor needs to be characterized. It has to be kept in mind that the wound healing experiment is carried out over a period of 50hrs with no supplementation of the various treatments, which could also indicate wearing off of the effect of the various treatments.

To account for the various inconsistencies, the wound healing experiment can be carried out with the DLC1 mutants created during the course of this project, namely DLC1-T301/S308A and DLC1-T301/S308D. Also, the RhoGAP activity assay can be carried out after a 50hr treatment to mimic the conditions used during this experiment. The interpretation of the combined results could give us a better insight into the GAP-dependency of the process as the two mutants have been shown to have difference in

RhoGAP activity. Use of various available truncation mutants of DLC1 would also delineate the role of the other domains in this process.

What is the mechanism by which EGF stimulation-mediated phosphorylation of DLC1 control its RhoGAP function?

There are a multitude of growth factors that are responsible for regulating cell process and dysregulation of the signalling cascades downstream of the growth factor receptors leads to cancer. EGF-mediated regulation of DLC1 is one of the first studies on effect of growth factors on DLC1s function. We have shown that EGF apart from priming DLC1 for PP2A-mediated dephosphorylation also phosphorylates other residues which are essential for DLC1s GAP activity. In this study we were unable to delineate the residues which upon EGF phosphorylation regulate the RhoGAP activity. A more high-throughput study would be required to do so. Phosphoproteomics combined with mutation studies can be used for this purpose.

DLC1 has been shown to be subject to various phosphorylation-mediated regulation which affects its GAP activity. This is as a result of the effect of direct phosphorylation or phosphorylation-mediated interactions with other proteins which then regulate the localization and hence its activity. Akt phosphorylation on S567 leads inhibition of DLC1 GAP function [Ko et al., 2010a]. On the other hand phosphorylation of residues S327 and S341 allows 14-3-3 interaction with DLC1 and inhibits its activity as is the case with Y442 phosphorylation which is necessary for interactions with Tensin and hence controls DLC1s localization [Qian et al., 2007; Scholz et al., 2008].

Identification of the residues on DLC1 will give us insights into possible interacting partners which might be responsible for regulating the localization of DLC1 and hence its activity. Also, it will be interesting to see

the RhoA binding ability of the phospho-mimic and phospho-defective mutant DLC1. It is possible that phosphorylation of DLC1 either opens up the structure or stabilizes the highly unstructured region of DLC1, allowing it to interact with RhoA leading to its inactivation.

CHAPTER 5

REFERENCES

5 REFERENCES

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