Role of N-WASP in skin cancer and role of N-WASP binding proteins in filopodia formation

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Role of N-WASP in skin cancer and role of N-WASP binding proteins in filopodia formation

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Abbreviations

A	Alanine
AD	DNA activation domain
APS	Ammonium Persulfate
ATP	Adenosine 5'-triphosphate
Arp2/3	Actin related protein complex
BSA	Bovine Serum Albumin
BD	DNA binding domain
BR	Basic region
cDNA	Complementary DNA
CRIB	Cdc42/Rac Interactive Binding Motif
CR16	Glucocorticoid-regulated gene product
Cdc42	Cell division cycle 42
C	Cofilin homology domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DTT	Dithiothreitol
EGFR	Epidermal Growth factor
ECM	Extracellular Matrix
E.coli	Escherichia coli
Ena/Mena	Enabled/Mammalian enabled
EDTA	Ethylenediamine Tetraacetic Acid
Erk	Extracellular Regulated Kinase

Eps8	Epidermal growth factor receptor kinase substrate 8
EMT	Epithelial-mesenchymal transition
FA	Focal adhesions
FC	Focal complex
FAK	Focal Adhesion Kinase
F-actin	Filamentous actin
FRET	Fluorescence resonance energy transfer
FBS	Foetal Bovine Serum
G-actin	Monomeric globular actin
GBD	GTPase Binding domain
GEFs	Guanine Nucleotide Exchange Factors
GFP	Green Fluorescent Protein
GTP	Guanosine Triphosphate
GRB2	Growth factor receptor bound protein-2
HRP	Horse Radish Peroxidase
Irs53	Insulin Receptor Substrate p53
IMD	IRSP53/MIM homology domain
LB	Luria – Bertani Medium
MAPK	Mitogen Activated Kinases
MCS	Multiple Cloning Site
mDia	Mammalian Diaphanous
MEFs	Mouse Emryonic Fibroblasts
N-WASP	Neural-WASP
NPFs	Nucleation promoting factors
PRR	Proline rich region
PBS	Phosphate Buffered Saline

PEG	Polyethylene glycol
PMSF	Phenylmethanesulfonyl fluoride
PCR	Polymerase Chain Reaction
PIP2	Phosphatidylinositol (4,5) Bisphosphate
Rac1	Ras-related C3 botulinum toxin substrate1
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT-PCR	Reverse Transcription PCR
SCC	Squamous Cell Carcinoma
SDS	Sodium Dodecyl Sulphate
SH2	Src homology 2
SH3	Src Homology 3
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Toca1	Transducer of Cdc42-dependent actin assembly
WAS	Wiskott Aldrich Syndrome
WASP	Wiskott–Aldrich-Syndrome Protein
WAVE	WASP-family verprolin homologous proteins
WBD	WASP binding domain
WIP	WASP-interacting protein
WIRE	WIP-Related
WICH	WIP and CR16 Homologous protein
V	Verprolin homology domain

Abstract

The actin cytoskeleton, made of polymerized actin together with the actin associated proteins, plays a critical role in many actin-based structures, including cell adhesion and cell motility. N-WASP promotes the formation of actin-rich structures such as filopodia, which affect cell adhesion and motility. Although N-WASP regulates cell migration and adhesion, function of N-WASP in cancer and metastasis has not been well characterized. N-WASP is expressed in the non-tumorigenic HaCaT cells however the expression was found to be low in the tumorigenic, non-metastatic epithelial cell line, HSC5 and high in the metastatic cell line, A5-RT3. A qPCR analysis of the SCC patient samples revealed a consistently low expression of N-WASP. Analysis of the adhesion properties of epithelial cells in the presence and deficiency of N-WASP revealed that N-WASP is important for cell adhesion and spreading on fibronectin. The knockdown of N-WASP expression in the cell lines, HaCaT and A5-RT3, respectively led to decrease in cell-ECM adhesion and an increase in cell migration. The overexpression of N-WASP in a tumorigenic cell line HSC-5 resulted in an increase in the cell adhesion, spreading on fibronectin and a decrease in cell migration. Altered localization and expression level of paxillin and vinculin in cells deficient in N-WASP was observed in both tumorigenic and non-tumorigenic epithelial cell lines, HaCaT and A5-RT3, respectively. Thus, the data suggests that deficiency of N-WASP in both tumorigenic and non-tumorigenic cell lines leads to decrease in cell-ECM adhesion to fibronectin accompanied by an increase in cell motility. Also, the varied expression level observed in tumorigenic and metastatic cell lines indicate a role for N-WASP in metastasis.

The role of the different domains of N-WASP in cell-ECM adhesion and migration were characterized using N-WASP^{-/-} MEFs. A defect in the cell-ECM binding and enhanced migration was observed in the N-WASP^{-/-} cells. The mutants, N-WASP^{ΔWH1} and the N-WASP^{Y256E} failed to restore the cell-ECM adhesion in the N-WASP^{-/-} (N-WASP^{ΔWH1}) and the N-WASP^{-/-} (N-WASP^{Y256E}) cells and the mutants did not inhibit cell migration in both cell lines. An increase in the paxillin localization and a decrease in the vinculin localization were also observed in the N-WASP^{-/-} (N-WASP^{ΔWH1}) and the N-WASP^{-/-} (N-

WASP^{Y256E}) cell lines. The above results hence suggest the importance of the WH1 domain and the site, N-WASP^{Y256} in cell-ECM adhesion and migration.

N-WASP interacts with the mammalian verprolins; WIP (WASP Interacting Protein), CR16 (Corticoid Regulated) and WIRE (WIp-RElated) in regulating the actin cytoskeleton. In order to characterize the function of CR16 independent of N-WASP, yeast two hybrid screen was carried out and IRSp53 (Insulin Receptor Substrate) was identified as a CR16 interacting protein. Expression of IRSp53 with CR16 in N-WASP^{-/-} mouse embryonic fibroblasts induced filopodia. The induction of filopodia is dependent on CR16–IRSp53 interaction as mutation in the SH3 domain of IRSp53 caused the CR16–IRSp53 interaction to be abolished as well as the ability to induce filopodia. Similarly, the Verprolin (V)-domain of CR16 is critical for IRSp53–CR16 interaction and for filopodia formation. Cdc42 regulates the interaction between CR16 and IRSp53, as mutations, which abolish Cdc42-IRSp53 interaction, lead to loss of IRSp53–CR16 interaction. Expression of Cdc42^{G12V} (active mutant) with CR16–IRSp53 caused significant increase in the number of filopodia per cell. Thus IRSp53 activity is controlled by CR16 by controlling the IRSp53–CR16 interaction to generate filopodia.

Chapter 1: Introduction

1.1 Skin cancer

Skin is the outer covering of vertebrates and is the largest organ in mammals. The mammalian skin consists of two primary layers, the epidermis and the dermis. This study involves the epidermal component, composed of keratinocytes and fibroblasts. The mammalian epidermis is a stratified tissue that is attached to the basement membrane. The cells present at the basal layer are proliferating undifferentiated cells that undergo differentiation as they move outward to the surface of the skin (Alonso and Fuchs, 2003). The epidermis is categorized into 4 major cell types, which include the keratinocytes, melanocytes, Langerhans cells and Merkel cells. The keratinocytes form the major portion of the epidermis and are characterized by the expression of keratin (Fuchs and Cleveland, 1998). This layer forms the sealant against environmental hazards such as microbes and chemicals. Melanocytes contain the pigment melanin, which filters the damaging UV light and also lends the skin a perceivable hue (Nordlund et al., 1998). Langerhans cells play the role of fighting against microbes and hence is a part of the immune response of the body. Merkel cells are the tactile cells, which are the most deep-seated and are involved in detecting the sensation of touch. The epidermis is also categorized into 5 layers: stratum basale, spinosum, granulosum, lucidum and corneum. The deepest layer consisting of a monolayer of keratinocytes, forms the stratum basale and a connection to the dermis below is made through desmosomes. The layer above the basale is the spinosum, which is made of 8-10 layers of keratinocytes. The role of this layer is to provide strength, support and elasticity. The granulosum contains cells, which have the keratohyalin granules. At the junction between the layers granulosum and the layer above, lucidum lamellar bodies are released containing proteins and lipids, which help form the hydrophobic sealant thus serving as a barrier. The stratum lucidum consists of 3-5 layers of dead keratinocytes, which serves as the thick skin at areas, which are prone to friction. The uppermost layer, which is shed and replaced by cells from underneath is formed of dead cells devoid of nucleus and organelles in the

cytoplasm. These cells are called corneocytes, which contain keratin in abundance and are filled with lipids, fatty acids and ceramides (William et al., 2005; James et al., 2009; Ovaere et al., 2009; McGrath et al., 2004).

The molecular events that lead to the conversion of a progenitor cell to a mature melanocyte involves EMT wherein the cell-cell adhesion changes occur and loss of E-cadherin expression is observed. The Snail/Slug transcription factor mediate repression of expression of E-cadherin. The conversion of a stem cell progenitor to a melanoblast and to a mature melanocyte involves the Wnt signaling which facilitates the change through β -catenin expression (Erikson et al., 1998; Dorsky et al., 1998; Dupin et al., 2000; Dunn et al., 2000). Genes that are important in the development stage of a melanocyte are the- mitf (microphthalmia transcription factor), c-kit, snail/slug, sox10, and endothelins (Uong et al., 2010).

1.2 Melanoma

Melanoma remains to be a highly incident form of skin cancer, being the sixth most common form of skin cancer in the United States. Melanoma has been found to be hetero-genetic in nature with several mutations (KO et al., 2011; Bis et al., 2013). One of the factors apart from genetic predisposition that leads to the development of melanoma is exposure to sun (Bandarchi et al., 2010). In most of the melanomas the most commonly seen mutations are in the CDKN2A (cyclin-dependent kinase inhibitor 2A) and CDK4 (cyclin-dependent kinase 4) (Bataille, 2003) while MC1R (melanocortin-1 receptor) and DNA repair genes are shown to be prognosis markers (Aitken et al., 1999; Bataille et al., 2000; Nagore et al., 2000; Bataille et al., 2003). Common mutations observed in malignant melanoma include the mutations in the receptor tyrosine kinases, mutations in NRAS/BRAF/ERK (neuroblastoma rat sarcoma/v-raf murine sarcoma homologue B/extracellular-related kinase) and phosphatidylinositol 3' kinase (PI3K)/PTEN pathways (Goel et al., 2006). PTEN/MMAC1 (phosphatase and tensin homologue/mutated in multiple advanced cancers 1) expression has been found to be higher in the cutaneous muscle, nerve and muscular arteries, comparatively lower levels were observed in the epidermis, in the sebaceous and eccrine glands. The PTEN/MMAC1 plays an important role in

facilitating apoptosis. Loss of expression of PTEN showed a correlation with progression of melanoma (Tsao et al., 2003). Increased expression of MAPK (mitogen-activated protein kinase; Ras-Raf-MEK-ERK) and the phosphoinositide 3-kinase-AKT (also known as protein kinase B/PKB) pathways, which are strongly associated with the progression of stages of melanoma are observed in a majority of the malignant melanomas (Smalley et al., 2003; Dhawan et al., 2002).

1.3 Non-melanoma skin cancers

There are two main forms of non-melanoma skin cancers (NMSCs) namely; basal or squamous cell carcinomas (BCC and SCC). BCCs are the most common and account for more than 70% of the NMSCs however they are rarely fatal or metastatic. BCCs are characterized by mutations in PTCH1 and p53 (Han et al., 2006; McGregor et al., 2002; Wilkening et al., 2007). Mutations in the p53 gene have shown to be associated with risk of development of BCCs. Variations in the MCR1 alleles are also associated with heightened risk of BCC. This risk association is found to be higher in patients with two alleles rather than persons with a single variant of the allele. Genetic changes in the tyrosinase and agouti signaling proteins are shown to increase the incidences of BCC (Gudbjartsson et al., 2008).

SCC is the second most common NMSC and is more devastating than BCCs which are rarely reported to be fatal (Humphreys, 2001). SCCs display the following aberrations- loss of the lowermost basement membrane, increase in number of cells that proliferate and a decrease in the number of cells that undergo terminal differentiation. SCCs have a tendency to develop as multiple tumor foci and generally have high chances of becoming metastatic (Nolen et al., 2011). The integrin expression is altered in SCCs such that there might be loss of the integrins or an increase in the expression of $\alpha 5\beta 1$ and $\alpha v\beta 6$. (Janes and Watt, 2006; Mariotti et al., 2001; Watt, 2002). SCC's exhibit

MALIGNANT CONVERSION OF HUMAN KERATINOCYTES

Figure 1.1: Schematic representation of the steps in the malignant conversion of the human keratinocytes, HaCaT. This flow chart details the generation of the cancerous and malignant cell lines. The cell lines used in this study are the metastatic tumor A5-RT3 and the malignant clone II-4. The malignant II-4 clone was obtained by tumorigenic conversion of the human HaCaT keratinocytes post transfection with the mutated val12-harvey-ras oncogene (HaCaT-Ras cells). HaCaT A-5 RT3 clone was obtained by recultivating *in vitro* the subcutaneous tumors derived from HaCaT-ras cells and re-transplanted three times *in vivo* (Mueller et al., 2001; Maillard et al., 2005).

unstable genomic integrity that allow for development of several mutations (Hoeijmakers, 2001). This genomic instability often results in inactivation of p53 which is induced by UVB rays. 58% of SCCs harbor mutations caused by UVB as shown by high incidence of UVB-induced cancer in p53^{-/-} mice (Brash et al., 1991).

Immunohistochemical studies showed increased p53 expression in cells with lesions that was attributed to the increased stability of the altered p53 proteins (Einspahr et al., 1999). EGFR and Fyn are aberrantly activated which lead to downregulation of p53 mRNA which is mediated by c-jun dependent mechanism (Kolev et al., 2008; Zhao et al., 2009). Decreased levels of inositol polyphosphate 5' phosphatase, which

leads to increased PI3k/Akt signaling, are also found to affect SCC development (Sekulik et al., 2010). Increased expression of SFKs (Src-family tyrosine kinase), EGFR (Epidermal growth factor receptor), Myc, and ATF-3 (Cyclic AMP-dependent transcription factor-3) are found to promote SCC (Kim et al., 2011; Toll et al., 2010).

Cancer development is a process that involves a multitude of steps, in which cells accumulate alterations in the genome and thereby eventually progress from a normal to a malignant phenotype (Aaronson, 1991). The cell line, HaCaT can maintain their non-tumorigenic phenotype for extended passages and are similar to normal human keratinocytes in their ability to grow and differentiate (Boukamp et al., 1988; Boukamp et al., 1997). The cell lines (Fig. 1.1), used in this study are: the metastatic tumour, A5-RT3 cell line and the malignant ras clone, II-4. The tumorigenic conversion of HaCaT was achieved by transfection with the mutated val-12-Harvey-Ras oncogene, which yielded clones that progressed as epidermoid cysts (benign tumors) after subcutaneous injections. Upon injecting into mice the cells that were grown *in vitro* from the established heterotransplants showed an increased invasive behavior due to the *in vivo* microenvironment that exerted the necessary selective pressure (Mueller et al., 2001). The malignant II-4 cell line was obtained from human HaCaT keratinocytes post transfection with the mutated val12-harvey-ras oncogene (HaCaT-Ras cells). The subcutaneous tumors that were derived by injecting HaCaT-ras cells into mice were re-transplanted three times to generate the II-4 cell line. The II-4 cell line was reported to result in well-differentiated SCCs when tested in mice whereas the HaCaT A-5 RT3 cell line resulted in SCCs that did not differentiate well leading to local lymph node metastases (Mueller et al., 2001; Maillard et al., 2005).

In normal epithelium the cells exist in tight cellular contact with the neighboring cells forming a highly organized sheet structure. The progression of a tumor to the metastatic stage involves the conversion of a cell to a more invasive form, which is facilitated by altered expression of several genes. One of the key changes seen include the changes in the actin cytoskeleton which leads to disruption of cell-cell

adhesions and formation of protrusive structures critical for invasion. This also leads to altered distribution of actin-associated proteins. The actin cytoskeleton aids in the formation of these protrusive structures such as invadopodia and pseudopodia (Reviewed by Nurnberg et al., 2011; Behrens et al., 1993; Volberg et al., 1991; Volberg et al., 1992).

1.4 Cytoskeleton

The cytoskeleton is made up of various filamentous structures consisting of actin filaments, microtubules and intermediate filaments. The actin cytoskeleton contributes to the functioning of a cell in two ways: structurally and functionally. Structurally it forms the framework and defines cell polarity. Functionally it contributes to processes such as formation of cell extensions, embryogenesis, wound healing, cell division, transport and position of organelles (Fletcher and Mullins, 2010).

1.5 Actin cytoskeleton

The actin cytoskeleton plays a critical part in maintaining the cell shape and facilitating the motility of the cells. The movement of the cell is based primarily on the re-organization of actin filaments, which leads to protrusions at the leading edge of motile cells in response to extracellular stimuli. The actin binding proteins control the actin dynamics by sequestering monomers, capping of barbed and pointed end filaments, severing of filaments and crosslinking of filaments (Pollard and Borisy, 2003). These proteins can be classified based on their functions as severing, capping, sequestering, nucleating, monomer binding and cross-linking proteins. Actin binding proteins such as profilin are involved in enhancing the exchange and addition of the ATP-monomers to the ends of actin assemblies and in turn regulating the dynamics of actin polymerization. Proteins that sequester G-actin such as thymosin- β 4 interact with G-actin to change the conformation, leading to inhibition of polymerization of actin and exchange of nucleotides (De La Cruz et al., 2000). Severing proteins such as cofilin and gelsolin regulate the quantity and distribution of the actin filaments (Disanza et al., 2005; Firat-Karalar and Welch, 2011; Pantaloni et al., 2001). The severing proteins, such as gelsolin cause the severing of filaments. The

crosslinking proteins assemble the filaments of actin into structures that are tight or loose (Carlier, 1998; Puius et al., 1998).

To initiate actin assembly free polymerisable filament ends are required which are generated by different mechanisms- uncapping of the ends of existent filaments, severing existent filaments and by de novo filament nucleation. The formation of actin nucleus in the formation of new actin filaments is one of the most crucial steps in actin polymerization. The nucleation is regulated by several actin nucleating factors and Arp2/3 complex (Goley and Welch, 2006). The Arp2/3 complex nucleates the generation of new actin filaments from the sides of preexisting actin filaments at an angle of 70° (Firat-Karalar and Welch, 2011).

Among these actin-binding proteins exist the nucleation and elongation factors. The main nucleation factors include the Arp2/3 complex and the formins (Machesky et al., 1994; Goode and Eck, 2007; Higgs, 2005; Pollard, 2007; Pryune et al., 2002; Zigmond et al., 2003). Recent discoveries include the nucleators present in eukaryotic cells, Spire (Quinlan et al., 2005) Cobl (Ahuja et al., 2007), VopL (Liverman et al., 2007), VopF (Tam et al., 2007), TARP (Jewett et al., 2006) and Lmod (Chereau et al., 2008). A nucleator is proposed to have two roles to play- 1). to facilitate the rapid growth of a filament at the barbed end and 2) To effectively seed actin monomers that are ATP bound (Reviewed by Chesarone and Goode, 2009).

One of the major actin nucleators which has been well studied is the Arp2/3 complex comprises of 7 subunits that include the 2 actin related proteins (Arp2 and Arp3) and 5 other proteins. At the resting state Arp2/3 complex remains inactive and is activated by actin filaments that are existent (Higgs & Pollard, 1999), ATP (Dayel, Holleran, & Mullins, 2001) and nucleation promoting factors. Activated Arp2/3 complex sparks off the formation of a filament from the side of a precursor filament at an angle of 70°. This generates a branched daughter filament. This actin polymerization activity leads to the assembly of actin cytoskeletal structures such as lamellipodia, focal adhesions and yeast endocytic patches (Reviewed

by Chesarone and Goode, 2009). The Arp2/3 complex performs two functions upon its activation- it nucleates new actin filaments (Ma et al. 1998b, Mullins & Pollard 1999, Rohatgi et al. 1999) and it crosslinks newly created filaments into Y-branched arrays at a branch angle of 70° (Blanchoin et al. 2000, Mullins et al. 1998).

The formins unlike the Arp2/3 complex are reported to facilitate the formation of linear filaments and cause the assembly of cytoskeletal filaments such as stress fibres, cytokinetic actin rings and actin cables (Faix et al., 2006; Goode et al., 2007). The formins are characterized by the presence of dimeric domains (FH2- formin homology), which bind to the barbed ends of the filaments by bridging two actin subunits. They promote the formation of spontaneous actin dimers and trimers (Pring et al., 2003; Tominaga et al., 2000) leading to formation of actin filaments.

Ena/VASP family members such as mammalian Ena (Mena), VASP and Ena-VASP-like (EVL) are a group of proteins that promote actin nucleation and polymerization (Gertler et al., 1996). These proteins are required for the generation of actin cytoskeletal structures with actin organization, which are dynamic in nature such as during axon guidance (Lanier et al., 1999; Goh, Cai, Cepko, & Gertler, 2002), platelet and T-cell activation (Krause et al., 2000) and fibroblast motility (Bear et al., 2000).

Newly discovered proteins Spire, Cordon bleu, and Leiomodin promote actin polymerization by another nucleation mechanism altogether which requires Spire to bind to four actin monomers through its four WH2 domains to form a precursor filament (Quinlan et al., 2005). Two studies have reported two different theories regarding the post nucleation effects of Spire. One study suggests that Spire binds to the side or pointed end of a filament thus allowing free barbed end elongation (Quinlan et al., 2005) while the other suggests that Spire binds with the barbed end and blocks elongation by profilin-actin (Le et al., 2007).

The ras-related family of small GTPases, Cdc42, Rac and Rho are involved in the regulation of the actin cytoskeleton critical for actin cytoskeletal structures such as cell adhesion and cell migration. The Cdc42

controls the microspike and filopodia formation. Rac plays a role in the generation of lamellipodia and ruffling. Filopodia are thin finger like projections, which are rich in actin and actin binding proteins. Lamellipodia are a result of tightly organized, parallel arrays of actin filaments that are polymerized at the leading edge of the cell (Small, 1981; Burridge et al., 1988). Stress fibres are long bundles of actin filaments that span the entire length of the cell and are connected to the ECM through the integrins and the focal adhesions (Heath and Dunn, 1978; Burridge et al., 1988; Gumbiner, 1993). Stress fibre formation is regulated by the Rho proteins.

1.6 Rho GTPases in cancer

Rho GTPases belong to the family of small GTPases that act as molecular switches that are active when GTP-bound and inactive when GDP-bound. Their activity is increased by the guanine exchange factors (GEFs) and down regulated by the GTPase activating proteins (GAPs) (Reviewed by Domino et al., 2011). On activation, the Rho GTPases bind to several effector molecules that activate the signaling cascade to direct cellular responses. Rho GTPases have been reported to play a role in regulation of actin and microtubule cytoskeleton in processes such as cell migration, embryonic development, cell polarity, cell adhesion and cell division (Jaffe and Hall, 2005). Apart from contributing to physiological processes, the Rho GTPases have been reported to play a role in cancer cell migration, metastasis, invasion, wound repair and inflammation (Aspenstrom et al., 2007; Jaffe and Hall, 2005).

Rho GTPases have been reported to be involved in almost all stages of cancer initiation and progression. The steps include the transformation of a quiescent cell to a cell with the ability to proliferate uncontrollably, with the ability to evade apoptosis and metastasize (Vega and Ridley, 2008). In epithelial cells, loss of the epithelial integrity takes place which leads to disruption of the cell-cell adhesions and cells become more invasive and migratory. This transformation is defined as the epithelial to mesenchymal transition (EMT) where the cells eventually acquire a more mesenchymal or fibroblast like property (Reviewed by Thiery et al., 2009).

The mammalian Rho GTPase family consists of 22 members and they are categorized into three subfamilies, Rho (RhoA, RhoB and RhoC), Rac (Rac1, Rac2 and Rac3) and Cdc42 (Cell Division Cycle-42). The extensively studied family members include the RhoA, Rac1 and Cdc42. The activation of Rac leads to actin polymerization, which forms lamellipodia, activation of Cdc42 leads to formation of filopodia or microspikes and activation of Rho generated stress fibres. The small GTPases of the Rho family act as molecular switches that cycle between their active state and inactive state. The active state is GTP bound while the inactive state is GDP bound and this conversion is catalyzed by the GEFs (Guanine nucleotide Exchange Factors) and GAPs (GTPase Activating Proteins). Rho GTPases when activated bind to their downstream targets and deliver to their functions in assembling actin cytoskeletal structures such as filopodia and lamellipodia (Boreux et al., 2007; Ridley AJ, 2006 and Oleksy et al., 2006).

Rho proteins have been studied in depth and mutations in them have rarely been the cause for tumors but alterations in their expression are often detected in tumors. Rho GTPases are up-regulated in some human tumors, including RhoA, RhoC, Rac1, Rac2, Rac3, Cdc42, Wrch2/RhoV and RhoF (Aronheim et al., 1998; Pulgar et al., 2005 and Gouw et al., 2005). According to a previous study overexpression of Rac1 in colorectal adenocarcinoma leads to accelerated tumorigenic progression and lack of it suppressed tumor formation (Espina et al., 2008). Cdc42 has been suggested to play two roles; promoting an invasive phenotype characteristic of the tumor metastases and acting as a tumor suppressor (Reviewed by Stengel and Zheng, 2011).

The RhoA activity has been shown to play a part in all stages of tumor progression with a role in increasing the growth rate of tumor cells and promoting the survival of the cells by evasion of apoptosis (Jaffe and Hall, 2005). A contradicting functional role has been identified where the protein is shown to disrupt epithelial organization during tumor progression as well as generate epithelial polarity, junction assembly and function (Braga and Yap, 2005; Wildenberg et al., 2006). Rho proteins promote epithelial

organization and polarity or EMT based on the GEF or the GAP protein involved in the activation (Labouesse , 2004).

RhoC is shown to be involved only in metastasis with no apparent activity involved in the promotion of EMT (Hakem et al., 2005). According to a gene expression-profiling study the gene (RhoC) has been shown to be up-regulated in metastatic stages of melanoma. Subsequently it has been proposed to be a marker for prognosis of cancers of different origins (Clark et al, 2000; Kleer et al., 2006). Knockdown of RhoC by RNAi shows that RhoC is important for invasion *in vitro* (Bellovin et al., 2006, Pille et al., 2005; Simpson et al., 2004).

RhoB unlike the RhoA and RhoC has shown to be down-regulated in human tumors. The protein has been proposed to act a tumor suppressor due to its expression in response to stress stimuli including DNA damage and hypoxia. RhoB suppresses tumor growth, cell migration and invasion. RhoB has been reported regulate the delivery of signaling proteins, including growth factors receptors and tyrosine kinase Src to specific intracellular compartments which in turn influences proliferation and invasion (Sandilands et al., 2007).

The activation of Rho GTPases by specific GEFs and GAPs is controlled in a spatiotemporal manner which defines the widely differing functional roles of the Rho GTPases in various aspects of cancer progression as seen in RhoA versus RhoB; RhoA promotes cell proliferation of the tumor cells while RhoB suppresses it (Jaffe and Hall, 2005, Sandilands et al., 2007). Deregulation of expression or activity of the GEFs, GAPs and effector proteins has been observed in cancers. Although the oncogenic form of the protein, Vav, (GEFs for Rac) has not been detected in human tumors, it has been implicated in human malignancies such as neuroblastoma, melanoma, pancreatic tumors and leukaemia (Katzav, 2007). The RhoGAP ARHGAP8 has been reported to be upregulated in colon and cervical tumors and RhoGDa expression is deregulated in various cancers (Jones et al., 2002). PAK and ROCK, downstream effectors of Rac/Cdc42 and Rho are upregulated in some cancers (Kamai et al., 2004; Salh et al., 2002). However

the effect of the altered expression level of the above proteins in influencing the Rho GTPase function in cancer is unclear and its exact role in tumor progression remains to be determined. So far there are few mouse models available for some of the Rho GTPases, the regulators and effector proteins (Walmsley, 2003 and Benninger, 2007). Cancer specific models in mice for the Rho GTPases will help elucidate the role and mechanism of the proteins in cancer.

Rho GTPases are known to regulate the actin dynamics in a cell thus influencing several cellular characteristics such as cell polarity, transcription, proliferation, vesicular trafficking, microtubule dynamics and cell migration (Myrto and Alan, 2004).

1.7 Cell migration

Cell migration is essential for development and tissue remodeling with a major role in promoting tumorigenesis and metastasis. Several genes such as Hox, p63, GATA genes, Mena, etc are involved in the regulation of cell migration, which have been studied and identified by targeted studies and genetic screens. Cell migration observed in normal wound healing and in invasion of tumor cells in malignancies are due to alteration in cytoskeleton dynamics and cell adhesions. (Horwitz & Parsons 1999; Webb et al. 2002). The two types of cellular migration that are generally observed are the single cell movement and the collective migration of a sheet of cells. The intercellular interactions are retained in the case of collectively migrating cells where they move as a sheet or a mass of cells of cells in a coordinated manner (Huttenlocher and Horwitz, 2011). In adherent cells the type of ECM, the strength of adhesion, the external stimuli, mechanical elasticity and dimensionality, and the assembly of the actin cytoskeleton affects the mode of migration (Reviewed by Ridley et al., 2003). The first step in cell migration involves the establishment of a polarized cell, wherein the cell has a defined leading edge and a trailing edge. The leading edge is characterized by actin polymerization, which results in a protrusion, extension of the plasma membrane, which attaches to the substratum and provides the traction for the movement of the cell body. Adhesion of the cell to the ECM takes place via integrin receptors, which links the ECM to the

actin cytoskeleton. The size of adhesion plaques usually vary from dot-like (nascent adhesions) to large and elongated (focal adhesions) (Petrie et al., 2009).

Cell migration is observed to be a cyclic process, which involves protrusion, adhesion and retraction. The process is initiated in cells as a response to migration promoting extracellular spatial cues (Lauffenberger et al., 2006). The response involves the conversion from an unpolarized to a polarized state and leads to the extension of protrusions in the direction of migration. These protrusions are driven by actin polymerization and can be broad sheet like structures called lamellipodia or thin finger like microspikes called filopodia. The cell protrusions are stabilized by cell adhesions to the underlying ECM or adjacent cells via cell-cell transmembrane receptors that are in turn linked to the actin cytoskeleton. These adhesions serve as sites, which provide the traction necessary for migration as the cell moves forward over them while the cell detaches at the rear end (Alexandrova et al., 2008; Choi et al., 2008).

The first step in cell migration begins with the establishment of cell polarity and protrusion. A polarized end is generated with a different molecular network of proteins in the front in comparison to the rear end. Directed cell migration is based on spatiotemporal cues; among the more extensively studied factors include the G-protein coupled receptors, growth factors, cytokines, receptors; which are cadherins, neuronal and immune receptors (Iglesias and Devreotes, 2008; Swaney et al., 2012). There are various adaptors and effectors that influence the polarity of a cell; one such among them is the initial GTPase implicated in the initial polarizing of the signals, Cdc42. Cdc42 is involved in repositioning of the nucleus and orienting the microtubules (Etienne-Manneville and Hall., 2002; Rodriguez, 2003). It is also involved in activating the nucleation of the actin networks through WASP/N-WASP family of proteins. The conjoined activation of Cdc42 and Rac leads to recruitment of other signaling adaptors that leads to clustering of the adhesive receptors and signaling molecules at the leading edge. Activated Rac and cdc42 in conjunction with WASP/WAVE family of protein along with the Arp2/3 complex generates branched actin assemblies at the leading edge, which forms actin cytoskeletal protrusions (Cory and Ridley, 2002; Welch et al., 2000 and Cory et al., 2002). Actin polymerization is regulated by proteins,

which sever existent filaments and cap growing filaments, which in turn controls the availability of activated actin monomers. Rac activity is inversely proportional to the activation of Rho. The Rho remains inactive at the front end while Rac mediates the protrusions (Rodriguez et al., 2003; Small and Kaverina, 2003). Localized activation of Cdc42 and Rac promotes PI3K activity and the production of PIP3 at the leading edge. Cdc42 promotes the polarization of the cell mediated by the re-orientation of the MTOC toward the cell front. Integrins and other adhesion effectors are activated by the PI3Ks, PKCs and/or Rap via talin and these adhesions stabilize the protrusions. Rac signaling recruits supplementary integrins and formation of adhesions. Nascent adhesions formed at the leading edge re-inforce the increased Rac, Cdc42 and PI3K activity at the leading edge while a gradient of low Rho activity at the frontal end of the cell and higher at the trailing end is observed. Stabilized adhesions provide for the tractional forces required for the protrusions over which the cell moves (Geiger et al., 2001; Beningo and Kaverina, 2001; Galbraith et al., 2002). This cycle of migration is completed once the adhesions disassemble and the rear retracts, which is controlled by pathways that include FAK (Focal Adhesion kinase), ERK (Extracellular signal-regulated kinase), Src (Sarc), and the protease calpain, and involve the controlled dynamics of the microtubules. Retraction at the rear requires Rho kinase and myosin. The adhesions are released at the rear which requires the activity of the Rho kinase (Turner et al., 2001; Webb et al., 2002). In short, cell migration is based on a number of factors such as the strength of cell-substratum adhesions, cell-cell adhesions, the type of substratum, external and internal spatial cues, and mechanical pliability (Reviewed by Ridley et al., 2003).

1.8 Cell-substratum contacts

Adherent and stationary cells remain firmly attached to the ECM below through distinct portions of the plasma membrane through cell-matrix junctions called focal adhesion plaques. At the sites of focal adhesions the transmembrane receptors such as the integrins bind to the ECM proteins such as collagens, laminins, fibronectin and vitronectin. On the cytoplasmic end bundles of microfilaments, the stress fibres

are linked to the integrins along with the cytoskeletal proteins. Thus the focal adhesions structurally connect the ECM and the actin cytoskeleton (Burrige et al., 1988). One of the first steps in the establishment of focal adhesions is integrin ligation which leads to the integrins to link with the actin cytoskeleton via focal adhesion proteins, such as vinculin, talin, α -actinin, paxillin and p125 focal-adhesion kinase, FAK (Yamada and Miyamoto, 1995). Other constituents that control the focal adhesion formation include the calcium-dependent protease; calpain II, protein kinase C, FAK and Src family tyrosine kinases (Beckerle et al., 1987; Burrige and Chrzanowska-Wodnicka, 1996; Jaken et al., 1989; Kaplan et al., 1994; Zamir and Geiger, 2001). In migrating fibroblasts microtubules and Rho GTPases regulates the turnover of focal adhesions (Ridley and Hall, 1992; Small et al., 1999).

1.9 Cell-cell adhesion

Individual cells are packed into three-dimensional organized tissues in animals. One of the other factors that influence cell migration include cell-cell adhesions, as during cancer metastasis the cells in tissues are required to break off cell-cell junction interactions in order to initiate cell migration. The proteins that are responsible for the cell adhesions (cell-cell or cell-ECM) are categorized as follows: 1) transmembrane glycoproteins present at the cell-cell interface such as cadherins and cell-matrix interface such as integrins. 2) The ECM proteins such as collagens are linked to the adhesion receptors. 3) the cytoplasmic plaque such as catenins form the adhesive link between the adhesion receptors and the actin cytoskeleton. These catenins are involved in the transduction of the signals from the substratum to the inside of the cell (Gumbiner, 1996).

The adhesive interactions between cells can be categorized as 1) gap junctions, which are also known as the communicating junctions 2) Junctions that seal are the tight junctions 3) Junctions that are anchoring in nature are adherens junctions and desmosomes. The adhesive contacts present between the individual cells plays a role in maintaining well-defined tissues.

Tight junctions: These are the most apically located intercellular junctions seen in the endothelial and epithelial cells which serve as barriers across cells that are selective and diffusive in nature. These junctions maintain a certain proportion of proteins and lipids between the basal and apical membrane thus performing the 'fence' function. These junctions also control the growth and differentiation potential of the cells (Balda and Matter, 1998; Tsukita et al., 1999). Tight junctions are noted to consist of two lipid opposing membranes that are juxtaposed together to form a belt like region which are in tight pairs. The proteins that are identified to form these junctions are categorized into two divisions 1) integral membrane proteins, such as occludin, claudin and junctional adhesion molecule, JAM and 2) peripheral membrane proteins (cytoplasmic plaque proteins), MAGUK (membrane-associated guanylate kinase) homologue proteins, such as ZO-1, 2, 3, cingulin, symplekin, 19B1, and AF-6. Apart from these there are several proteins involved in signaling (protein kinases, heterotrimeric G-proteins and small GTP-binding proteins) that participate in the formation of these junctions (Tsukita et al., 1999).

Desmosomes and gap junctions: They are responsible for facilitating connections that are firm and tight between the epithelial cells and cardiac myocytes. (Schwartz et al., 1995). The desmosomes are comprised of several transmembrane adhesive glycoproteins and proteins that are present at the cytoplasmic plaque. They link themselves to cytokeratins present within cells (Garrod, 1993).

Gap junctions: These are structures present between cells that facilitate the diffusion of the ions and small molecules in a passive manner through aqueous intercellular channels (connexons) between the cytoplasm of the adjacent cells (Kumar and Gilula, 1996). These connections are seen in most tissues except skeletal muscle cells and erythrocytes.

Cadherin based adherens junctions: These junctions are characterized by the presence of cadherin molecules, these cadherin molecules of adjacent cells interact in a calcium dependent manner and are tethered to the actin cytoskeleton via catenins. (Geiger and Ginsberg, 1991; Ozawa et al., 1990; Rimm et al., 1995; Takeichi, 1995). Cadherins are subcategorized into classic cadherins based on the cell type -E; epithelial, P; placental, N; neuronal cadherins (Yap et al., 1997).

Classical cadherins consist of 2 domains- extracellular and cytoplasmic domain, which are conserved. The extracellular part is found to link with cadherins of adjacent cells and cytoplasmic part interacts with proteins within cells which participate in the formation of the junction. E-cadherin is responsible for facilitating the assembly of adherens junctions and controls the assembly of desmosomes and tight junctions (Gumbiner et al., 1988b; Wheelock and Jensen, 1992).

C-terminal part of E-cadherin of the cytoplasmic domain binds to the α -catenin through the β -catenin or to γ -catenin (Fig.1.2). Through another site in the transmembrane domain it also interacts with the p120ctn, a catenin, which is a substrate of v-Src kinase (Reynolds et al., 1994; Thoreson et al., 2000; Yap et al., 1998). β -catenin tethers the cadherin receptors to the actin cytoskeleton (Fig. 1.2) and relays signals from cell adhesions through Wnt proteins of the Wnt/Wingless signalling pathway thus promoting development, differentiation, proliferation and morphogenesis (Wodarz and Nusse, 1998).

In the E-cadherin complex formation, the proteins α -catenin, α -actinin, vinculin, ZO-1, spectrin connect with the actin cytoskeleton via direct and indirect interactions (Yamada and Geiger, 1997). The focal adhesion-associated adaptor protein, vinculin has been reported to mediate the tethering of the adherens junction complex to the actin cytoskeleton via α -catenin (Wang and Gerdes, 1999). The establishment of firm and tight adhesions is dependent on the formation of adherens junctions in tissues while in epithelial cells, the adherens junction fastens the adhesion by tethering the actin cytoskeleton to the sites of firm adhesions. The E-cadherin depleted cells suffer weakened cell-cell adhesions due to other affected junctional proteins, which is seen in tumor cell invasions and metastasis of epithelial cancers (Birchmeier and Behrens, 1994).

1.10 Vinculin and Paxillin

A focal adhesion is defined as the close cellular contact that is formed between the cell and the extracellular matrix between the integrins, the F-actin cytoskeleton and with the extracellular adhesion

molecules. This focal adhesion complex can disassemble or mature into larger focal adhesions (Burridge et al., 1988).

Paxillin is an adaptor protein that localizes at focal adhesion complexes observed at the ends of stress fibres and is involved in tethering other signaling proteins, such as vinculin and talin at the complex. Paxillin promotes cell migration by increasing the FA turnover and has been shown to play a role in the embryonic development. Paxillin is involved in the regulation of cell migration and cell spreading (Schaller, 2001). Paxillin expression is elevated in breast cancer cells (Cai et al., 2010). Paxillin over-expression has been observed during early stages of lung cancer development (Mackinnon et al., 2011). In addition paxillin was found to localize at matrix degrading structures such as the invadopodia (Bowden et al., 1999). Paxillin over-expression is observed in the osteosarcoma cell line, in high metastatic sublines of HuO9 (M112 and M132) in comparison with the low metastatic sublines of HuO9 (L12 and L13) (Azuma et al., 2005).

Vinculin was identified as a paxillin binding protein that localizes at the ends of stress fibres and is involved in linking the actin cytoskeleton to the focal adhesion. Vinculin deficient cells show impaired cell spreading and migration. The focal complex formation is impaired which leads to disruption in the formation of focal adhesions. In addition to reduced focal adhesions, the formation of stress fibres and actin polymerization decreases which affect cell adhesion and spreading (Xu et al., 1998a). Vinculin has been shown to play a critical role in the formation of adherens junctions (Kaiser et al., 1993) and impaired adherens junction complex formation is observed upon knocking down vinculin expression. The reduction in the expression of vinculin causes a reduced interaction with E-cadherin required for the adherens junction (Peng et al., 2010). Vinculin- β catenin interaction has been found to alter the expression level of E-cadherin, which is required for cell-cell adhesion (Peng et al., 2010). Vinculin plays a pivotal role in regulating both, the adherens junction and focal adhesions with some striking differences. β -catenin plays a vital role in the formation of adherens junctions in binding to vinculin and hence influencing the expression level of E-cadherin at intercellular junctions however it is not involved

in the formation of focal adhesions. On the other hand talin is required for the activation of vinculin and is essential for the assembly of focal adhesions but it plays no role at the adherens junction complex formation (Kaiser et al., 1993; Peng et al., 2010).

1.11 E-cadherin and the actin cytoskeleton

Cadherins mediate cell-cell adhesions in a calcium dependent manner through their ectodomains. The transmembrane protein, cadherin links with other focal adhesion proteins present in the cytoplasmic plaque (catenins, vinculin), which in turn are tethered to the actin cytoskeleton to maintain mechanical stability of the adherens junctions (AJs). The primary role of these junctions is to maintain firm adherence between cells in the epithelium and homeostasis within tissues (Nelson, 2008). Changes in cadherin-dependent adhesions lead to responsive rearrangements in the actin organization connected to the sites of cell-cell adhesions (Yamazaki et al., 2007). The epithelial cells suffer weakened cell-cell interactions due to the depletion of the marginal actin bundle and the reorganization of the E-cadherin-based adherens junctions in promoting motile behavior and metastasis of tumors during cancer progression. Thus, the structural and dynamic coordination between cadherins, actin cytoskeleton, and proteins that regulate the actin structures may change during neoplastic transformation (Ayollo et al., 2009)

Figure 1.2: The cadherin-catenin complex. The Ca^{2+} ions are required to form homophilic interactions. The intracellular domain of E-cadherin links with the α and β catenins to form the cadherin-catenin complex. β -Catenin binds α -catenin, which is tethered to the actin cytoskeleton and the several actin-regulatory proteins including α -actinin, vinculin, and formin-1.

The adherens junctions (AJs) form the key structure of epithelial sheets. Adherens junctions are in continuous process of formation and disruption for the maintenance of epithelial compactness of structure (Baum et al., 2008). Epithelial cadherin belongs to the cadherin family of adhesion molecules that mediates the changes in the adhesive contact between epithelial cells. E-cadherins contain an extracellular domain, which mediates interaction with the neighbouring cells and a cytoplasmic, which binds several proteins that link it to the actin cytoskeleton (Fristrom, 1988; Gumbiner et al., 1988a). One such link involves the binding of the E-cadherin to the β -catenin, which interacts with the α -catenin. The α -catenin in turn binds actin and other actin binding proteins (Bershadsky, 2004; Yonemura et al., 2010).

Mechanical signals that are generated at the cell-cell junctions are transduced across the extracellular domain of the E-cadherin, which is linked to the actin framework by the cytoplasmic tail, to the nucleus where gene expression is altered (Balda and Matter, 2009; Okada et al., 2007). Junctional E-cadherin-catenin complexes regulate the proper functioning of the epithelia. The junctions maintain structural integrity by providing strong adhesive links and coordinate cell polarity (Baum and Georgiou, 2011). The formation and the disassembly of adherens junctions involve the AJ complexes, the actin cytoskeleton, the regulators and the Rho family of GTPases- Rho, Rac and Cdc42. The interaction takes place both ways such that the extracellular domain transduced signals across the E-cadherin to the Rho GTPases in order to facilitate changes in the cell structure and polarity and the Rho GTPases regulate the AJs (Lampugnani et al., 2002). In addition to Rho GTPases N-WASP depletion was found to affect the formation of cell junctions, assembly pattern of F-actin and E-cadherin in epithelial cells (Tetsuhisa et al., 2006).

1.12 Wiskott Aldrich Syndrome (WAS) and WASP

Wiskott Aldrich Syndrome (WAS) is an X- linked disease characterized by thrombocytopenia, immunodeficiency and eczema (Thrasher, et al 2000). Various non-erythroid hematopoietic cells are affected, with severe defects in platelets and lymphocytes (Remold-O'Donnell et al., 1996). The underlying defect in this disease was clarified in 1994 by the identification of the WAS gene that encodes the Wiskott Aldrich Syndrome Protein (WASP) (Derry et al., 1994). The protein, WASP is expressed exclusively in hematopoietic cells (Miki et al., 2003).

1.13 WASP family of proteins and their domain structures

The WASP family of proteins consists of five members, namely WASP, N-WASP, WAVE1, 2 and 3. N-WASP was identified and was reported to share ~50% amino acid identity to WASP (Miki et al., 1996). In contrast to WASP, N-WASP is expressed ubiquitously, but the highest expression was observed in

nerve cells, and thus it was named Neural-WASP (N-WASP). The GBD (GTPase binding domain) binds to GTP-bound Cdc42, which leads to N-WASP activation (Symons et al., 1996).

Figure 1.3: Schematic diagram illustrating the various domains of WASP family of proteins. The WH1 domain binds to WIP (WASP interacting protein). The basic region (BR) binds to PIP₂. The GTPase binding domain (GBD) binds to GTP-bound Cdc42. The proline rich region (PRR) binds to many SH3 domain containing proteins and profilin.

N-WASP contains multiple domains that allow for interactions with multitude of effectors and adaptors that facilitate the integrations of signaling cascades to actin cytoskeleton remodeling. The WH1 domain at the N-terminal of N-WASP (Fig. 1.3) interacts with proline rich sequences of the mammalian verprolins WIP/WIRE/CR16 (Reviewed in Anton and Jones, 2006). The interaction between WIP and N-WASP results in the formation of WIP/N-WASP complex that prevents N-WASP activation via Cdc42 (Martinez et al., 2001). hnRNPK, a member of the heterogenous nuclear ribonucleoprotein complex interacts with the WH1 domain of N-WASP and has been observed to have a negative regulatory role in the induction of filopodia formation and cell spreading (Yoo et al. 2006).

The basic region and the GBD are located centrally. The basic region is involved in binding to negatively charged phospholipids, such as PIP₂. The binding of HSP90 (Heat Shock Protein 90) has been reported to facilitate the Src-mediated phosphorylation, which leads to further activation of N-WASP (Park et al. 2005). The small GTPases Cdc42, Tc10 and RhoT have been reported to bind at the GBD and activate N-WASP to regulate the Arp2/3 complex activation thus activating actin polymerization (Higgs and Pollard 2000; Rohatgi et al. 2000; Abe et al. 2002). mDab1 binds at the region before the basic region of N-

WASP through a NRFY (Asn-Arg-Phe-Tyr) sequence close to the CRIB motif of N-WASP (Suetsugu et al. 2004). IQGAP1, a protein involved in cell-ECM adhesion, migration and cytokinesis binds and activates N-WASP by interacting with the basic region and CRIB motif in a Cdc42-like manner (Le Clainche et al. 2007).

Proline rich motif present in N-WASP interacts with the SH3 domain of many proteins, which activates N-WASP. These proteins include the following- WISH, Grb2, Nck1, Nck2, cortactin (Kowalski et al. 2004), intersectin-I (Hussain et al. 2001), CrkII (Tang et al. 2005), Abi1 (Innocenti et al. 2005), Abp1 (Pinyol et al. 2007) and profilin (Mimuro et al. 2000). Among them Grb2, Nck1, Nck2 and CrkII all contain both SH2 and SH3 domains. The SH2 domain containing proteins bind and stabilize tyrosine (Tyr256) phosphorylated N-WASP to maintain and enhance its activation. Certain SH3 domain containing proteins activate N-WASP in collaboration with another protein, for example- Cdc42 functions with Grb2 (Rohatgi et al. 2000; Carrier et al. 2000), and Nck1 works together with PIP₂ (Prehoda et al. 2000). The BAR domain proteins are another group of proteins that interact with the N-WASP through their SH3 domains which include the syndapins/pascins (Qualmann et al. 1999), CIP4 (Tian et al. 2000), FBP17 (Tsujita et al. 2006), Toca-1 (Ho et al. 2004), Pstpip1, Pstpip2 (Wu et al. 1998) and Nostrin (Legg et al. 2007); the N-BAR proteins Amphiphysin (Yamada et al. 2009), Tuba (Kovacs et al. 2006) and Endophilin (Otsuki et al. 2003); the I-BAR protein IRSp53 (Lim et al. 2008); and the PX-BAR protein SNX9 (Yarar et al. 2007). The interaction of N-WASP with BAR domain proteins links membrane remodeling with actin dynamics thus facilitating their role in vesicle fission (Tsujita et al. 2006; Itoh et al. 2005). N-WASP has been reported to be involved in mediating endocytosis in conjunction with BAR proteins in cooperation with dynamin (Plomann et al. 1998; Qualmann et al. 1999).

The C-terminus of N-WASP harbors the VCA domain consisting of a verprolin homology region (V), a cofilin homology/central region (C) and an acidic region (A). The VCA region is responsible for binding

proteins involved in actin polymerization including the Arp2/3 complex, G-actin and F-actin (Machesky et al. 1999). The VCA domain binds and activates the Arp2/3 complex (Rohatgi et al., 1999) and is the minimal domain essential for activation of Arp2/3 complex to promote actin polymerization (Rohatgi et al., 1999; Machesky et al., 1999).

The binding of the actin monomers to the V domain and F-actin to the central C domain are required for the Arp2/3 complex activation, which interacts with the A domain thus converting the Arp2/3 complex to its active conformation. Upon activation the Arp2/3 complex promotes actin polymerization assembling the nucleus for actin nucleation. FBP11 has also been reported to bind to the V domain of the VCA region and co-expression of N-WASP and FBP11 has been implicated in nuclear accumulation of N-WASP, which reduces EGF induced filopodia formation (Mizutani et al. 2003).

1.14 N-WASP: regulation and biological role

The N-terminal of WASP and N-WASP is characterized by the presence of a WH1 domain, which is central for the regulation of their stability and activity as this domain binds to the mammalian verprolins-WIP, CR16 and WIRE (Ramesh et al., 1997; Ho et al. 2001; Kato et al. 2002). The binding of WIP to WASP plays a role in stabilizing WASP by preventing degradation by calpains (Chou et al. 2006; de la Fuente et al. 2007; Sawa and Takenawa 2006). The binding of WIP to the WH1 domain of N-WASP has been shown to prevent the activation of N-WASP through Cdc42 (Martinez-Quiles et al. 2001). The presence of N-WASP/WIP complex in the xenopus oocytes has been demonstrated where the N-WASP/WIP complex prevents Cdc42 mediated N-WASP activation. A ternary complex with Toca1 led to release the autoinhibited state of N-WASP allowing for the activation of N-WASP through Cdc42 in the presence of WIP or CR16 (Ho et al. 2004).

The major regulatory mechanism seen in N-WASP and WASP is the autoinhibition, which is relieved by a multitude of effectors. In the resting state N-WASP exists in a closed conformation where the GBD is bound to the C-terminal VCA domain which makes the VCA domain unavailable to stimulate actin

polymerisation machinery (Kim et al. 2000; Miki and Takenawa 1998; Rohatgi et al. 2000). The GBD of N-WASP binds to Cdc42, which relieves the autoinhibition. The Cdc42-GBD interaction leads to an open conformation (Kim et al., 2000), which unmask the VCA domain, which can now interact with the Arp2/3 complex hence initiating actin polymerization (Miki and Takenawa 1998; Rohatgi et al. 1999). There are two more Cdc42 related small GTPases, Tc10 and RhoT which also interact with the GBD to activate N-WASP (Abe et al. 2003). The basic region also binds phosphoinositides which induce maximal induction of Arp2/3- mediated actin assembly in synergy with Cdc42 (Rohatgi et al. 2000; Kim et al. 2000; Prehoda et al. 2000).

N-WASP is characterized by the poly-proline sequence to which many SH3 domain containing proteins bind and activate N-WASP. These proteins relieve the autoinhibited state and hence leading to the open conformation. These include the Nck1, Nck2, Grb2, Profilin, WISH, cortactin, intersectin-1 and some BAR domain proteins (Carrier et al. 2000; Rohatgi et al. 2001; Fukuoka et al. 2001; Kowalski et al. 2005; Hussain et al. 2001). Toca1, which is a F-BAR protein binds to N-WASP through its SH3 domain to activate the N-WASP/WIP or N-WASP/CR16 complex in the presence of GTP bound Cdc42 (Ho et al. 2001). Another protein Abi1 that belongs to the Scar/WAVE complex has been shown to interact with N-WASP by its SH3 domain. Abi mediates activation of N-WASP through Cdc42 and not PIP2 thus regulating actin based vesicle trafficking and receptor endocytosis (Innocenti et al. 2005).

N-WASP is regulated by numerous kinases such as the Src family tyrosine kinases Fyn and Lck, FAK (focal adhesion kinase), Abl and Ck2 (casein kinase 2). Tyr256 lies (equivalent to Tyr 253 in mouse N-WASP) (Suetsugu et al. 2002) in the GBD region of N-WASP and is located within a binding sequence for Src family SH2 domains domains (Yaffe et al. 2002). In the folded conformation of N-WASP the site is inaccessible to protein kinases and phosphatases. PIP2 is one of the activators that converts the closed conformation of N-WASP into an open one thus facilitating phosphorylation of Tyr256 in N-WASP by Fyn in PC12 cells which is required for neurite outgrowth (Suetsugu et al. 2002). The other activators present that facilitate phosphorylation of the Tyr256 are Grb2 and GTP bound Cdc42 (Torres and Rosen

2003). The phosphorylated GBD binds SH2 domain containing proteins, which stabilize N-WASP in an open conformation and promote Arp2/3 mediated actin polymerization (Torres and Rosen 2003). This is supported by the finding, in a recent study of *Shigella* invasion where N-WASP is activated by the *Shigella* protein IcsA on the pathogen surface leading to phosphorylation of Tyr256 by Abl kinases. The phosphorylation of Tyr256 leads to stabilization of active N-WASP, which is required for efficient actin comet tail formation and elongation (Burton et al. 2005). A second phosphorylation site was identified at Tyr175 (equivalent to Tyr172 in mouse N-WASP), which is phosphorylated by Abl. Mutation at Tyr256 led to the Abl mediated phosphorylation of N-WASP to be abolished in the presence of Cdc42-GTP and mutation of both Tyr256 and Tyr175 was required to block Abl mediated phosphorylation of N-WASP in the presence of Grb2. This suggests that tyrosine phosphorylation of N-WASP is affected by the binding of different activators of N-WASP which affects its subsequent cellular role. Phosphorylation of Tyr256 alone can keep N-WASP in a partially activated state compared with basal levels even in the absence of activating ligands (Torres and Rosen 2003). FAK directed phosphorylation of Tyr256 prevents nuclear localization of activated N-WASP thus increasing the N-WASP activity to promote actin polymerization in the cytoplasm (Wu et al. 2004).

Serine residue, Ser485 in N-WASP in the junction between C and A motifs of the VCA domain is a CK2 phosphorylation site, which regulates binding with Arp2/3 complex (Cory et al. 2003). Constitutive activity of N-WASP was observed upon phosphorylation at the Ser485 that resulted in an increase in the affinity of the VCA domain for the Arp2/3 complex up to 7 fold (Cory et al. 2003). Taken together, phosphorylation of tyrosine and serine residues has a crucial part in regulation of WASP/N-WASP activity and its function to activate the Arp2/3 complex for actin filament assembly.

Cell migration plays an important role in several biological processes such as embryonic development, placental development and angiogenesis (Reviewed by Ridley et al., 2003). The actin cytoskeleton is connected to the ECM via the integrins at these cell adhesion sites and N-WASP is involved in facilitating changes in the actin cytoskeleton through the Arp2/3 complex. N-WASP has been identified

as an essential protein in mice (Lommel et al., 2001; Snapper et al., 2001) and has been shown to play a crucial part in cell-ECM adhesion and migration as depletion of the protein leads to cell adhesion defects and enhanced migration (Misra, et al., 2007). Cell migration has an important role in promoting tumorigenesis and metastasis due to its functions in development and tissue remodeling (Simpson et al., 2008). N-WASP was down regulated in breast cancer patients. Overexpressing N-WASP in breast cancer cells led to decreased cell motility, invasiveness of the cells and tumor growth (Martin et al., 2012). Hence decreased N-WASP expression seen in breast cancer patient samples may cause reorganization within the actin cytoskeleton that results in elevated migratory ability of the tumor cells, which marks the beginning of a metastatic cascade (Martin et al., 2008). A decrease in the N-WASP expression was observed in human colon cancer. The N-WASP expression showed an inverse relation with the disease progression and invasiveness of the cancer (Martin et al., 2012). N-WASP expression was also found to be associated with the tumorigenesis of esophageal squamous cell carcinoma (Wang et al., 2010).

In eukaryotic cells, regulated actin polymerization controls the following- the motility of the cells, contractility and maintenance of cell polarity. Protrusion of the front end of the cell represents the initial response of a cell to external stimuli, which are characterized as the actin rich structures: flat broad lamellipodia and thin long filopodia (Small et al., 1999). Lamellipodia and filopodia are characterized by very different actin organization and are regulated by different signaling pathways (Hall, 1998; Svitkina and Borisy, 1999). N-WASP along with Cdc42 has been shown to localize at filopodia and microspikes (Miki et al., 1998; Miki and Takenawa, 1998).

Malignant and invasive tumor cells when cultured on an ECM substratum, invade through the matrix by protrusions containing proteolytic enzymes that degrade the underlying ECM (Chen, 1989; Kelly et al., 1994; Mueller and Chen, 1991). These active structures are termed as invadopodia and are rich in actin and actin associated proteins such as cortactin (Ayala et al., 2006; Bowden et al., 2006). Cortactin binds and activates N-WASP via the SH3 domain. N-WASP being a regulator of the actin machinery initiates actin polymerization required for the formation of, invadopodia (McNiven et al., 2000; Mitzutani et al.,

2002; Kinley et al., 2003). N-WASP and one of its activators, Cdc42 has been reported to be critical for the formation of invadopodia (Lorenz et al., 2004; Yamaguchi et al., 2005). Overexpression of cortactin in normal and malignant cells increases, cell migration and invasive behavior is enhanced. Cortactin increases migration in an N-WASP dependent manner (Kowalski et al., 2005). In carcinoma cells EGF receptor signaling regulates invadopodia formation through N-WASP-Arp2/3 pathway indicating that N-WASP is downstream member of EGF signaling (Yamaguchi et. al., 2005). Recently, it was shown that formation of invadopodia is dependent on EGF stimulation (Philippar et al., 2008). Knockdown of N-WASP in MTLn3 cancer cells showed inhibition of invadopodia formation which coincide with the loss of protrusion formation in the direction of EGF stimulation, indicating that N-WASP works downstream of EGFR signaling for chemotatic migration (Desmarais, et al, 2009). These reports clearly suggest the function of N-WASP in tumor cell migration and chemotaxis. In response to EGF, N-WASP regulatory proteins such as Cdc42, Nck and WIP localize in invadopodia. During invadopodia formation, a focal adhesion protein such as FAK interacts with N-WASP, indicating a role for N-WASP in cell-ECM adhesion and migration (Yamaguchi et. al., 2005).

In a recent study, N-WASP expression was observed to have an inverse relation with keratinocyte proliferation. However wound healing and epidermal differentiation were unaffected in the absence of N-WASP (Lyubimova et al., 2010). In another study, N-WASP deficient mice showed no defects in the re-epithelialization of wounds. N-WASP was also identified as a regulator of hair follicle cycling (Lefever et al., 2010).

1.15 The Mammalian Verprolins

The human verprolin family is known to bind to the WH1 domain of WASP family of proteins. The three verprolins include the WASP interacting protein (WIP), glucocorticoid regulated gene-product (CR16), and WIP-related (WIRE, also known as WIP and CR16 homologous protein, WICH). The verprolins can influence the actin cytoskeleton independently of the Wiskott–Aldrich syndrome protein (WASP) family

of proteins (Reviewed by Aspenstrom, 2006). The N-terminal proline rich motif of the verprolins contains one or two motifs involved in binding to profilin which in turn binds to actin monomers (Fig. 1.4). The consensus profilin-binding motif, XPPPPP in which X most often is represented by a Gly, Ala, Leu, Ser or an Ile, consist of a few copies in the N-terminus of all mammalian verprolins. The three human verprolins are proline-rich (27–29%), and apart from binding to profilin, the proline-rich motifs have been reported to interact with SH3 domain containing proteins (Aspenstrom, 2005).

Figure 1.4: Schematic representation of the human verprolin family: WIRE, CR16 and WIP. The domain organization here shows the Profilin binding domain, Verprolin homologous domain, the Proline rich region and the WASP binding domain.

The gene that encodes the protein, “corticosteroids and regional expression 16” (CR16) is regulated by glucocorticoids (Masters et al., 1996) and is a member of the Wiskott–Aldrich syndrome protein (WASP)-interacting protein (WIP) family (Ho et al., 2001). The protein was later identified as an N-WASP associated protein that was found to bind tightly to N-WASP in bovine brain extracts by pull down assays. CR16 localization was observed in hippocampal neurons and the tips of the growth cone (Ho et al., 2001). However the role of CR16 in filopodia generation has not been characterized in depth. CR16 has been implicated in male-specific sterility based on the results obtained from CR16 knockout mice (Suetsugu et al., 2007). In another study, decrease in the expression of CR16 was observed in men with idiopathic azoospermia as compared to in healthy men (Xiang et al., 2011).

WIP was first identified as a proline rich protein, which binds to WASP in a yeast two-hybrid screen (Ramesh et al., 1997). WIP carries out its function to influence the actin cytoskeleton through interaction with WASP family of proteins by WASP binding domain at the C-terminus. It has been reported that around 95% of the WASP is present in the lymphocytes as a complex with WIP (Sasahara, et al., 2002) while WIRE and CR16 have been reported to form a tight complex with N-WASP (Ho et al., 2001, Kato et al., 2002). WIP inhibits Cdc42 activation by N-WASP indicating that one of the major functions of WIP is to stabilize WASP/N-WASP in a closed and inactive conformation (Martinez-Quiles et al., 2001).

Both WIP and WIRE are expressed ubiquitously however CR16 expression is restricted to brain, heart, lung, testis and colon (Ramesh et al., 1997; Weiler et al., 1996; Aspenstrom, 2002; Kato et al., 2002). Both WIP and WIRE stabilize the cellular F-actin (Nartinez-Quiles et al., 2001; Ramesh et al., 1997) by inhibiting actin de-polymerization in a concentration dependent manner (Kato et al., 2002; Martinez-Quiles et al., 2001). WIRE has been reported to cross link actin filaments through the central proline rich region rather than the WASP binding domain (Kato and Takenawa, 2005). In agreement with the above finding, WIP was found to co-localize with actin stress fibres and filopodia (Vetterkind et al., 2002) while WIRE was observed to localize along with the bundles of actin filaments as well as in F-actin constituting protrusions at the edges of cells independent of WASP (Aspenstrom, 2004). WIRE induces the assembly of thick actin fibers in fibroblast cells as well as in the cross-linking of actin filaments resulting in straight bundled actin filaments. However, in the presence of WASP, actin cross-linking activity of WIRE can be altered (Kato et al., 2002). Therefore, though WASP family of proteins is the major regulator and integrator of the Verprolin family of proteins, they play an important part in influencing changes in the actin cytoskeleton, which is independent of WASP family of proteins.

Based on data obtained using in-vitro actin polymerization assay, it was suggested that WIP prevents WASP/N-WASP from promoting actin polymerization through the Arp2/3 complex (Martinez- Quiles et al., 2001). Later it was suggested that phosphorylation of WIP by PKC θ causes the disassembly of WIP from WASP which allows WASP to be activated by the Arp2/3 complex (Sasahara et al., 2002).

However, in our laboratory it has been seen that WIP and WASP function as a complex (Rajmohan et al., 2006). WIP and WIRE have been reported to function in conjunction with N-WASP to form filopodia/actin-microspikes (Kato et al., 2002; Martinez-Quiles et al., 2001). The role of WIP or WIRE in N-WASP induced filopodia formation is still not clear. WIRE promotes peripheral protrusions such as filopodia and lamellipodia in PDGF treated fibroblast (Aspenström, 2002). WIRE is also required for the localization of WASP in PDGF treated fibroblasts (Aspenström, 2002). WIRE was observed to localize along the bundles of actin filaments as well as in F-actin containing protrusions at the cells edge independent of WASP or N-WASP. It has been shown that the expression of mutant WIRE, which is unable to interact with WASP, induces reorganization of actin filaments (Aspenstrom, 2004). WIRE cross-links F-actin resulting in straight bundled actin filaments, and induces the formation of thick actin fibers in cultured fibroblasts. This actin cross-linking activity is independent of WASP, but modified by the presence of WASP (Kato et al., 2005). The role of WIRE in inducing filopodia along with IRSp53 and independent of N-WASP was recently published (Misra et al., 2010).

1.16 IRSp53

Insulin receptor tyrosine kinase substrate p53 (IRSp53), is a multifunctional adaptor protein enriched in the central nervous system. IRSp53 is regulated by Rho family of small GTPases, Rac1 and Cdc42 and provides a molecular link between these GTPases and the actin cytoskeleton regulators, Wiskott-Aldrich syndrome protein (WASP) family verprolin homologous protein 2 (WAVE2) and mammalian enabled (Mena), which are involved in the formation of lamellipodia and filopodia. Active Cdc42 binds to the half-CRIB motif, whereas Rac1 binds to the unique N-terminal domain of IRSp53. It has been proposed that Rac1 or Cdc42 relieves the SH3 domain of IRSp53 present at the C-terminus which is masked by the N-terminus of IRSp53 thus making it available for other interactors to bind (Yamagishi et al., 2004). IRSp53 interacts directly with N-WASP via the SH3 domain, which is required for generation of filopodia as IRSp53 did not generate filopodia in N-WASP knockout (KO) fibroblasts (Lim et al., 2008).

Figure 1.5: Schematic representation of the domain organisation of IRSp53. The domains shown here include the I-BAR domain, a partial CRIB (Cdc42-Rac interactive binding) motif, the SH3 domain, WW domain binding site and a PDZ binding site. The site directed mutants used in the study are indicated below the domains .

Several studies have suggested a role for IRSp53 in lamellipodia formation and in filopodia formation in mesenchymal cells, branched neurites and dendritic spines in neuronal cells (Tada et al., 2006; Nakagawa et al., 2003). This dual function of IRSp53 is mediated through its interaction with WAVE2 and Rac1, which is required for lamellipodia formation and interaction with Cdc42 and Mena, which regulate filopodia formation (Lim et al., 2008; Nakagawa et al., 2003). Role of IRSp53 in filopodia as well as in lamellipodia formation can be further explained by the finding that IRSp53 localizes at the tips of both lamellipodia and filopodia (Nakagawa et al., 2003). Recently, it has been shown that IRSp53 interacts with N-WASP and induces filopodia (Lim et al., 2008). IRSp53 has been shown to exist in an auto-inhibited conformation as a result of the binding between the N-terminus of IRSp53 (1-178 aa) and the central region (180-317 aa). Cdc42 upon binding with IRSp53 leads to changes in conformation (Krugmann et al., 2001). Thus auto-inhibitory structure of IRSp53 and its activation by Cdc42 may be similar to that found in WASP and N-WASP (Kim et al., 2000).

IRSp53 is an effector of both, Cdc42 and Rac and is involved in the generation of filopodia and lamellipodia (Nakagawa et al., 2003). The I-BAR domain is involved in F-actin bundling, which facilitates the membrane dynamics (Fig. 1.5). The I-BAR domain of IRSp53 interacts with Cdc42 to facilitate the generation of filopodia (Lim et al., 2008; Scita et al., 2008). A recent study revealed that IRSp53 activity is controlled by Cdc42 by influencing the WIRE-IRSp53 interaction to form a complex that localizes at the membrane where filopodia is generated (Misra et al., 2010).

1.17 Objectives

Members of the Wiskott Aldrich syndrome family proteins, such as N-WASP and WASP influence the actin cytoskeleton by Arp2/3 complex activation, which advances actin polymerization by forming the nucleus critical for actin polymerization. N-WASP interactors have been studied extensively however the function of N-WASP in skin cancer has not been well characterized. Epithelial cells are organized into tissues, which are held tightly by intercellular, junction complexes. During wound healing, embryonic development and during metastasis of tumors the intercellular interactions are weakened/lost causing them to become motile. The objectives of this study are:

- 1) To determine the expression level of N-WASP in human skin cancer samples from patients.
- 2) To characterize the function of N-WASP in cell-ECM and cell-cell adhesion in normal skin cells, malignant (non-metastatic) and metastatic skin cancer cells.
- 3) To identify the domains of N-WASP required for cell-ECM adhesion using N-WASP^{-/-} MEFs.
- 4) To characterize role of CR16 independent of N-WASP in filopodia formation.

2. Materials and Methods

2.1 *Escherichia coli* strain

The *Escherichia coli* strain DH5 α was used for transforming the plasmids and in DNA subcloning experiments.

DH5 α F[']/endA1 hsdR17(r_k-m_k⁺) supE44 thi-1 recA1 gyrA

(Nal_r)relA1 Δ (lacZYA-argF)_{U169} (F80lacZ!M15) Hanahan, 1983

2.2 Preparation of *E. coli* competent cells (CaCl₂ method)

A bacterial colony was picked from LB plate and inoculated in 2 ml of LB medium and grown overnight at 37°C. The culture was diluted to an OD₆₀₀ of 0.05 in 50 ml of LB and incubated at 37°C to an OD₆₀₀ of 0.8. The culture was then transferred to sterile centrifuge falcons and stored in ice until further use. The cells were spun at 2908 g at 4°C for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 25 ml of ice-cold sterile 100 mM MgCl₂. The cells were then spun at 2908 g for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was re-suspended in 5 ml of sterile ice cold 100 mM CaCl₂ for 2 hours to become transformation competent. 2 ml of sterile ice cold 50% glycerol was added to the cell suspension. The cells were later stored at -80°C as 200 μ l aliquots in eppendorf tubes. The cells and the reagents were always maintained at 4°C.

2.3 Transformation of DNA into *E. coli*

50 μ l of competent cells were added into an eppendorf tube. The plasmid DNA (1 μ l) was added to the competent cells and incubated on ice for 30 minutes. The tube was then incubated at 42°C in water bath for 90 seconds and then immediately put on ice for 5 minutes. The cells were then plated on LB agar containing antibiotic and incubated at 37°C.

2.4 Isolation of plasmid DNA from *E. coli*

A single bacterial colony was inoculated in 3 ml of LB containing antibiotic and grown overnight at 37°C. The cells were spun at 2908 g at 4°C for 5 minutes. The plasmid DNA was isolated from the pellet according to the manual provided in the plasmid purification kit (Qiagen).

2.5 Strains and Media

Yeast strain PJ69. YPUAD (Yeast extract, peptone, uracil, adenine, and dextrose) is supplemented with 40µg/ml adenine and 20µg/ml uracil. SD (Selective dropout medium) minimum media and YPD were prepared according to (Munn et al., 1995). *S. cerevisiae* cells were transformed using the lithium acetate protocol (Thanabalu and Munn, 2001).

2.6 Transformation of yeast cells

Several colonies of yeast cells were inoculated in 25 ml of YPUAD and allowed to grow overnight at 24°C. The overnight culture was diluted to an OD600 of 0.2 and the cells were allowed to grow until they reached an OD600 of 0.8. Several steps of spin-down and washing were performed. Culture after reaching the OD of 0.8 was then transferred to 50 ml centrifuge tubes and spun at 1000X g for 5 minutes. Supernatant was discarded; pellet was washed with 30 ml of distilled H₂O and spun again at 1000X g for 5 minutes. Again the supernatant was discarded; pellet was re-suspended in 1 ml of distilled H₂O and transferred to a 1.5 ml eppendorf tube. This time the cell suspension was spun at maximum speed in a micro-centrifuge for 15 seconds. The supernatant was discarded again and the pellet was re-suspended in 250 µl of 100 mM LiAc.

The 250 µl the pellet was redistributed 50 µl aliquots in eppendorf tubes and employed for transformation. The cells were spun at the maximum speed in a micro-centrifuge for 15 seconds and the supernatant was discarded. To the pellet 240 µl of 50 % PEG, 36 µl of 1 M LiAc, 73 µl of distilled H₂O, 5 µl of sonicated salmon sperm DNA and 1 µl of plasmid DNA were added. The transformation tubes were vortexed for 1 minute, incubated at 24°C for 30 minutes and given a heat shock at 42°C for 20

minutes. The cells were spun again at 3670 g for 15 seconds, the supernatant was discarded and the pellet was re-suspended in 400 µl of distilled H₂O. 100µl of this cell suspension was plated on selective media plates and incubated at 24°C until the colonies reached sufficient growth for further analysis.

2.7 Yeast two hybrid screening

Yeast two hybrid screening is one of the most widely used techniques for identification of novel protein-protein interactions. The bait gene was expressed as a fusion to the GAL4 DNA-binding domain (BD), while yeast cDNA library were fused with GAL4 activation domain (AD) (AD library). When bait BD fused, protein interacts, the GAL4-BD and GAL4-AD were brought into proximity, thus activating transcription of two reporter genes (ADE2 and HIS3).

We optimized the 3-AT concentration using a series of SD/Trp-/His- plates containing 3AT at different concentrations in order to prevent the auto activation. The optimal concentration of 3AT was found to be 3mM.

2.8 Agarose gel electrophoresis (DNA)

1% agarose gel containing 0.4 µg/ml of EB was prepared. Once the gel was polymerized, it was soaked in 1X TAE buffer in different lanes 20 µl of DNA sample was loaded. The electrophoresis was performed to resolve the sample at 100 V. The electrophoresis was stopped when the DNA sample mixed with the dye run halfway through the gel. UV light was used to visualize the DNA bands.

2.9 DNA extraction from the agarose gel

The DNA sample resolved in the agarose gel was first visualized under UV light was used to visualize the DNA sample, resolved by the electrophoresis. The bands of desired molecular weight were excised out with a scalpel and placed in an eppendorf tube. The DNA was extracted according to the manual provided in the plasmid purification kit.

2.10 Construction of recombinant plasmids

Restriction enzymes were used to digest the purified DNA fragments and the reaction tube was prepared according to the instructions supplied by the manufacturer. DNA sample together with restrictions enzymes were incubated at 37°C for 2 hours however in the case of restriction enzymes having star activity such as BamHI and EcoRI, the restriction enzyme digestion was performed only up to 1.5 hours. Agarose gel electrophoresis was used to resolve the restriction enzyme digested products DNA fragments of interest were excised and purified from the agarose gel. 2 µl of each of the purified DNA fragment was mixed with loading dye and distilled H₂O and subjected to agarose gel electrophoresis in order to verify the presence of sufficient quantities of DNA fragment enough for the ligation reaction.

Purified insert DNA was ligated with linearized vector DNA by using T4 DNA ligase and the ligation reaction mixture was made according to the instructions supplied by the manufacturer. The ligation reaction mixture was incubated at 24°C for 4 hours and then introduced into competent *E. coli* cells by following the transformation protocol for *E. coli*.

Table 2.1: Plasmids that were constructed that were used for the experiments

N-WASL SiA	EBV-YFP	Cop-CR16-6XHis	Cop-CR16-EGFP
EBV-N-WASP-YFP	EBV-YFP	Cop-EGFP	Cop-CR16
Cop (Empty vector)	Cop-CR16ΔV	Cop-N-WASP¹⁻²⁷⁶	NYFP-N-WASP- CYFP-N-WASP mammalian sensor
Cop-CR16ΔV-EGFP	Cop-WIP- EGFP		

Table 2.2: Plasmids created by others in the laboratory

Cop-WIRE-6XHis	Cop-IRSp53-6XHis	Cop-IRSp53-shRNA	EBV-NWASP ^{Y256F}
Cop-IRSp53	EBV-YFP	Cop-Cdc42-shRNA	EBV-N-WASP ^{ΔC19}
Cop-IRSp53 ^{I267N}	Cop-Cdc42	Cop-Rac1	EBV-N-WASP ^{ΔVCA}
Cop-IRSp53 ^{2A}	Cop-Cdc42 ^{G12V}	Cop-Rac1 ^{G12V}	EBV-N-WASP ^{Y256E}
Cop-IRSp53 ^{4A}	Cop-Cdc42 ^{T17N}	Cop-Rac1 ^{T17N}	EBV-N-WASP ^{ΔV}
EBV-N-WASP	EBV-NWASP ^{ΔWH1}		

2.11 Verification of recombinant plasmid constructs**2.12 Restriction digestion**

Restriction digestion method was used to confirm the presence of the recombinant plasmid. Specific restriction enzymes were used for the digestion of recombinant DNA that target unique sites on the plasmid at regions flanking the cloned DNA insert. Agarose gel electrophoresis was performed on the digested DNA products and the size of the Recombinant DNA fragments were examined on the basis of respective molecular weight.

2.13 Polymerase chain reaction (PCR)

Polymerase chain reaction was performed to specifically amplify DNA from very small amount of DNA.

PCR reactions were performed in 0.2 ml tubes. Following are the volumes of reagents used-

DNA Template	0.5 μ l
Taq DNA polymerase	0.5 μ l
10X Reaction buffer	10 μ M
Forward primer	1 μ l (10 μ M)
Reverse primer	1 μ l (10 μ M)
distilled H ₂ O	37 μ l

PCR reaction was performed as follows:

Denaturation 30 sec, 95°C

Annealing 30 sec, 65°C

Elongation 30 sec, 72°C

These 3 steps were repeated 3 times.

Denaturation 30 sec, 95°C

Annealing 30 sec, 55°C

Elongation 30 sec, 72°C

These 3 steps were repeated 30 times

The PCR reaction was cycled 30 times for optimal amplification of the target fragment. The primers used in this study are listed in Appendix 2A and 2B. The PCR products were analysed by Agarose gel electrophoresis. 10X Taq DNA polymerase Buffer

Tris HCL (pH 9) 100mM

KCl 500mm

MgCl₂ 20mM

2.14 DNA quantification

To quantify the DNA concentration with minimal consumption of sample the Nanodrop was used. Nanodrop can measure as little as 1 µl of DNA, significantly lower down the sample volume. 1 µl of DNA sample was diluted in 49 µl of TE buffer and in separate eppendorf reference solution (negative control) was prepared. Concentration of the DNA sample was calculated by using Nanodrop. Software will generate the standard curve using the DNA concentrations along with the negative control and gives the DNA concentration in ng/µl. OD₂₆₀/OD₂₈₀ ratio was checked to confirm the purity of DNA sample. OD₂₆₀/ OD₂₈₀ less than 1.8 indicate impurity caused by proteins and/or other matter capable of absorbing UV light. OD₂₆₀/ OD₂₈₀ higher than 2.0 indicates possibility of RNA contamination in the DNA sample. OD₂₆₀/ OD₂₈₀ between 1.8 and 2.0 indicate the absorption due to DNA thus all DNA samples which had OD₂₆₀/ OD₂₈₀ between 1.8 to 2.0 were chosen for further experiments.

2.15 Commercial Vectors

DNA subcloning was performed by using pUC19 and pUC18 vectors. The vectors pACT2 and pAS2-1, which express the Gal4p Activation domain and the Gal4p Binding domain, were expressed in pACT2 and pAS2-1 vectors respectively in Yeast Two Hybrid assays. For yeast two hybrid assay proteins were expressed in the YCplac and YEplac (Gietz et. al., 1988) series of plasmids in *Saccharomyces cerevisiae* strains.

The following plasmids were constructed by the author of the thesis that were used for this study.

Table 2.3 Expression vectors used in the study

Vector	Resistance	Selective marker	Expression Host
pACT2	Amp	Leu	<i>S.cerevisiae/E.coli</i>
pAS2-1	Amp	Trp	<i>S.cerevisiae/E.coli</i>
Cop	Amp	-	Mammalian cells
EBV	Amp	Puromycin	Mammalian cells

2.16 Cell culture reagent and Plastic ware

Cell culture media additives were purchased from Hyclone or Gibco. Plastic wares were bought from costar, falcon and Eppendorf.

2.17 Antibodies

The antibodies used in this study are listed below (Table 2.1 & 2.2) and the dilutions used for the western blot experiments are specified. The secondary antibodies conjugated with Horseradish peroxidase (HRP).

2.18 Primary antibodies

Antibodies purchased: anti-GFP antibody and anti-GAPDH from Clontech, anti-IRSp53 from Santacruz, anti-Cdc42 from Upstate, anti-Rac1 from BD Biosciences, anti E-cadherin from BD were purchased from Invitrogen. BD Biosciences; Alexa fluor 488-conjugated goat anti-mouse immunoglobulin, alexa fluor Immunoglobulin and Alexa Fluor 488-conjugated phalloidin.

2.19 Secondary Antibody

Secondary antibodies were used for western blot and immunofluorescence. Their dilutions for western blot and immunofluorescence are given in table 2.3.

2.20 Enzymes and kits used for molecular biology

The restriction endonucleases used in cloning and subcloning experiments were from New England Biolabs (NEB) (Ipswich, MA, USA), Fermentas (Hanover, MD, USA) or Roche (Indianapolis, IN, USA). For PCR amplification, *pfu* DNA polymerase from Stratagene (La Jolla, CA, USA) and the DNA polymerase from BIOTOOLS (Madrid, Spain) were used. For ligation of the DNA fragments the T4 DNA ligase was from Roche (Indianapolis, IN, USA). Site directed mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA).

Plasmid DNA was isolated from *Escherichia coli* cells by using QIAprep Spin Miniprep Kit (Qiagen) (Valencia, CA, USA) and the QIAquick Gel extraction Kit (Qiagen) (Valencia, CA, USA) was used for DNA extraction from Agarose gels. The QIAquick PCR Purification Kit (Qiagen) (Valencia, CA, USA) was used for the purification of PCR fragments.

Table 2.4: Primary antibodies and the dilutions used for Western blot analysis and immunofluorescence.

Antibody	Company	Dilution for WB	Blocking conditions
E-Cadherin	BD	1:1000	5% milk
Vinculin	Singma	1:1000	3% milk
Paxillin	Sigma	1:1000	3% milk
Rac1	Sigma	1:2000	3% milk
IRSp53	Santacruz	1:500	3% milk
Cdc42	Upstate	1:1000	3% milk
N-WASP	SANTacruz	1:5000	5% milk

2.21 Chemicals and reagents

DNA loading ladder was from NEB (Ipswich, MA, USA). The dNTP (dTTP, dATP, dGTP and dCTP) mixtures were purchased from CHEMICON. Protein molecular weight marker was from Axygen Biosciences (Hercules, CA, USA). 1, 4-Dithiothreitol (DTT), Phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail tablets were from Roche (Indianapolis, IN, USA). The X-gal (1-Bromo-4-Chloro-3- indolyl-b-D-galactopyranoside) was from BioRad (Hercules, CA, USA). Isopropyl-Thio-B-D-galactopyranoside (IPTG) was from Promega (Madison, WI, USA). Ampicillin was purchased from Invitrogen Lifescience technology (St. Louis, Mo, USA). AbI PRISM DNA sequencing kit (Big Dye) was purchased from Applied Biosystems (Foster City, CA, USA). Ni²⁺-NTA resins were purchased from Invitrogen by life technology (UK). The Alexa-488-conjugated phalloidin was from Molecular

Table 2.5 Secondary antibodies and the dilutions used for Western blot analysis and immunofluorescence.

Antibody	Company	Dilution for WB
Mouse anti-rabbit IgG	Sigma	1:20,000
Rabbit anti-mouse IgG	Sigma	1:20,000
Alexa-488 Rabbit anti-mouse IgG	Molecular Probs	-
Alexa-594 Rabbit anti-mouse IgG	Molecular Probs	-
Donkey anti-goat anti-IgG	Jakson Immuno	1:5,000

Probes (Eugene, OR, USA). Nitrocellulose membrane was from Bio-Rad (Hercules, CA, USA). Unless otherwise stated, all the chemicals were of analytical grade and purchase from Sigma Aldrich Chemical Company Ltd. (St. Louis, Mo, USA).

2.22 Buffers and solutions

TAE (Tris-acetate-EDTA, 50X)

50X stock solution was prepared by dissolving 242 g of Tris base, 57.1 ml of glacial acetic acid and 100ml of 0.5 M EDTA (pH 8.0) in 1000 ml of dd H₂O.

Agarose gel

1% agarose was made by suspending 10 g of agarose in 1000 ml of 1X TAE buffer and microwaved for 10 min. The agarose solution can be maintained in liquid form at 60°C by storing in an oven.

DNA loading buffer

6X DNA loading buffer was made by adding bromophenol blue (0.25%), xylene cyanol FF (0.25%) and sucrose (40%) in dd H₂O. The solution was stored at 4°C.

SDS

10 g of SDS was dissolved in 100 ml of distilled H₂O

Tris-glycine electrophoresis buffer

15.1 g of Tris, 94 g of glycine and 50 ml of 10% SDS stock solution were dissolved in 1000 ml of distilled H₂O, the pH of the solution was adjusted to 8.3.

2X SDS-PAGE gel-loading buffer

2X buffer had 100 mM Tris-Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% Bromophenol blue and 20% glycerol. The solution was stored at 4°C.

10% Ammonium persulphate

1 g of ammonium persulphate was dissolved in 10 ml of distilled H₂O and the solution was stored at 4°C.

Membrane transfer buffer

3.03 g of Tris, 14.4 g of glycine and 200 ml of methanol were dissolved in 800 ml of distilled H₂O.

1X Tris buffered saline (TBS)

TBS had 20 mM Tris and 500 mM NaCl, the pH was adjusted to 7.5.

Wash solution

5 ml of 10% Triton X-100 solution was added to 995 ml of 1X Phosphate buffered saline.

Blocking solution

0.5 g of non-fat dry milk was dissolved in 10 ml of 1X Phosphate buffered saline and stored at 4 °C until further use.

1M Tris buffer

121.12 g of Tris was dissolved in 800 ml of distilled H₂O and the pH of the solution was adjusted to 7.5 or 8.0 using HCl and then the volume was made up to 1000 ml with distilled H₂O.

5M NaCl

292 g of NaCl was dissolved in 1000 ml of distilled H₂O and the solution was autoclaved.

10X TE buffer

TE buffer had 0.1 M Tris-HCl and 10 mM EDTA, the pH was adjusted to 7.5 and autoclaved.

50% PEG 3350

50 g of PEG 3350 was dissolved in 100 ml of sterile deionized H₂O. The solution was warmed in a microwave to enable the PEG 3350 to dissolve faster.

10X LiAc

1 M lithium acetate solution was prepared in distilled H₂O, the pH adjusted to 7.5 and autoclaved.

Lysis buffer for His-Pull down

50 mM Tris (pH8), 100 mM NaCl, 5% NP40, 200 mM PMSF

Equilibration Buffer for Ni-NTA beads

50mM Tris (Ph8), 100 mM NaCl

Elution buffer for Ni beads

50 mM Tris (Ph8), 100 mM NaCl, 250 mM imidazole

2.23 Culture conditions

The following cell lines were all grown at 37°C cell culture incubator with 5% CO₂ supplied for the optimal maintenance of pH of the cell.

2.24 Culturing of N-WASP^{+/+} and N-WASP^{-/-} MEFs

The mouse embryonic fibroblasts (MEFs) were obtained from snapper et al and were immortalized by using transient transfection with Cre recombinase expression vector (Snapper, S.B., et al 2001). The cells were grown in Dulbeccos modified eagle medium (DMEM) purchased from Invitrogen (USA) with gultamine, high D-glucose, supplemented with 10% FBS (Hyclone), 1% Penicillin, 1% Streptomycin at 37 Dulbeccos modified eagle medium (DMEM) purchased from Invitrogen (USA) and 5% CO₂ on plasticware.

2.25 Culturing of keratinocytes

The cells were grown in the Dulbeccos modified eagle medium (DMEM) purchased from Invitrogen(USA) with gultamine, high D-glucose, supplemented with 10% FBS (Hyclone), 1% Penicillin, 1% Streptomycin at 37 °C and 7.5% CO₂ on plasticware.

Culturing of HaCaT

HaCaT cells were passaged after reaching a confluency of 70%. The cells were incubated in 3mL of trypsin for 5-10 mins at 37 °C. The trypsin was then neutralized using media. The cells were pelleted at

47 g for 5 mins and re-suspended in 4mL of media before seeding onto culture flasks. The seeding density was a 30%.

Culturing of A5-RT3

The A5-RT3 cells were passaged at a confluency of 90-100%. The A5-RT3 cells were first incubated in 3mL of EDTA for 10 mins at 37°C followed by trypsinisation for less than 5mins in 3mL of trypsin. The trypsinisation is followed by neutralization by using the media. The cells were then pelleted down at 47 g for 5 mins. The pellet was resuspended in 4 mL of media and then seeded at a confluency of 30-40%.

Culturing of HSC-5

The HSC-5 cells were grown for 4 days until the confluency of 80%-90% was reached. These cells are first incubated in 3mL of EDTA for 10 mins at 37°C followed by trypsinisation for 5 mins. The trypsin is neutralized with 4 mL of media and cells were pelleted down. The cells were then seeded at a confluency of 40%.

Culturing of II-4

The II-4 cells were grown to a confluency of 80% and passaged. The cells were treated with 3mL of EDTA for 10 mins and then incubated at 37°C in 3 mL of trypsin for 5 mins . The trypsin was neutralized using the media. The cells were then pelleted down and resuspended in fresh media. The resuspended cells were seeded at a confluency of 40%.

Culturing of 293T

The 293T cells were grown and maintained in DMEM supplemented with 10% FBS and 1% Penicillin and Streptomycin. The cells are incubated in 1ml trypsin for a few mins outside at room temperature. The cells were neutralized with media and 1ml of the cells were seeded onto a 10mL petridish.

2.26 Freezing and thawing of mammalian cells

The cells are grown and then harvested at a high confluency. The cells are incubated in trypsin at 37°C in the cell culture incubator. The trypsin is neutralized with media and pelleted down. The pellet is re-suspended in freezing media (10% DMSO + 90% FBS). The mixture is then aliquoted into freezing vials. The vials are then placed at -80°C overnight. The vials are then transferred and stored permanently in liquid nitrogen.

In order to culture a new batch of frozen cells, the vial containing the mixture of cells and freezing media are placed at room temperature in the cell culture laminar hood. The frozen pellet is thawed using media and seeded into a culture flask containing media.

2.27 Culture of mammalian cells prior to microscopic analysis and transfection

Cells were maintained at 37°C, 5% CO₂ in Deulbecco's Modified Eagle's Medium (DMEM) purchased from Invitrogen with glutamine, supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin). Transfection was carried out using PEI according to the manufacturer's recommendations. Transfected cells were incubated for 36 hours before analysis.

Cells prior immunohistochemistry were maintained at the desired confluency (approximate 70%). The cells are seeded and grown for 24 hours prior to immunohistochemistry.

2.28 Immunofluorescence in mammalian cells

Cells were washed thrice with 1X PBS. The cells are then fixed with 3.7% formaldehyde for 20 mins. The cells were washed thrice right after, with 1X PBS after fixing and permeabilized with Triton-X-100. Permeabilization was followed by washing with 0.1% triton X- PBS and then cells were blocked with 1% BSA for half an hour. After blocking, cells were then incubated with primary antibody for a period of 1 hour. After primary incubation cells are washed thrice with 0.1% triton X-PBS, followed by secondary antibody incubation. The secondary antibody incubation was done for 45mins-1 hour. Washing for three

times, with 0.1% triton-PBS, follows the secondary antibody incubation. After staining coverslips with the cells are mounted on the slide using the DAPI mounting medium.

2.29 Relative filopodia Index tabulation

The fluorescence images taken on the metamorph software were assessed for the lengths of the filopodia. The cells with filopodia were counted based on two criteria: the length of the filopodia ranged between 8-20 μ m and cells with filopodia, more than 5 were counted. Based on the above criteria the number of cells with filopodia were counted and expressed as a percentage.

The experiments were performed thrice. Average values were calculated and standard deviation was obtained for the values. A t-test was done to quantify the significance of the difference in the test versus the control sample by Microsoft excel.

2.30 SDS polyacrylamide gel electrophoresis

Thick and thin gel plates are cleaned and dried off any lint or particle. The plates are aligned and fitted onto the stand. The following proportions of the reagents are used for resolving and stacking portions of the gel.

Table 2.5: Reagents used for resolving gel

Distilled water	Polyacrylamide mix	1.5mMTris pH 8.8	10%SDS	10% Ammonium persulfate	TEMED
4mL	3.3 mL	2.5 mL	0.1mL	0.1mL	0.004mL

Table 2.6: Reagents used for stacking gel.

Distilled Water	Polyacrylamide mix	1.5mMTris pH 8.8	10% SDS	10%Ammonium persulfate	TEMED
3.3mL	0.830 mL	0.630 mL	0.05mL	0.05mL	0.005mL

2.31 Western Blot for mammalian cell lysate

Cell lysate from 5×10^6 cells was prepared by lysing in buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 10% glycerol, 1 mM EDTA, 1mM phenylmethylsulfonyl fluoride, and 0.1mM EDTA on ice for 1 hr. Lysates were boiled in 5x Laemmli dye for 5 mins. Proteins were then resolved by 10% SDS-PAGE gel and transferred onto nitrocellulose membrane. They were then probed overnight with the appropriate primary antibody. The probing is followed by three washed with 0.1% triton-PBS. The blot is probed with the secondary antibody for 1 hour. The probing is followed by three washed with 0.1% triton-PBS. The secondary antibody is conjugated with horse-radish peroxidase (HRP) and detected with Amersham ECL PlusTM western blotting detection reagent.

Table 2.7: Composition of Lysis buffer used for lysis of mammalian cells to extract the protein.

<u>Chemical</u>	<u>Stock concentration</u>	<u>Final concentration</u>	<u>For 10 ml of lysis buffer</u>
Na-deoxycholate	10 %, 1 g / 10 ml	1 %	1000 µl
TritonX-100	10 %, 1 ml / 10 ml	1 %	1000 µl
SDS	20 %, 200 g / 1 l 10,	1%	100 µl
HEPES pH 7.4	1 M, 23.83 g / 100 ml	50 mM	500 µl
NaCl	5 M, 146.1g /500 ml	150 mM	300 µl
EDTA-free proteinase inhibitors (Roche)			- 1 tablet (according to the protocol of the manufacturer)
EGTA	50 mM, 1.2 g / 50 ml	1mM	200 µl
Na ₃ VO ₄	100 mM, 0.092 g / 5 ml	1mM	100 µl
MgCl ₂	1 M, 10.2 g / 50 ml	1.5 mM	15 µl
Na ₄ P ₂ O ₇	100 mM, 0.45 g / 10 ml	10 mM	1000 µl
NaF	1 M, 0.42 g / 10 ml	100 mM	1000 µl
glycerol 96 % 10 % 1150 µl	96 %	10 %	1150 µl

2.32 His tag pull-down

300µl of Ni-agarose beads are aliquoted and spun down at the lowest speed for 4 min. The beads are washed with distilled water. The beads are then incubated on a rocker with equilibration buffer for 1-2 hours, at 4°C. After this the cell lysate is added to the beads and incubated on the rocker for 1-2 hours at 4°C. The beads are then washed in elution buffer with different concentrations of imidazole ranging from (10mM-500mM). Incubate in the elution buffer each time for 5-10 mins followed by centrifugation. The supernatant is collected to determine the concentration of imidazole that is optimum for the pull-down of the concerned protein. In our case the concentration of 200mM was found to be optimum. After collecting the supernatant a TCA precipitation of the protein is carried out and eventually the pellet is

resuspended in the loading dye. 15-20 μ l of the sample is loaded onto a SDS gel and then selective antibodies are used to probe the different proteins.

36 hours of post-transfection, transfected cells were washed once with 1X PBS and subsequently trypsinized with 1 ml of trypsin for and incubated for 2 min at 37°C. Transfected cells were collected by washing with 3 ml of DMEM medium and spun for 11.98 g for 4 minutes. Supernatant was discarded and the pellet was re-suspended with 1X PBS and the cell suspension was spun again at 11.98 g for 4 minutes. The supernatant was discarded and the pellet was subjected to glass bead lysis to obtain the total protein lysate. The Ni-NTA beads were then prepared for the pull down assay by washing the beads thrice with equilibration buffer. The pull down was then carried out by adding the beads to the total protein lysate and the beads were incubated at 4°C for 1 hour. The beads were then spun at 11.98 g for 5 minutes and the supernatant was discarded. The beads were subsequently washed 3 times with wash buffer and finally the proteins bound to the beads were eluted out by TCA (Trichloroacetic acid) precipitation method. Subsequently, TCA precipitation was also performed for flow through as well as for whole cell lysate. TCA was added in the protein sample with in equal volume of 20% TCA (250 μ l). Incubated on ice for 10 minutes and subsequently spun down at 11.98 g for 10 minutes to pellet. Pellet was washed with ice-cold acetone and kept on ice for 10 minutes. After incubation pellet was spun down at 11.98 g for 10 minutes and aspirated and air-dry. Protein pellet was re-suspended in 60 μ l of 2XSDS-PAGE loading dye together with 1M DTT and boiled at 100°C for 5 minutes. Pull down sample was either stored at -20°C or run immediately on SDS-PAGE gel subsequently western blot was performed to visualize the proteins bands.

2.33 Immunoblotting and chemiluminescence

After blotting the membrane was removed from the transfer cassette and Ponceau S solution (Sigma) was used to detect the size protein bands. Subsequently, the membrane was washed with the 1X PBS for 10 min and blocked in the 3-5% skim milk solution in PBS for 30 min at room temperature. Primary antibody was incubated with the membrane overnight with appropriate dilution (for dilutions see Table:

2.6) for overnight at 4°C. After the incubation with primary antibody, membrane was washed 3 times 5 minutes per wash. Subsequently, membrane was incubated in appropriate secondary antibody (for dilutions see Table: 2.7) conjugated with Horseradish Peroxidase (HRP) for 1 hour at room temperature. The membrane was then washed and the protein bands were detected with PICO (Pierce). Prior to the detection of protein, membrane was washed again with PBS and detection solution (ECLTM Western blotting detection reagent, Amersham Biosciences) was used as per the instructions of manufactures. Chemo luminescence method was use for the detection of protein. In this method hydrogen peroxide oxidized the Luminol, which is conjugated with secondary antibody. Membrane was sealed under transparent plastic cover and exposed to ECL film (Fujifilm). The exposed film was developed in a developing machine.

2.34 Transient knockdown of mammalian genes by RNAi

Plasmid that expressed shRNA in pFIV vector (Open Biosystems) targeting mouse IRSp53 (GCACTGAAGAAATACCAAA), WIRE (TTTCTCCCTGTTGTAGGCTTG), WIP (AACCGCCAACAGGGATAATGA) and Cdc42 (GCCTATTACTCCAGAGACT) were constructed. The plasmid was transfected into N-WASP^{-/-} MEF or N-WASP^{+/+} mouse embryonic fibroblasts and the cells were harvested 48 µg post transfection and lysed for Immuno blot analysis to determine the level of knockdown.

2.35 Generation of stable cell lines

Stable sublines of the keratinocytes: HaCaT, A5-RT3 and HSC-5 cells were microporated with plasmids to knockdown the expression level of N-WASP in HaCaT and A5-RT3 and overexpression of N-WASP in HSC-5 cells. Microporation of the plasmids was followed with selection using puromycin of different concentrations for the three cell lines over 3-4 passages: HaCaT- 800ng/mL, HSC-5-1000ng/mL and A5-RT3-800ng/mL. The cells were checked for expression of N-WASP by western blot.

In order to identify the functions of N-WASP the different domains were eliminated to obtain the plasmids with genes expressing the truncated versions of N-WASP. The plasmid was tagged with a puromycin and ebna. The plasmids were transfected and selection was carried out with puromycin to establish stable cell lines expressing the different domains of N-WASP protein. The cells were grown in DMEM containing penicillin and streptomycin and puromycin ($1\mu\text{g}/\mu\text{L}$). The cells were harvested after several passages to obtain a culture of cells expressing the protein. The expression was checked by western blot.

2.36 Cell Spreading assay

Wells were coated with $1.41\mu\text{g}/\text{mL}$ fibronectin in 1% BSA for 2 μg . The wells were then blocked with 1% BSA for 30mins. Cells were incubated in trypsin for the required number of minutes depending on the cell line. The trypsin containing the cells, were neutralized using additional media and pelleted down. The cells were counted and the required number of cells, were serum starved for 30mins. The cells were pelleted down and the required number of seeded onto the wells. Images of the cells were taken at three different time points of 10, 30 and 60 minutes of incubation in the incubator. The cell area was calculated using the metaview software to compare the spreading. Images were taken using the metaview software with the 40X objective.

The experiments were performed thrice. Average values were calculated and standard deviation was obtained for the values. A t test was done to quantify the significance of the difference in the test versus the control sample by Microsoft excel.

2.37 Cell Adhesion assay

The wells were coated with $1.41\mu\text{g}/\text{mL}$ fibronectin in 1% BSA for 2 hours. The wells were blocked with 1% BSA for 30 mins. The cells were trypsinized and serum starved for 30mins in the incubator. The serum starvation of the cells was followed by incubation in Calcein AM for 20 mins. The cells were then

pelleted down and re-suspended in media. The desired volume of suspended cells was seeded so as to obtain equal number of cells in each of the wells. The cells were incubated for 30mins in the incubator. The number of cells in the wells was measured as fluorescence units by the tecan machine.

The experiments were performed thrice. Average values were calculated and standard deviation was obtained for the values. A t test was done to quantify the significance of the difference in the test versus the control sample by Microsoft excel.

2.38 Scratch assay

The cells were allowed to grow to 100% confluency. Three scratches were drawn in each well. The media was changed after drawing the scratches and after 15minutes the assay was set up. Wound healing was monitored by time-lapse microscopy over 18 hours. The videos obtained were tracked for the distance covered by the migration of the cells to close the gap created by the scratch

2.39 RNA Extraction

The RNA extraction from samples embedded in paraffin was done using the RecoverAllTM Total Nucleic Acid Isolation by ambion. The paraffin embedded samples are de-paraffinized using Xylene. After discarding the xylene, protease digestion is carried out by adding the given digestion buffer and protease followed by a 3hours incubation. After which the nucleic acid isolation is done and washed with ethanol followed by passing the mixture through a filter cartridge. Lastly, the nuclease digestion and final nucleic acid purification is carried out by incubating in a DNase mix followed by washing with wash buffers and finally eluted using the elution solution.

2.40 Reverse Transcription

1-2.5µg of RNA and 0.5µg/µl of random primers (Invitrogen) were mixed and made up to 11µl with nuclease free water. This mixture is incubated at 70°C for 5 min. Followed by the heating, the tubes are placed on ice for 5min. A reaction mixture containing 4µl reaction buffer (Invitrogen), 2µl dNTPs, 2 µl

of nuclease free water is prepared. 8 µl of this mixture is added to each of the tubes placed on ice. This is followed by incubation at 37°C, 5min. Then the tube is placed on ice followed by the addition of 0.5µl of reverse transcriptase (Invitrogen) to each tube. The entire mixture is then incubated for 60min at 42°C followed by heat inactivation at 70°C, 5min. The cDNA prepared is placed at -20°C.

2.41 Real time PCR

The following preparation is followed for each sample setup: 12.5µl SYBR green Master, 0.25 µl of cDNA prepared, and 12.25µl of diluted primers (forward and reverse) (50µM in 25µl). The samples are prepared in qPCR tubes from Qiagen. The tubes are placed in the 7500 ABI machine and the following reaction cycle is set.

Tabulation is done by the comparative C_T method: The average cycle threshold (Ct) values are obtained and applied in the following equation to compare the gene expression in two different samples.

$$\begin{array}{ll} \mathbf{Ct_{Target\ gene} = Ct_{N-WASP}} & \mathbf{Ct_{Tumor} = SCC} \\ \mathbf{Ct_{Endogenous\ control} = Ct_{MRPL}} & \mathbf{Ct_{Reference} = Perilesional} \end{array}$$

The following steps are to be followed:

Step A : Normalization to the endogenous control

$$\mathbf{Ct_{Target\ gene} - Ct_{Endogenous\ control} = \Delta Ct}$$

$$\mathbf{1. Ct_{tumor(N-WASP)} - Ct_{tumor(MRPL)} = \Delta Ct_{Tumor}}$$

$$\mathbf{2. Ct_{Perilesional(N-WASP)} - Ct_{Perilesional(MRPL)} = \Delta Ct_{Reference}}$$

Step B : Normalization to reference sample

$$\mathbf{Ct_{Tumor} - Ct_{Reference} = \Delta \Delta Ct}$$

$$\mathbf{(Ct_{tumor(WASL)} - Ct_{tumor(MRPL)}) - (Ct_{Perilesional(wasl)} - Ct_{Perilesional(MRPL)}) = \Delta \Delta Ct}$$

2.42 Microscopy

In order to visualize the morphology of mammalian cells as well as fluorescent images microscopy was

performed. DIC and Fluorescence images were acquired using Olympus IX51 fitted with Cool SNAPHQ camera and analyzed using Metamorph software (Molecular Devices). Images were further processed with Adobe photoshop. Phase contrast live cell imaging was performed in a closed chamber filled with 5% CO₂, 40% humidity at 37 °C using Olympus IX51 fitted with Cool SNAPHQ camera.

To perform live cell imaging, prior to fluorescence live imaging, cells were cultured and transiently transfected with GFP constructs. For fluorescence live cell imaging, only GFP transfected cells were selected for imaging. All the experiments were in triplicate and repeated at least three times.

Chapter 3

3.0 Function of N-WASP in cell-ECM adhesion and migration in fibroblasts and skin carcinomas.

3.1 Introduction

The actin cytoskeleton provides the supportive framework and force for the cell shape change, the motility of the cells and organization within the cytoplasm. The cell adhesion turnovers involving the attaching of a cell to the matrix and detaching of a cell off the matrix is required for cell migration (Ridley et al., 2003). The cells attach to the ECM via the integrins, heterodimeric transmembrane receptors, which connect the extracellular matrix to the actin cytoskeleton through focal adhesions (Schwartz et al., 2000). The proteins that bind to the integrins that include the focal adhesion kinase (FAK), paxillin, and vinculin are present in the focal adhesion complexes (FA) (Jockusch et al., 1995). Fibroblasts, which are depleted of FAK/paxillin have display reduced focal adhesion turnover (Mitra et al., 2005) while depletion of vinculin results in an increase in the turnover of focal adhesions (Saunders et al., 2006 and Xu et al., 1998). Phosphorylation of N-WASP at the Tyr256 located in the GBD is facilitated by FAK, which keeps N-WASP in the cytoplasm, indicating the role of FAK in controlling the intracellular localization of N-WASP (Wu et al., 2004). The function of N-WASP in fibroblast cell adhesion and cell spreading has been studied in mouse fibroblasts and Chinese hamster ovary cells, which showed that N-WASP promotes cell adhesion (Misra et al., 2007; Yoo et al., 2006). A study by Wang et al showed that N-WASP expression was related to lymph node metastases and has been suggested to be a candidate biomarker for the diagnosis and prognosis of ESCC (Esophageal squamous cell carcinoma) (Wang et al., 2010). Another study showed that N-WASP acts as a putative tumor suppressor as the expression level of N-WASP was found to be lesser in the breast cancer tissues (Martin et al., 2008). Claudin-5 was implicated in promoting metastasis through the N-WASP signaling cascade in breast cancer cells (Escudero Esparza et al., 2012). The N-WASP expression was found to have an

inverse relation with the invasiveness of the cells and adhesion (Martin et al., 2012). N-WASP expression was reduced in colon tumors. N-WASP acts as a tumor suppressor in colorectal cancer however the function of N-WASP in colon tumor progression, metastasis and the underlying mechanisms remain to be determined (Wang et al., 2012). A direct correlation was shown between N-WASP expression and invadopodia formation in metastasis (Gligorijivec et al., 2012). The above-mentioned reports on N-WASP correlation with cancer suggests that the loss of N-WASP may affect cytoskeletal dynamics of a cell, which affects epithelial cell migration, adhesion and spreading. The changes in the expression and localization of the focal adhesion proteins may also occur hence vinculin and paxillin, cell-cell junction protein- E-cadherin were also studied. In addition, in this study the role of different domains of N-WASP that may affect the cell adhesion, migration and spreading characteristics in mouse embryonic fibroblasts (MEFs) were also explored.

3.2 Expression of N-WASP is altered in both tumorigenic and metastatic epithelial cell lines

Increased motile behavior is a pre-requisite for successful metastasis. Therefore characterizing proteins involved in the regulation of cancer cell migration could lead to a better understanding of this process. In breast cancer cells, Phosphoinositide 3-kinase (PI3K), small Rho GTPases and N-WASP have been reported to regulate changes to the actin cytoskeleton during cell migration (Martin et al., 2008). However the function of N-WASP in skin cancer has not been characterized. The first step in order to investigate the correlation of N-WASP expression with tumorigenicity, a western blot was performed with the cell lysates of tumorigenic and metastatic epithelial cell lines (Fig. 3.1). The cell lines used were HaCaT and its tumorigenic clones, A5-RT3 and II-4. In addition western blot was performed using the cell lysate of a SCC cell line, HSC-5 (Human Squamous Cell Carcinoma-5). In all the three tumorigenic cell lines the expression level was found to vary. The II-4 and A5-RT3 cell line displayed an increase in the expression level of N-WASP in comparison with the non-tumorigenic, HaCaT. However the HSC-5 cell line showed a decrease in the expression level of N-WASP (Fig. 3.1).

Figure 3.1: Altered the expression level of N-WASP in normal, malignant and metastatic cell lines. HaCaT, A5-RT3, II-4 and HSC-5 cells were lysed and the cell extract was used to determine the protein concentration using the Bradford's assay. Equal amount of the protein was loaded and resolved by polyacrylamide gel electrophoresis. Proteins were transferred onto the nitrocellulose membrane and probed for the respective antibodies, anti- N-WASP and anti-GAPDH.

3.3 Cell-ECM adhesion is reduced in the two skin cancer cell lines: A5-RT3 and HSC-5

In the previous section, HaCaT and the three skin cancer cell lines were analyzed for N-WASP expression, which showed that the expression level of N-WASP reduced in HSC-5 cell line in comparison to the N-WASP expression in HaCaT (Fig. 3.1). However a higher expression was observed in the A5-RT3 in comparison to HaCaT. N-WASP expression is critical for cell-ECM adhesion (Misra et al., 2007). Hence the cell adhesion assay was performed to characterize cell-ECM binding of the cell lines (Fig. 3.2). The percentage of cells that adhered was found to be the lowest in the HSC-5 ($31\% \pm 1\%$) cell line in comparison to the non-tumorigenic HaCaT ($79\% \pm 1\%$) cell line. The low binding efficiency observed in HSC-5 corresponded to the low N-WASP expression observed in HSC-5 ($P < 0.001$). The A5-RT3 cell line showed a higher percentage of cells that adhered as compared to HSC-5. However the percentage of cells that adhered observed in the A5-RT3 ($49\% \pm 1.7\%$) cell line was significantly lower in comparison to the HaCaT cell line ($P < 0.01$). The binding percentage observed in the II-4 ($75\% \pm 1\%$) was found to be similar as the HaCaT cell line ($79\% \pm 2\%$).

Figure 3.2: Cell-ECM binding is reduced in the three skin cancer cell lines II-4, A5-RT3 and HSC-5. The four cell lines (HaCaT, II-4, A5-RT3 and HSC-5) were grown under normal conditions of 37°C and 5% CO₂. The cells were trypsinized and incubated in Calcein AM dye. The cells were seeded onto wells coated with fibronectin and were allowed to bind for 30mins. The cells that adhered were measured using a fluorescence tecan fluorescence reader. The experiment was carried out in triplicates. The fraction of cells that were found to bind to fibronectin was expressed as a percentage. Statistical significance is indicated relative to control cells, HaCaT. Significance for data: ** $P < 0.01$ and *** $P < 0.001$ (Student's *t*-test).

3.4 Expression of N-WASP is reduced in the Squamous cell carcinoma (SCC) and Basal cell carcinoma (BCC) patient samples

II-4 and the A5-RT3 cell lines were generated by overexpressing the Ras oncogene in HaCaT cells and further propagated by injecting into mice which led to the tumorigenic conversion of the HaCaT cell line into the above aforementioned cancerous cell lines. These cell lines have been cultivated *in vitro* and hence might harbor unknown genetic changes. To investigate the expression level of N-WASP *in vivo* we made the use of patient skin cancer samples along with their paired perilesional samples to avoid any patient-to-patient variations in the expression level of N-WASP. RNA was extracted from paraffin embedded samples by using the Qiagen kit. The extraction involved the dissolution of the paraffin using xylene and protein was removed by proteinase K digestion. After digestion of the protein, samples were incubated at 80°C for 15 minutes to reverse the formalin crosslinking. Genomic DNA, including small DNA fragments found in paraffin embedded samples, was then effectively removed using DNase. Finally, concentrated RNA was purified using RNeasy Mini Elute spin columns. RNA was

converted to cDNA by the reverse transcription and the cDNA was used to perform qPCR. The endogenous internal control used was MRPL4 (mitochondrial ribosomal protein subunit L4). The fold difference was calculated using the relative quantification method. In order to confirm the results observed in the western blot in Fig. 3.1, N-WASP expression was initially checked in 3 patient SCC samples and 1 BCC sample. The N-WASP expression was reduced in both SCC and BCC samples (Fig. 3.3). Reduced expression level of N-WASP has been reported in both cancerous and metastatic breast cancer tissues (Martin et al., 2008). A recent study on colorectal cancer also showed reduced N-WASP expression in colon tumor samples (Martin et al., 2012). The lower expression level of N-WASP in the patient derived SCC cell line, HSC-5 cells in comparison to the other cell lines, reflects the low expression level of N-WASP in the patient SCC samples.

Figure 3.3: Expression level of N-WASP is reduced in the squamous cell carcinomas. Paraffin embedded patient skin cancer samples were processed for RNA extraction. cDNA obtained by reverse transcription of RNA was used to further perform qPCR. The endogenous control used was MRPL4 (mitochondrial ribosomal protein subunit L4). The relative quantitation method was used to tabulate the fold difference. The graph shows the variation in the expression level of N-WASP in SCC samples and a BCC sample (sample numbered as 4) with respect to their perilesional controls.

On finding a reduced expression level of N-WASP in the initial analysis of 4 skin cancer samples (Fig. 3.3), a further investigation of the expression level of N-WASP was done on a larger sample number

upon obtaining the bioethics approval (Fig. 3.4). The N-WASP expression level was observed to be consistently lower in all the patient samples as compared to their perilesional controls (Fig. 3.4).

Figure 3.4: Reduced expression level of N-WASP observed in SCC patient samples. The paraffin embedded skin cancer samples of 33 patients were obtained and RNA extraction was performed. The cDNA obtained by reverse transcription was used for qPCR analysis. The fold difference in the N-WASP expression in the SCC samples in comparison to the peri-lesional control was measured by relative quantification method. The endogenous control used was MRPL4.

3.5 Generation of N-WASP knockdown HaCaT cells

The qPCR data from paraffin embedded samples show that N-WASP expression is down regulated in the tumors in comparison to their paired perilesional controls. HaCaT cells were established from normal human skin and hence have been used extensively in research laboratory as a normal skin cell line. In order to understand the function of N-WASP in cell-ECM adhesion and migration in a non-tumorigenic epithelial cell line, HaCaT cells were used. A N-WASP specific shRNA (puromycin plasmid) was used to knockdown the N-WASP expression in HaCaT cells, the resultant cell line with reduced expression level of N-WASP will be referred to as HaCaT^{N-WASP KD} in this thesis. HaCaT cells were also transfected with a YFP expressing plasmid (puromycin plasmid), referred to as HaCaT^{CTR}. A western blot analysis

followed by quantification shows the decrease in the expression level of N-WASP in HaCaT^{CTR} in comparison to the HaCaT^{N-WASP KD} cells (Fig. 3.5 A and B).

Figure 3.5: Knockdown of N-WASP expression using N-WASP specific shRNA. HaCaT cells were microporated with a plasmid that expressed N-WASP specific shRNA. The cells were selected using puromycin and lysed. 30µg of the cell lysate was used to perform a western blot. The lysate was resolved by polyacrylamide gel electrophoresis and a nitrocellulose membrane transfer was done. The proteins on the membrane were probed with anti-N-WASP and anti-GAPDH primary antibodies. B. Densitometry quantification shows the level of N-WASP expression normalized against GAPDH in HaCaT^{CTR} in comparison to the HaCaT^{N-WASP KD}. Statistical significance is indicated relative to control cells expressing empty vector (HaCaT^{CTR}). Significance is indicated as stars: ** $P < 0.01$ (Student's *t*-test).

3.6 Reduced N-WASP expression results in reduced cell-ECM adhesion

The function of N-WASP in cell-ECM adhesion has been previously characterized using N-WASP^{-/-} and N-WASP^{+/+} mouse embryonic fibroblasts (MEFs) (Misra et al., 2007). To understand the function of N-WASP in cell-ECM adhesion in the epithelial cells, HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were used in a cell adhesion assay. The wells of a 96 well plate were coated with fibronectin, which served as the ECM and blocked with BSA. Cells were trypsinised and incubated in Calcein AM for duration of 30 minutes. The cells were seeded onto the wells and allowed to bind to the ECM for 30 minutes. The cell-ECM adhesion was quantified using the Tecan fluorescence reader. The adhesion assay revealed a decrease in the percentage of HaCaT^{N-WASP KD} cells that bound to the ECM as compared to the HaCaT^{CTR} cells (Fig. 3.6). This suggests that the reduction in the N-WASP expression led to a decrease in the cell-ECM

adhesion. The above result is consistent with the previous results; N-WASP^{+/+} mouse embryonic fibroblasts adhere better as compared to the N-WASP^{-/-} mouse embryonic fibroblasts (Misra et al., 2007).

Figure 3.6: Knockdown of N-WASP expression causes reduced cell adhesion. HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were grown under normal conditions of 37°C and 5% CO₂. The cells were trypsinized and incubated in Calcein AM dye. The cells were seeded onto wells coated with fibronectin and were allowed to bind for 30mins. The cells that adhered were measured using a fluorescence tecan fluorescence reader. The experiment was carried out in triplicates. The fraction of cells that were found to bind to fibronectin was expressed as a percentage. Statistical significance is indicated relative to control cells expressing empty vector (HaCaT^{CTR}). Significance is indicated as stars: ****P*<0.001 (Student's *t*-test).

3.7 knockdown of N-WASP expression in HaCaT cells results in reduced cell spreading

Cell spreading is tightly linked with cell adhesion as well-defined cell spreading depends on integrity and assembly of actin cytoskeleton in terms of focal adhesion assembly and cell contractility. To further confirm the adhesion characteristics of the cells observed earlier, the HaCaT^{CTR} cells and the HaCaT^{N-WASP KD} cells were analyzed for their ability to spread on fibronectin coated coverslips. The cells were trypsinised, washed with PBS and seeded onto wells coated with fibronectin and blocked with BSA. The cells were monitored at 10, 30 and 60 minutes intervals for their ability to spread on the ECM. The ability of the cells to spread was found to correlate with N-WASP expression. The HaCaT^{N-WASP KD} cells had a rounded morphology even after 30 minutes as compared to the HaCaT^{CTR} cells, which had a more flattened morphology (Fig. 3.7A).

A.

B.

Figure 3.7: N-WASP is required for efficient cell spreading in HaCaT cells. A. HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were trypsinised and seeded onto wells of a 96 well plate treated with fibronectin. The cells were observed for their morphology and spreading at time intervals of 10, 30 and 60 minutes after seeding them onto the fibronectin coated wells. Bar represents 20µm. B. The surface area of the cells was measured at each of these time points using metaview software. Statistical significance is indicated relative to control cells expressing empty vector (HaCaT^{CTR}). Significance is indicated as stars: * $P < 0.05$ (Student's t -test).

At 60 minutes time point the cells showed a completely flattened morphology in the HaCaT^{CTR} cells in comparison to the HaCaT^{N-WASP KD} cells. In conclusion, the cells with reduced N-WASP expression

displayed reduced spreading on fibronectin, which is consistent with the reduced cell adhesion observed earlier (Fig. 3.6 and 3.7).

3.8 Knockdown of N-WASP expression in HaCaT cells leads to increased cell migration

Cell adhesion performs a significant role in cell migration. Changes in cell-ECM adhesion characteristics affect cell migration (Hynes, 2002). In order to study changes in the cell migration resulting from knockdown of N-WASP expression in HaCaT cells, a scratch assay was performed. The HaCaT^{N-WASP KD} and the HaCaT^{CTR} cells were grown to 100% confluence in a 35mm petri-dish. A line was drawn in the middle of the culture using a pipette tip. The cells were placed back in the incubator and monitored for wound closure at different time points of 0 hr and 15 hours. As seen from the image (Fig. 3.8) the HaCaT^{N-WASP KD} cells closed the gap more efficiently in 15 hours as compared to the HaCaT^{CTR} cells (Fig. 3.9).

Figure 3.8: Reduced expression level of N-WASP leads to increased cell migration. HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were seeded onto wells of a 6 well plate and grown to a 100% confluence. A line was drawn in the middle of the culture using a pipette tip. The scratch assay images were taken at time points of 0 and 15 hours. Bar represents 20µm.

Figure 3.9: Comparison of the scratch widths indicates increase in cell migration in knockdown cells. The scratch width of the healing scratch wound at the time points was measured using metaview software. Statistical significance is indicated relative to control cells expressing empty vector (HaCaT^{CTR}). Significance is indicated as stars: ** $P < 0.01$ (Student's t -test).

3.9 knockdown of N-WASP expression in HaCaT cells leads to alteration in expression and localization of vinculin and paxillin.

Epithelial cells arranged compactly by both cell-cell and cell-ECM adhesions. While the cell-cell adhesion is facilitated by E-cadherin, cell-ECM contacts are maintained through integrin-ECM binding at the sites of nascent adhesions formed, which are made up of complexes of several proteins including vinculin and paxillin (Li and Sakaguchi, 2002). Data from published literature suggests that cells with increased motility had reduced vinculin patches (Misra et al., 2007; Ridley et al., 2003) thus HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were analyzed for localization of vinculin patches (Fig. 3.10). The HaCaT^{N-WASP KD} cells showed a decrease in the number of vinculin patches (Fig. 3.10B). It is possible that the reduced vinculin patches observed in the HaCaT^{N-WASP KD} cells are due to a decrease in the vinculin expression thus the vinculin expression was analyzed which showed that the expression of vinculin was reduced in HaCaT^{N-WASP KD} in comparison to in HaCaT^{CTR} cells (3.11A). The number of paxillin patches (Fig. 3.12) and the expression of paxillin (Fig. 3.13) was found to be the opposite to that of vinculin with

an increase in its number per cell in the HaCaT^{N-WASP KD} cells as compared to the HaCaT^{CTR} cells (Fig. 3.13 and 3.14A) and the expression of paxillin was increased in HaCaT^{N-WASP KD} cells.

A.

B.

Figure 3.10: Knockdown of N-WASP expression in HaCaT cells resulted in reduced localization of vinculin patches. HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were seeded onto coverslips and were fixed using formaldehyde. Fixed cultures were permeabilized and incubated with anti-vinculin as the primary antibody followed by anti-mouse (alexa fluor, 594nm) as the secondary antibody. The HaCaT^{CTR} and HaCaT^{N-WASP KD} cells appear yellow due to the YFP fluorescence as the expressed plasmid is tagged with YFP. The cells were observed under 40X oil lens. Bar represents 20μm. B. Graph represents the comparison of the number of vinculin patches. Statistical significance is indicated relative to control cells expressing empty vector (HaCaT^{CTR}), indicated as stars: ** $P < 0.01$ (Student's *t*-test).

A.

B.

Figure 3.11: Knockdown of N-WASP expression in HaCaT cells resulted in reduced expression of vinculin and reduced number of vinculin patches per cell. A. Knockdown leads to a reduction in the expression of vinculin in the HaCaT^{N-WASP KD} cells. HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were lysed and 30μg of protein was resolved on the POLYACRYLAMIDE GEL ELECTROPHORESIS, transferred onto a membrane and probed with anti-vinculin and anti-GAPDH (loading control) primary antibodies. Densitometry quantification shows the level of Vinculin expression normalized against GAPDH in HaCaT^{CTR} in comparison to the HaCaT^{N-WASP KD}. B. The number of patches per cell in both cell lines were quantified in a total of 30 cells per experiment. Statistical significance is indicated relative to control cells expressing empty vector (HaCaT^{CTR}), indicated as stars: * $P < 0.05$, ** $P < 0.01$ (Student's *t*-test).

A.

B.

Figure 3.12: Knockdown of N-WASP expression in HaCaT cells shows an increase in the number of paxillin patches. HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were seeded onto coverslips in wells of a 6 well plate. The cells were fixed using formaldehyde. Fixed cultures were permeabilized and incubated with anti-paxillin as the primary antibody followed by anti-mouse (alexa fluor, 594nm) as the secondary antibody. The cells were observed under 40X oil lens for the paxillin patches. The cells appear yellow due to the YFP fluorescence as the expressed plasmid is tagged with YFP. Bar represents 20μm.

A.

B.

Figure 3.13: Knockdown of N-WASP expression in HaCaT shows an increase in the expression of paxillin and the number of paxillin patches as compared with the HaCaT^{CTR} cells A. Knockdown leads to a reduction in the expression of paxillin in the HaCaT^{N-WASP KD} cells. HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were lysed and 30μg of the cell lysate was used to carry out western blot. The lysate was resolved by polyacrylamide gel electrophoresis and a nitrocellulose membrane. The proteins on the membrane were probed with anti-paxillin and anti-GAPDH antibodies. Densitometry quantification shows the level of paxillin expression normalized against GAPDH in HaCaT^{CTR} in comparison to the HaCaT^{N-WASP KD}. B. The number of patches per cell in both HaCaT^{CTR} and HaCaT^{N-WASP KD} were quantified in a total of 30 cells per experiment, which was performed thrice. Statistical significance is indicated relative to control cells expressing empty vector (HaCaT^{CTR}). Significance is indicated as stars: * $P < 0.05$ (Student's t -test).

3.10 knockdown of N-WASP expression causes a reduction in cell-cell contact

Epithelial cells are maintained in place by cell-cell and cell-ECM interaction. In the previous section we checked for the function of N-WASP in cell-ECM adhesion by checking the distribution and expression of vinculin or paxillin. In epithelial cells, cell-cell interactions are mediated by E-cadherin, which influences a multitude of cell behavior including cell-cell adhesion, differentiation and tissue development. The function of N-WASP in E-cadherin localization in HaCaT^{N-WASP KD} cells was checked and a decrease in the localization of E-cadherin was observed at the junctions (Fig. 3.14A). A western blot was carried out to check for E-cadherin expression and a decrease in expression of E-cadherin was found in HaCaT^{N-WASP KD} cells, which explained the decrease in the localization (3.14A and B). E-cadherin expression is often used as a marker for the conversion of epithelial cells to mesenchymal cells prior to metastasis (Onder et al., 2008). Hence a decrease in the expression of E-cadherin (Fig. 3.14A) resulting from decreased N-WASP expression indicates that N-WASP might play a role in EMT (epithelial mesenchymal transition).

A.

B.

Figure 3.14: N-WASP is required for E-cadherin localization and expression. A. HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were grown to a 70% confluence on wells with coverslips in 6 well plates. Cells were fixed using formaldehyde. Fixed cultures were permeabilized and incubated with anti-E-cadherin as the primary antibody followed by anti-mouse (alexa fluor, 594nm) as the secondary antibody. Bar represents 20μm. B. N-WASP knockdown leads to a reduction in the expression of E-cadherin in the HaCaT^{N-WASP KD} cells. HaCaT^{CTR} and HaCaT^{N-WASP KD} cells were grown under normal growth conditions of 37°C and 5% CO₂. 1) Cell lysis was carried out and 30μg of protein was resolved on the POLYACRYLAMIDE GEL ELECTROPHORESIS, transferred onto a membrane and probed with anti-E-cadherin and anti-GAPDH (loading control) primary antibodies. 2) Densitometry quantification shows the level of E-cadherin expression normalized against GAPDH in HaCaT^{CTR} in comparison to the HaCaT^{N-WASP KD}. Statistical significance is indicated relative to control cells expressing empty vector (HaCaT^{CTR}). Significance is indicated as stars: * $P < 0.05$ (Student's *t*-test).

3.11 Generation of a stable subline of HSC-5 expressing N-WASP

HSC-5 is a human squamous carcinoma cell line, which was established from a patient sample (Hozumi et al., 1990). The immune-blot analysis (Fig. 3.1) suggests that the expression level of N-WASP is down regulated in the human squamous cell carcinoma (HSC-5). In order to understand the contribution of N-WASP to the adhesion and migration characteristics of the HSC-5 cell line, two stable sublines were generated by transfection: HSC-5 cells with plasmids over-expressing N-WASP, HSC-5^{N-WASP} and HSC-5 cells expressing the empty vector, HSC-5^{CTR}. Selection was carried out using puromycin over 3

passages. Cell lysis was carried out for western blot analysis to determine the expression level of N-WASP. An increase in the N-WASP expression was observed for the HSC-5^{N-WASP} cells in comparison to the HSC-5^{CTR} cells (Fig. 3.16).

Figure 3.15: HSC-5^{N-WASP} shows heightened expression level of N-WASP. HSC-5^{CTR} and HSC-5^{N-WASP} cell lysates were analyzed by an immunoblot as described in Fig. 3.5. The GAPDH expression level shows equal loading of the proteins hence indicating that the change in the expression level of N-WASP is not due to variations in the loading of the cell lysates. B. Densitometry quantification shows the level of N-WASP expression normalized against GAPDH in HSC-5^{CTR} in comparison to the HSC-5^{N-WASP}. Statistical significance is indicated relative to control cells expressing empty vector (HaCaT^{CTR}). Significance is indicated as stars: *** $P < 0.001$ (Student's *t*-test).

3.12 Over-expression of N-WASP in HSC-5 cells leads to increased cell- ECM Adhesion

The function of N-WASP in cell-ECM adhesion has been studied in the N-WASP^{-/-} and N-WASP^{+/+} mouse embryonic fibroblasts where the loss of N-WASP led to a decrease in the cell-ECM adhesion (Misra et al., 2007). Knocking down N-WASP expression in HaCaT cells using shRNA also caused a reduction in the cell-fibronectin adhesion. HSC-5 cells show reduced N-WASP expression and reduced cell adhesion suggesting that the reduced N-WASP expression could be the cause of reduced cell-ECM adhesion. The HSC-5 subline that was generated was used to study cell-ECM adhesion in an adhesion assay. The wells of a 96 well plate were coated with fibronectin and blocked with BSA to block non-specific binding. The cells were trypsinised, and incubated in Calcein AM before seeding them onto the fibronectin coated wells. The binding assay was carried for 30 minutes before quantifying with a tecan

fluorescence tecan fluorescence reader. The adhesion assay revealed an increase in the percentage of cells that bound to the fibronectin in the HSC-5^{N-WASP} as compared to the HSC-5^{CTR} cells which, suggests that the increase in the N-WASP expression resulted in an increase in cell-ECM adhesion of the cells (Fig. 3.17).

Figure 3.16: N-WASP overexpression in HSC-5 cells leads to enhanced cell-ECM binding. HSC-5^{CTR} and HSC-5^{N-WASP} cells were grown under normal conditions of 37°C and 5% CO₂. Adhesion assay was carried out as described in Fig. 3.6. The fraction of cells that were found to bind to fibronectin was expressed as a percentage. Statistical significance is indicated relative to control cells expressing empty vector (HSC-5^{CTR}). Significance is indicated as stars: *** $P < 0.001$ (Student's *t*-test).

3.13 Increased expression of N-WASP enhances cell spreading

The cells interact with the underlying ECM through specific cell receptors, integrins. The integrins bind to specific motifs in the ECM proteins thereby mediating the ability of the cells to bind to the specific matrix. Once cells attach onto a surface they first flatten and eventually deform extensively to adopt the specific morphology (Cuvelier et al., 2007; Lauffenburger and Horwitz, 1996). The alterations in the cell-ECM adhesion characteristic of the cells on overexpressing N-WASP were observed previously (Fig. 3.17). In order to further characterize the extent of cell spreading and the changes caused due to the overexpression of N-WASP were analyzed by a spreading assay. The cells were trypsinised and seeded onto wells that were coated with the ECM, fibronectin. The cells were allowed to spread and monitored at 10 minutes, 30 minutes and 60 minutes intervals. The HSC-5^{N-WASP} cells displayed increased spreading

as compared with HSC-5^{CTR} cells (Fig. 3.18 A and B), suggesting that increased N-WASP expression led to increased ability of the cells to spread.

3.14 Over-expression of N-WASP in HSC-5 retards cell motility

Cell migration is a process with various steps and it has an important role in the progression of various diseases. Data from previous section suggests that loss of N-WASP expression in the HaCaT cells resulted in an increase in the cellular motility (Section 3.16). To study the function of N-WASP in the motility of the cells in HSC-5 cells that express reduced N-WASP expression, the HSC-5^{N-WASP} and the HSC-5^{CTR} cell lines were grown to confluence and a line was drawn in the middle of the culture. The cells were observed for the time taken to close the wound and pictures were taken under 40X objective in the microscope at the respective time points. The HSC-5^{N-WASP} cells were found be slower in closing the wound in comparison to the HSC-5^{CTR} cells (Fig. 3.19). This difference in the time taken to close the gap indicates the decrease in the motility of the HSC-5^{N-WASP} cells as a result of N-WASP overexpression.

A.

B.

Figure 3.17: N-WASP overexpression in HSC-5 leads to enhanced cell spreading. A. HSC-5^{CTR} and HSC-5^{N-WASP} cells were trypsinised and seeded onto wells of a 96 well plate treated with fibronectin. Spreading assay was carried out as described in 3.8. Bar represents 20 μm. B. The surface area of the cells was measured at each of these time points using metaview software. Statistical significance is indicated relative to control cells expressing empty vector (HSC-5^{CTR}). Significance is indicated as stars: **P* < 0.05 (Student's *t*-test).

A.

B.

Figure 3.18: Increased expression of N-WASP in HSC-5 cells results in reduced cell migration. A. Confluent monolayers of both cell lines HSC-5^{CTR} and HSC-5^{N-WASP} were used and a line was drawn in the middle of the culture. The scratch was monitored for the time taken by the cells to close the wound and images were taken at time points of 0 and 15 hours using 40X objective. Bar represents 20 μm. B. Scratch widths were measured in both HSC5^{CTR} and HSC5^{N-WASP} at time points of 0 and 15 hours. The experiment was performed thrice. Statistical significance is indicated relative to control cells expressing empty vector (HSC-5^{CTR}). Significance is indicated as stars: ** $P < 0.01$ (Student's t -test).

3.15 An increase in the expression of N-WASP alters the expression and localization of vinculin and paxillin

Vinculin and paxillin are focal adhesion proteins that play a critical role in the focal adhesions assembly and turnover. Vinculin knockout mice display a defect in the formation of focal adhesions, cell adhesion and cell spreading. Overexpression of vinculin was shown to promote cell adhesion (Goldmann and Ingber, 2002; Xu et al., 1998). In order to understand the mechanism by which the cell adhesion and migration characteristics of the cells are regulated, cells were immunostained to visualize the localization of vinculin and paxillin. The expression of the proteins was analyzed by western blot. The HSC-5^{N-WASP} displayed an increase in the number of vinculin patches per cell and a decrease in the number of paxillin patches per cell as shown in the immunostained figures (Fig. 3.20 and 3.21). The patches were counted in a total of 90 cells. The western blot showed an increase in the expression of vinculin (Fig. 3.20B) and a decrease in the expression of the paxillin (Fig. 3.21B). The increase in the expression of vinculin has been implicated in increased adhesion and according to published results vinculin when overexpressed might restore cell adhesion and cell spreading defects by facilitating recruitment of cytoskeletal proteins to the focal adhesions at the site of integrin binding (Ezzell et al., 1997). The previous sections show an increase in cell-ECM adhesion and spreading in HSC-5 cells overexpressing N-WASP (Sections 3.12 and 3.13). Overexpression of N-WASP in this section has shown to cause an increase in the expression of vinculin and an increase in the localization of vinculin patches. The increase in the expression of vinculin hence explains the increase in the observed cell adhesion and spreading.

A.

B.

C.

D.

Figure 3.19: N-WASP overexpression leads to an increase in the expression and localization of vinculin. A. HSC-5^{N-WASP} and HSC-5^{CTR} cell lines were grown to a 70% confluence, on wells with coverslips in 6 well plates. Immunostaining was carried out as described in Fig. 3.11. Bar represents 20μm B. The number of patches per cell was measured in 30 cells and the mean value is shown. The experiment was performed thrice. Statistical significance is indicated relative to control cells expressing empty vector (HSC-5^{CTR}). Significance is indicated as stars: ** $P < 0.01$ and *** $P < 0.001$ (Student's *t*-test). C. Equal amount of protein of HSC-5^{N-WASP} and HSC-5^{CTR} cell lines were loaded and resolved by polyacrylamide gel electrophoresis, transferred onto a membrane and probed with anti-vinculin and anti-GAPDH primary antibodies. D. Densitometry quantification shows the level of vinculin expression normalized against GAPDH in HSC-5^{CTR} in comparison to the HSC-5^{N-WASP}.

A.

B.

C.

D.

Figure 3.20: Decrease in the expression and localization of paxillin observed as a result of an overexpression of N-WASP. A. HSC-5^{N-WASP} and HSC-5^{CTR} cells were trypsinized and seeded onto wells of a 6 well plate with coverslips. Immunostaining was carried out as described in Fig. 3.13. . Bar represents 20μm B. The number of patches per cell was measured in 30 cells and the mean value is shown. The experiment was performed thrice. Statistical significance is indicated relative to control cells (HSC-5^{CTR}) expressing empty vector. Significance is indicated as stars: * $P < 0.05$ and ** $P < 0.01$ (Student's t -test). C. Equal amount of protein of HSC-5^{N-WASP} and HSC-5^{CTR} cell lines were loaded and resolved by polyacrylamide gel electrophoresis, transferred onto a membrane and probed with anti-paxillin and anti-GAPDH primary antibodies. D. Densitometry quantification shows the level of paxillin expression normalized against GAPDH in HSC-5^{CTR} in comparison to the HSC-5^{N-WASP}.

3.16 Over-expression of N-WASP in HSC-5 causes increased E-cadherin at cell-cell junction

One of the critical intercellular adhesion proteins, E-cadherin, maintains the morphological structure of normal epithelial cells. E-cadherin is a transmembrane glycoprotein and an epithelial-specific member of

the cadherin family of intercellular adhesion molecules, which mediates a cell-cell interaction through calcium- dependent homophilic interaction of its extracellular domain. Cytoplasmic domain of E-cadherin interacts with the α and β catenins and p120 to form complexes, which interacts with the actin cytoskeleton to maintain epithelial integrity (Kim et al., 2011).

The disruption of the above interactions of the complex leads to a disruption in the signaling mechanism that regulates cellular growth and differentiation. Data from previous section (Fig. 3.14) suggests that reduced expression of N-WASP in HaCaT causes disruption of this complex and hence leading to reduced cellular contact. However the effect of overexpression of N-WASP in cells expressing lower amounts of N-WASP has not been shown. In order to study the change in the cell-cell contact due to overexpression of N-WASP, HSC-5^{N-WASP} and HSC-5^{CTR} cells were probed for E-cadherin. The immunostaining showed an increase in the localization of the protein at the junctions. The western blot displayed an increase in the expression of E-cadherin (Fig. 3.21 B). Densitometry of the western blot was done which shows an increase in the expression level of E-cadherin in HSC-5^{N-WASP} cells in comparison to the HSC-5^{CTR} cells (Fig. 3.21C).

A.

B.

C.

Figure 3.21: N-WASP promotes the formation of prominent E-cadherin belts. A. HSC-5^{N-WASP} and the HSC-5^{CTR} cell lines were grown to a 70% confluence, on coverslips in 6 well plates. Immunostaining was carried out as described in Fig. 3.10. The stained cells were observed under 40X oil lens for the E-cadherin localization at the cell-cell junctions. Bar represents 20μm B. Cell lysates of HSC-5^{N-WASP} and HSC-5^{CTR} cell lines were loaded in equal amount and resolved by polyacrylamide gel electrophoresis, transferred onto a membrane and probed with anti-E-cadherin and anti-GAPDH primary antibodies. C. Densitometry quantification shows the level of E-cadherin expression normalized against GAPDH in HSC-5^{CTR} in comparison to the HSC-5^{N-WASP}. Statistical significance is indicated relative to control cells expressing empty vector (A5-RT3^{CTR}). Significance is indicated as stars: ***P*<0.01 (Student's *t*-test).

3.17 Generation of N-WASP knockdown A5-RT3 cells

HaCaT-Ras cells were established by transfecting the human HaCaT keratinocytes with the mutated val12-harvey-ras oncogene. The A-5 RT3 cell line was established by re-cultivation *in vitro* of subcutaneous tumors derived from HaCaT-ras cells and re-transplanted three times *in vivo* (Mueller et al., 2001; Maillard et al., 2005). A5-RT3 cells have been reported to be highly metastatic and the immunoblot suggests a higher N-WASP expression as compared to the other non-tumorigenic cell line (HaCaT), benign non-metastatic cell line (HSC-5) and cancerous cell line (II-4). The metastatic cancerous cell line, A5-RT3 displayed a higher N-WASP expression as in comparison to the non-metastatic, cancerous HSC-5. In order to understand the role played by N-WASP in a metastatic cancerous cell line, sublines of A5-RT3 cells were generated with the N-WASP expression knocked down. The knockdown was confirmed by western blot (Fig. 3.23A). A densitometric analysis indicates the decrease in the level of N-WASP expression in A5-RT3^{CTR} in comparison to the A5-RT3^{N-WASP KD} (Fig. 3.22).

Figure 3.22: Knockdown of N-WASP expression in the A5-RT3 cells. A. A5-RT3^{N-WASP KD} and the A5-RT3 cells were grown under normal conditions of 37°C and 5% CO₂. Cell lysis was carried out and the cell extract was used to determine the protein concentration by Bradford's assay. Western blot was carried out as described in Fig. 3.5. B. Densitometry quantification shows the level of N-WASP expression normalized against GAPDH in A5-RT3^{CTR} in comparison to the A5-RT3^{N-WASP KD}. Statistical significance is indicated relative to control cells expressing empty vector (A5-RT3^{CTR}). Significance is indicated as stars: **P*<0.05 (Student's *t*-test).

3.18 Knockdown of N-WASP in A5-RT3 causes reduced cell-ECM adhesion

The integrin heterodimers connect the ECM to the intracellular cytoskeleton. Knockdown of N-WASP in HaCaT cells caused reduced cell-ECM adhesion and overexpressing N-WASP in HSC-5 caused increased cell-ECM adhesion hence the alterations in the cell-ECM adhesion, cell spreading and migration characteristics on knocking down the N-WASP expression in metastatic and cancerous A5-RT3 cell line were further analyzed. An adhesion assay was performed to investigate the changes in the cell adhesion characteristics of the A5-RT3^{N-WASP KD} and A5-RT3^{CTR} cells. The cells were grown to confluence and trypsinised. The cells were incubated in Calcein AM and were seeded onto wells coated with fibronectin. The cells were allowed to bind over half hour before measuring the cell number using a fluorescence tecan fluorescence reader. The percentage of cells that bound to the fibronectin were found to be reduced in the A5-RT3^{N-WASP KD} cells as compared to the A5-RT3^{CTR} cells (Fig. 3.23). This difference in the ability of the cells to bind in cells with reduced N-WASP expression indicates the change in the adhesion property of the cells.

Figure 3.23: The binding efficiency reduced due to N-WASP knockdown in the A5-RT3 cells. A5-RT3^{N-WASP KD} and the A5-RT3^{CTR} cells were trypsinized and seeded onto wells of a 96 well plate treated with fibronectin. Binding assay was carried out as described in Fig. 3.6. The fraction of cells that adhered were tabulated and expressed as a percentage. Statistical significance is indicated relative to control cells expressing empty vector (A5-RT3^{CTR}). Significance is indicated as stars: *** $P < 0.001$ (Student's *t*-test).

3.19 Knockdown of N-WASP in A5-RT3 cells leads to poor cell spreading

Cell spreading is a fundamental event wherein once the cell adheres to the surface, the contact area between the cell and the substrate increases when the actin polymerization increases (Chamaraux et al., 2005). In order to confirm the reduction seen in the cell-ECM adhesion of the cells as a result of a knock down in N-WASP expression, a spreading assay was done to observe the efficiency with which the cells spread. The cells were trypsinized and seeded onto wells coated with fibronectin and blocked with BSA before seeding the cells onto the wells. The inability of the cells to spread well is supported by the reduced binding efficiency of the cells as seen previously. The cells expressing a lower level of N-WASP were found to spread less efficiently as compared to the control cells transfected with an empty vector (Fig. 3.24).

A.

B.

Figure 3.24: N-WASP is required for cell spreading in A5-RT3 cells. A. A5-RT3^{N-WASP KD} and A5-RT3^{CTR} cells were trypsinized and seeded onto wells of a 96 well plate treated with fibronectin. Spreading assay was carried out as described before in Fig. 3.7. . Bar represents 20 μm B. The surface area covered by the A5-RT3^{N-WASP KD} and the A5-RT3^{CTR} cells at different time points (10, 30 and 60 minutes) were measured using the metaview software. Statistical significance is indicated relative to control cells expressing empty vector (A5-RT3^{CTR}). Significance is indicated as stars: *P < 0.05 (Student's *t*-test).

3.20 Knocking down N-WASP expression in A5-RT3 cells leads to increased cell motility

Cell spreading and cell migration are interlinked processes that involve tight regulation of actin cytoskeleton and require extension of plasma membrane. Thus any alteration in the machinery involved in membrane protrusion would affect both cell spreading and migration. In order to study the function of N-WASP in affecting the cell migration characteristics in skin cancer, A5-RT3^{N-WASP KD} and A5-RT3^{CTR} cell lines were used in a cell migration assay wherein the cells were grown to a confluence and a line was drawn in the middle of the culture. The cells were then monitored for the time taken by the cells to close the wound and images were taken using 40X objective. The A5-RT3^{N-WASP KD} cells were found to close the gap faster than the A5-RT3^{CTR} cells (Fig. 3.25).

3.21 Knockdown of N-WASP leads to altered distribution and expression of the focal adhesion proteins, vinculin and paxillin

The focal adhesion complex consists of several proteins including vinculin and paxillin. The loss of vinculin has been shown to affect cell functions such as the formation of focal complexes and hence affects cell adhesion and spreading (Goldmann and Ingber, 2001). The paxillin knockout mouse displays abnormal focal adhesions and reduced cell migration (Hagel M et al., 2002). To investigate the underlying reason for the observed altered behavior in the cell adhesion and migration properties of the cell caused due to a reduction in the N-WASP expression, the expression of vinculin and paxillin was seen by a western blot (Fig. 3.26B and 3.27B). The expression level of vinculin had decreased while there was an increase in the paxillin expression in the A5-RT3^{N-WASP KD} cells in comparison with the A5-RT3^{CTR} cell line. This decrease in the vinculin may have caused the decrease in the cell adhesion and spreading as observed earlier (Sections 3.6, 3.7 and 3.10). Also, the localization of the vinculin patches was lower in accordance with the decrease in its expression (Fig. 3.26). The expression of paxillin was found to have increased and likewise there was an increase in the localization of the number of patches per cell as compared to the A5-RT3^{CTR} cells (Fig. 3.27 A and B). The increase in paxillin localization and

expression suggests the increase in the FA turnover and hence an increase in cell migration as seen earlier (Section 3.8 and 3.9).

A.

B.

Figure 3.25: Knockdown of N-WASP expression leads to an increase in migration. Confluent monolayers of both cell lines A5-RT3^{CTR} and A5-RT3^{N-WASP KD} were used to perform scratch assay as described in Fig. 3.8. Bar represents 20μm B. The scratch widths were measured using the metaview software in both A5-RT3^{N-WASP KD} and A5-RT3^{CTR} cells at time points of 0 and 15 hours. Statistical significance is indicated relative to control cells expressing empty vector (A5-RT3^{CTR}). Significance is indicated as stars: ** $P < 0.01$ (Student's t -test).

A.

B.

C.

D.

Figure 3.26: Knockdown of N-WASP expression results in increased expression and localization of vinculin. A. A5-RT3^{CTR} and A5-RT3^{N-WASP KD} cells were trypsinized and seeded onto wells of a 6 well plate. The cells appear yellow due to the YFP fluorescence as the expressed plasmid is tagged with YFP. Immunostaining was carried out as described previously in Fig. 3.10. Bar represents 20μm. B. A5-RT3^{CTR} and A5-RT3^{N-WASP KD} cells were lysed and the protein concentration was measured by bradford's assay. Western blot was carried out as described before in Fig. 3.5 using anti-vinculin and anti-GAPDH. Densitometry quantification shows the level of vinculin expression normalized against GAPDH in A5-RT3^{CTR} in comparison to the A5-RT3^{N-WASP KD}. C. The number of vinculin patches per cell was counted for 30 cells and The experiment was performed thrice. Statistical significance is indicated relative to control cells expressing empty vector (A5-RT3^{CTR}). Significance is indicated as stars: * $P < 0.05$, ** $P < 0.01$ (Student's t -test). D.

A.

B.

B.

D.

Figure 3.27: Knockdown of N-WASP expression results in reduced expression and localization of paxillin. **A.** A5-RT3^{N-WASP KD} and A5-RT3^{CTR} cells were seeded onto coverslips in a 6 well plate. The cells appear yellow due to the YFP fluorescence as the expressed plasmid is tagged with YFP. Immunostaining was carried out as described before in Fig. 3.10. Bar represents 20μm. **B.** A5-RT3^{CTR} and A5-RT3^{N-WASP KD} cells were lysed western blot was carried out as described before in 3.5 using anti-paxillin and anti-GAPDH. Densitometry quantification shows the level of N-WASP expression normalized against GAPDH in A5-RT3^{CTR} in comparison to the A5-RT3^{N-WASP KD}. **C.** The number of paxillin patches per cell was counted for 30 cells and The experiment was performed thrice. Statistical significance is indicated relative to control cells expressing empty vector (A5-RT3^{CTR}). Significance is indicated as stars: **P*<0.05 (Student's *t*-test). Densitometry quantification shows the level of paxillin expression normalized against GAPDH in A5-RT3^{CTR} in comparison to the A5-RT3^{N-WASP KD}. Statistical significance is indicated relative to control cells expressing empty vector (A5-RT3^{CTR}).

3.22 Knockdown of N-WASP in A5-RT3 cells alters the expression and localization of the intercellular adhesion protein, E-cadherin

The expression of the junction protein, epithelial cadherin has shown to be altered in metastatic cancers. In order to understand the function of N-WASP in acting as a tumor suppressor, A5-RT3^{N-WASP KD} and A5-RT3^{CTR} cells were lysed and the expression level of the protein E-cadherin was analyzed by western blot (Fig. 3.29A). A densitometry graph shows the relative band intensity which shows the decrease in the level of expression of E-cadherin in A5-RT3^{CTR} in comparison to the A5-RT3^{N-WASP KD} (Fig. 3.29B). The expression level of E-cadherin showed a decrease as observed in the western blot (Fig. 3.29) and the immunostaining revealed a decrease in the localization of the protein at the junctions. Hence the above results suggest that the expression of the protein, E-cadherin is altered due to the N-WASP knockdown.

A.

B.

Figure 3.28: Decreased localization and expression of E-cadherin in N-WASP knockdown cells. A. A5-RT3^{CTR} and A5-RT3^{N-WASP KD} cells were trypsinized and seeded onto wells of a 6 well plate. The cells appear yellow due to the YFP fluorescence as the expressed plasmid is tagged with YFP. Immunostaining was carried out as described before in Fig. 3.10. Bar represents 20 μm. B. A5-RT3^{CTR} and A5-RT3^{N-WASP KD} cells were lysed, the cell extracts were loaded in equal amounts and resolved by polyacrylamide gel electrophoresis. 1) The proteins were probed for using anti-E-cadherin and anti-GAPDH. 2) Densitometry quantification shows the level of E-cadherin expression normalized against GAPDH in A5-RT3^{CTR} in comparison to the A5-RT3^{N-WASP KD}.

3.23 Generation of stable sublines of N-WASP^{-/-} mouse embryonic fibroblasts expressing N-WASP mutants

In previous studies it has been shown that N-WASP has an important function in performing cell adhesion and migration in mouse embryonic fibroblasts (Misra et al., 2007). N-WASP has several functional domains the role of individual domains in cell-ECM adhesion and migration has not been characterized. N-WASP is characterized by the presence of the following domain regions: WH1, Basic region, GBD region, proline rich region and the VCA region. These domains each of them interact with a plethora of proteins, which are involved in delivering to a multitude of functions. The WH1 domain is important in binding to the mammalian verprolins among other proteins that enable it to contribute to the

actin dynamics (Volkman et al. 2002; Kato et al. 2002; Ho et al. 2001). The basic region and the GBD interact with PIP₂ and Cdc42 respectively, that convert the closed conformation of N-WASP to an open one. This region hosts the phosphorylation sites; upon phosphorylation other activators are attracted to the site that promote its activation to lead to an open conformation (Miki and Takenawa 1998; Rohatgi et al. 1999). The proline rich region binds SH3 domain containing proteins, which relieve the autoinhibited state of N-WASP (Rohatgi et al. 2001). The SH3 domain containing proteins mediate changes in the cytoskeleton dynamics through N-WASP to form structures such as podosome and invadopodia formation through the N-WASP-cortactin interaction (Kowaliski et al., 2004; DesMarais et al., 2009). The above regions with their established functional roles serve the basis for selecting mutations and deletions in these regions to study their role in cell-ECM adhesion and migration.

The WH1 domain of N-WASP binds the mammalian verprolins and the complex in turn has been known to prevent Cdc42 mediated activation of N-WASP (Martinez-Quiles et al. 2001; Ho et al. 2004). Activation of N-WASP leads to an open conformation of N-WASP due to the unmasking of the VCA domain. The VCA domain is primarily involved in the activation of the actin polymerization machinery through the binding of actin monomers and the Arp2/3 complex. Actin nucleation is proposed to play an important role in cell-ECM adhesion and migration. Hence the above two domains were considered as important candidates in the study of the function of the N-WASP domains in cell-ECM adhesion and migration.

Conversion of the closed state of N-WASP to the open state requires the binding of interactors at the CRIB motif and the basic region. N-WASP is phosphorylated by the Src family kinases, FAK and Btk. Tyrosine 256 is present in the GBD of N-WASP is unable to bind protein kinases in the folded conformation. Upon binding of activators such as Grb2 or activated Cdc42, the site is made available to bind tyrosine kinases that phosphorylate the Y256. Upon phosphorylation of Y256 the site forms a

docking site for the SH2 containing proteins, which stabilizes the binding of Arp2/3 and maintains actin polymerization (Torres and Rosen, 2003; Wu et al., 2003; Caron, 2003). The mutation of tyrosine 256 to glutamic acid (Y256E) mutant is capable of inducing microspikes in cos-7 cells and neurites in primary neurons. Y256E is also essentially found to be located in the cytosol (Suetsugu et al., 2002). Mutation of tyrosine 256 to glutamic acid mimics the negative charge introduced by tyrosine phosphorylation of this tyrosine residue under normal conditions. Mutation of tyrosine 256 to phenylalanine (Y256F) abolishes tyrosine phosphorylation. The phosphorylation of the Y256 leads to destabilization of the autoinhibited structure of N-WASP (Wu et al., 2004). Hence in this study the phosphomimicking, Y256E and the phosphodisruptive, Y256F mutants were used to analyze the effects of the phosphorylation of this site upon N-WASP mediated cell adhesion and cell migration roles in vitro.

The binding of the actin monomers to the V domain and F-actin to the central C domain are required for the Arp2/3 complex activation which interacts with the A domain thus converting the Arp2/3 complex to its active state. Since the binding of actin monomers to the V and the C domain is pivotal for the binding of the Arp2/3 complex to the A domain deletion mutants of V and C19 were used to study changes in the adhesion and migration characteristics of these cells.

In order to identify and characterize the N-WASP domains critical for the regulation of cell adhesion and migration, the role of different domains of N-WASP were studied in mouse embryonic fibroblasts N-WASP^{-/-} (MEFs). The N-WASP^{-/-} mouse embryonic fibroblasts were transfected with plasmids expressing N-WASP or N-WASP mutants to identify the domains responsible for the regulation of cell adhesion and migration (Fig. 3.30). The transfected cells were selected using puromycin to kill off cells that did not harbor the expression vector. The cells after selection for two weeks were then used for further experiments. The generated stable cell lines were analyzed for cell adhesion and migration. Listed below are the N-WASP mutants that were stably expressed in the N-WASP^{-/-} cells:

Figure 3.29: Schematic diagram showing N-WASP and its mutants. Plasmids expressing the above mutants (N-WASP^{ΔVCA}, N-WASP^{ΔWH1}, N-WASP^{ΔC19}, N-WASP^{ΔV}, N-WASP^{Y256E}, N-WASP^{Y256F}) and full length N-WASP were stably expressed in N-WASP^{-/-} MEFs.

The plasmids expressing the respective proteins were transfected in the N-WASP^{-/-} cells to generate 6 stable cell lines: N-WASP^{-/-}(N-WASP^{Y256E}), N-WASP^{-/-}(N-WASP^{Y256F}), N-WASP^{-/-}(N-WASP^{ΔVCA}) (without the V1 and V2 domains of N-WASP), N-WASP^{-/-}(N-WASP^{ΔWH1}), N-WASP^{-/-}(N-WASP^{ΔV}) (without the V1 domain of N-WASP), N-WASP^{-/-}(N-WASP^{ΔC19}), and N-WASP^{-/-}(N-WASP^{YFP}). In addition to these generated stable sublines, two additional cell lines were used for the experimental

Figure 3.30: N-WASP expression in the sublines used as controls in this study. N-WASP^{+/+} and N-WASP^{-/-} mouse embryonic fibroblasts were grown under normal conditions of 37° C and 5% CO₂. Cell lysis was carried out followed by measurement of the protein concentration by bradford's assay. The protein samples were loaded equally and resolved in an POLYACRYLAMIDE GEL ELECTROPHORESIS.

assays: N-WASP^{-/-} and N-WASP^{+/+}. To confirm the expression of the mutants and deletion constructs, cells were lysed for western blot analysis (Fig. 3.30 and 3.31).

Figure 3.31: N-WASP expression and its mutants in N-WASP^{-/-} MEFs. The N-WASP^{-/-} cells were transfected with plasmids expressing N-WASP with domain deletions and point mutations as shown in fig. 3.30. N-WASP^{-/-} mouse embryonic fibroblasts transfected with the plasmids were selected and grown under normal conditions of 37° C and 5% CO₂. Cell lysis was carried out and the protein concentration was measured by Bradford's assay. Equal amount of the protein samples were loaded and resolved by polyacrylamide gel electrophoresis.

3.24 The WH1 domain of N-WASP is required for cell-ECM adhesion

The function of N-WASP in cell-ECM adhesion and migration has been well characterized in the fibroblasts. N-WASP promotes cell adhesion and the loss of the protein causes increased cellular motility (Misra et al., 2007). The 8 stable cell lines expressing N-WASP and its mutants were used to study the adhesion characteristics of the cells by an adhesion assay.

The N-WASP^{+/+} (65%±2.5%) cells in comparison to the N-WASP^{-/-} (37%±1%) cells, which showed a low percentage of cells that adhered in the cell adhesion assay. Hence loss of N-WASP expression leads to a cell adhesion defect. The restoration of N-WASP expression, restores the cell-ECM binding as seen from the graph, the observed percentage of cells that adhered in the N-WASP^{-/-} (N-WASP) is 57%±1.4%. The N-WASP^{-/-} (YFP) cells (35%±4%) showed similar cell-ECM adhesion as as the N-WASP^{-/-} cells which was a confirmation that the vector by itself caused no change in the adhesion property of the cell and mimicked the N-WASP^{-/-} cells in adhering to the extracellular matrix (Fig. 3.32).

On restoring the N-WASP expression in N-WASP^{-/-} cells the adhesion defect was restored to a 57%±1.4% (Fig. 3.32). However expressing the mutant N-WASP^{ΔWH1}, the cell adhesion defect of N-WASP^{-/-}(N-WASP^{ΔWH1}) failed to revert and the difference in the percentage of cells that adhered was observed to be statistically significant at 33%±1% in comparison to the N-WASP^{-/-}(N-WASP) cells (65%±2.5%) (P<0.01). Hence the WH1 domain of N-WASP is critical for cell adhesion. On expressing N-WASP^{ΔVCA} mutant in the N-WASP^{-/-} cells, the cell adhesion defect was restored and a binding efficiency of 50%±3.5% was observed, suggesting that the VCA domain is not critical for cell adhesion. The VCA domain has a role in regulating actin dynamics by binding and activating the Arp2/3 complex, which in turn enhances cell migration.

The N-WASP^{-/-} cells expressing N-WASP^{ΔV} and N-WASP^{ΔC19} display a binding efficiency of 41%±1.8% and 58%±2.1% respectively. The V domain binds to actin monomers, hence the absence of the V domain in the VCA region of N-WASP impairs the ability of N-WASP to bind actin monomers. The V domain is also found to affect cell adhesion, as the cell adhesion defect upon expressing the N-WASP^{ΔV} mutant is not restored in the N-WASP^{-/-} cells. This indicates that N-WASP^{ΔV} mutant acts as the non-functional form of N-WASP, which explains the low percentage of cells that adhered observed in the cell-ECM adhesion assay. The N-WASP^{ΔC19} expressing cells showed a high binding percentage of 58%±2.1% thus indicating that the acidic domain is not important in cell-ECM adhesion as the cell adhesion defect observed in the N-WASP^{-/-} cells is restored on expressing the N-WASP^{ΔC19} mutant. The above results suggest the WH1 domain of N-WASP is required for cell adhesion.

Figure 3.32: The WH1 domain of N-WASP is required for cell-ECM adhesion. The stable cell lines expressing N-WASP with domain deletions (N-WASP^{ΔWH1}, N-WASP^{ΔVCA}, N-WASP^{C19} and N-WASP^{ΔV}) were grown under normal conditions of 37°C and 5% CO₂ in a cell culture incubator. The cells were trypsinized and seeded onto wells of a 96 well plate treated with fibronectin. The cells were allowed to adhere for duration of 30 mins. The cells that adhered were quantified using the tecan fluorescence reader. The fraction of cells that were found to bind to the fibronectin from the total number of cells that were seeded was expressed as the percentage of cells that adhered to the fibronectin. Statistical significance is indicated relative to control cells expressing N-WASP^{-/-}(N-WASP). Significance is indicated as stars: ***P*<0.01 (Student's *t*-test).

3.25 Expression of N-WASP^{Y256E} does not restore cell-ECM adhesion in N-WASP^{-/-} cells.

Phosphorylation, a post-translational modification, also regulates the activity of N-WASP. N-WASP is phosphorylated at a conserved tyrosine residue Y256 present in the GBD region, in response to varying extracellular spatial cues. Tyrosine phosphorylation causes the relief of the auto inhibited state and it is predicted to stabilize the open conformation of WASP and N-WASP, significantly enhancing their activity toward the Arp2/3 complex (Caron, 2003).

The study of the function of the N-WASP domains showed that the WH1 domain is important for cell-ECM adhesion (Fig. 3.33). Apart from domain deletions, point mutants of N-WASP were used to study the function of N-WASP in cell-ECM adhesion. Cell lines expressing the N-WASP^{Y256E} and N-WASP^{Y256F} mutants were generated in the N-WASP^{-/-} cells. Binding assay showed that the expression of

the point mutant, N-WASP^{Y256E} failed to restore the cell adhesion defect observed in the N-WASP^{-/-} cells. The percentage of cells that adhered was lower in the N-WASP^{-/-}(N-WASP^{Y256E}) cells (40%±1.1%) in comparison to the N-WASP^{+/+} cells (65%±2.5) (Fig. 3.34). The N-WASP^{-/-}(N-WASP^{Y256E}) cells behaved similar to the N-WASP^{-/-} cell line as the percentage of cells that adhered displayed by the N-WASP^{-/-}(N-WASP^{Y256E}) cell line (40%±1.1%) was nearly the same as the N-WASP^{-/-}(37%±1%) and the N-WASP^{-/-}(YFP) cell lines (35%±4%). The N-WASP^{-/-}(N-WASP^{Y256F}) cells (60%±0.245) restored the cell adhesion as the percentage of cells that adhered was nearly the same as the N-WASP^{+/+} cells (65%±2.5) and the N-WASP^{-/-}(N-WASP) cells (57%±1.4%). The N-WASP^{Y256E} is a phospho-mimic and N-WASP^{Y256F} mutant prevents phosphorylation of N-WASP. The N-WASP^{-/-}(N-WASP^{Y256E}) cells displayed reduced cell-ECM adhesion (Fig. 3.34). Tyrosine 256 lies within the GTPase-binding domain (GBD) of N-WASP, the phosphorylation of which is known to sustain actin polymerization by stabilizing the Arp2/3 binding through SH2-containing proteins (Torres and Rosen, 2003). The phospho-mimic mutant expressed in the N-WASP^{-/-} cell line, N-WASP^{Y256E} may thus be promoting cell migration and causing decreased cell adhesion. On the other hand the Y256F mutation promotes the closed conformation of N-WASP, which results in reduced actin polymerization and hence causing increased adhesion.

Figure 3.33: Expression of N-WASP^{Y256E} failed to restore cell-ECM adhesion in N-WASP^{-/-} cells. The stable cell lines expressing N-WASP with mutations (N-WASP^{Y256E} and N-WASP^{Y256F}) were grown under normal conditions of 37°C and 5% CO₂ in a cell culture incubator. The cells were trypsinized and seeded onto wells of a 96 well plate treated with fibronectin. The cells were allowed to adhere for duration of 30mins. The cells that adhered were quantified using the tecan fluorescence reader. The fraction of cells that were found to bind to the fibronectin from the total number of cells that were seeded was expressed as the percentage of cells that adhered to the fibronectin. Statistical significance is indicated relative to control cells expressing N-WASP^{-/(N-WASP)}. Significance is indicated as stars: ***P*<0.01 (Student's *t*-test).

3.26 The N-WASP^{ΔWH1} and the N-WASP^{ΔV} mutant failed to restore the cell spreading defect in the N-WASP^{-/-} cells

Cell adhesion requires a cell to attach and spread, both events are mediated through the formation of focal adhesion complexes via the integrins that externally connect with the underlying ECM and internally with the actin cytoskeleton (Birchmeier and Behrens, 1994). In order to confirm the cell adhesion results (Fig. 3.33 and 3.34) a spreading assay was done to determine if the cells that adhere well, also spread as efficiently. The spreading assay was done in a 96 well plate with wells that were coated with fibronectin and blocked with BSA. The cells were trypsinized and seeded onto the wells before monitoring their spreading at intervals of 10, 30 and 60 minutes.

The N-WASP^{+/+} MEF cells that displayed a binding efficiency of 65% were found to show a more efficient spreading as compared to the N-WASP^{-/-} mouse embryonic fibroblasts that displayed a binding efficiency of 37% (Fig. 3.33 and 3.35). Similarly the control cells, N-WASP^{-/(YFP)} did not spread as

efficiently as the N-WASP^{-/-}(N-WASP), which had completely spread in 60 minutes (Fig. 3.35A). Hence N-WASP^{-/-} cells, which showed a cell adhesion defect, also displayed a cell-spreading defect. The N-WASP^{-/-}(N-WASP^{ΔWH1}) cell line that showed a lower binding efficiency also showed reduced ability to spread in 60 minutes in comparison with the cells expressing N-WASP^{+/+}(N-WASP). Hence the expression of the mutant N-WASP^{ΔWH1} failed to restore the cell-spreading defect observed in the N-WASP^{-/-} cells. The N-WASP^{-/-}(N-WASP^{ΔVCA}) cell line was observed to have a more flattened morphology in comparison with the N-WASP^{-/-}(YFP) cells however not better than the N-WASP^{+/+} cells and the N-WASP^{-/-}(N-WASP) cells. Hence the expression of the N-WASP^{ΔVCA} in the N-WASP^{-/-} cells partially restores the cell-spreading defect. The N-WASP^{-/-}(N-WASP^{ΔV}) cell line displayed lesser spreading with a less flattened morphology at 60 minutes as compared to the N-WASP^{+/+} cells, which had adopted a completely spread out morphology. Without the V1 domain, the N-WASP^{-/-} cells expressing the N-WASP^{ΔV} failed to restore the observed cell-spreading defect observed in N-WASP^{-/-} cells. The cell line expressing N-WASP^{-/-}(N-WASP^{ΔC19}) did spread efficiently when compared to the N-WASP^{+/+} cells. Hence the loss of the 19 amino acids in the A region of the N-WASP^{ΔC19} mutant expressed in the N-WASP^{-/-} cells leads to the reversion of the cell spreading defect observed in the N-WASP^{-/-} cells. Hence it can be concluded that the WH1 domain and V domain of N-WASP are required for cell adhesion and spreading.

A.

B.

Figure 3.34: The WH1 domain is critical for efficient cell spreading. A. The stable cell lines expressing the different N-WASP mutants (N-WASP^{ΔWH1}, N-WASP^{ΔVCA}, N-WASP^{ΔC19} and N-WASP^{ΔV}) were grown and trypsinized. The cells were seeded onto wells of a 96 well plate treated with fibronectin. Images of the cells were taken at time points of 10, 30 and 60 minutes to observe differences in the ability of the cells to spread. B. Surface area of the cells was measured using the metaview software at the time points- 10, 30 and 60 minutes. Statistical significance is indicated relative to control cells expressing N-WASP^{-/-} (N-WASP). Significance is indicated as stars: **P<0.01 (Student's t-test).

3.27 N-WASP^{Y256E} mutant affects the ability of the cells to spread efficiently

The stable cell lines expressing the N-WASP mutants, that earlier showed a cell adhesion defect also showed a cell spreading defect (Fig. 3.35). The expression of the N-WASP mutants with the different domain deletions in the N-WASP^{-/-} cells showed that the WH1 domain is important for the cell-ECM binding. A spreading assay was also done using stable cell lines expressing the N-WASP domain deletions, where the absence of the domain inhibited the ability of the N-WASP^{ΔWH1} mutant to restore the cell spreading defect in the N-WASP^{-/-} cells.

The adhesion assay using the N-WASP^{-/-}(N-WASP^{Y256E}) cells showed that the expression of the N-WASP^{Y256E} mutant failed to restore the cell-spreading defect in the N-WASP^{-/-} cells. The N-WASP^{-/-}(N-WASP^{Y256F}) cells were found to have spread completely with a flattened morphology in 60 minutes in comparison with the N-WASP^{-/-}(N-WASP^{Y256E}) cells (Fig. 3.36). The N-WASP^{-/-}(N-WASP^{Y256E}) cells expressing the N-WASP^{Y256E} mutant did not spread as well in comparison with the N-WASP^{+/+} and the N-WASP^{-/-}(N-WASP) cells. The N-WASP^{-/-}(N-WASP^{Y256F}) cells were found to spread as well as the N-WASP^{+/+} cells (Fig. 3.36). Thus the expression of the N-WASP^{Y256F} mutant restored the cell spreading and adhesion defect in the N-WASP^{-/-} cells however the N-WASP^{Y256E} mutant did not restore the spreading defects. The above results suggest that the N-WASP^{Y256E} mutant affects cell adhesion and cell spreading. This suggests that the non-phosphorylated N-WASP promotes cell adhesion.

A.

B.

Figure 3.35: The phospho-mimic mutant N-WASP^{Y256E} does not restore the cell-spreading defect of N-WASP^{-/-} cells. A. The stable cell lines expressing the N-WASP with mutations at Y256E and Y256F were grown and trypsinized. The cells were seeded onto wells of a 96 well plate treated with fibronectin. Images of the cells were taken at time points of 10, 30 and 60 minutes to observe differences in the ability of the cells to spread. B. Surface area of the cells was measured using the metaview software at the time points- 10, 30 and 60 minutes. Statistical significance is indicated relative to control cells expressing N-WASP^{-/-} (N-WASP). Significance is indicated as stars: **P < 0.01 (Student's *t*-test).

3.28 WH1 domain of N-WASP plays an inhibitory role in cell migration

In order to further analyze the cell adhesion and migration characteristics a cell migration assay was performed wherein the cells were grown to a 100% confluence and a line was drawn in the middle of the culture. The cells were then monitored for the time taken to close the scratch and images were taken at the respective time points (0 and 14 hours). The N-WASP^{-/-} cells were found to close the wound most efficiently by 14 hours. The N-WASP^{+/+} cells took the longest to close the wound. The controls that were used for this study, the stable cell line-expressing the empty vector, N-WASP^{-/-}(YFP) showed a similar efficiency in closing the wound compared with the N-WASP^{-/-} cells and the stable cell line expressing N-WASP, N-WASP^{-/-}(N-WASP) similarly took a longer time in closing the wound similar to the N-WASP^{+/+} cells. Hence the expression of N-WASP inhibits cell migration. The expression of N-WASP^{ΔWH1} in the N-WASP^{-/-} cells inhibit cell migration as in the absence of the WH1 domain there is no inhibitory effect observed on cell migration hence closing the wound faster as compared to the N-WASP^{+/+} cells (Fig. 3.36).

The loss of interaction with other binding partners at the WH1 domain may have led to relieving the inhibitory effect thus causing an increase in cell migration or the actin polymerization activity of the WH1 domain independent of the VCA domain may. Hence the above observation suggests that the WH1 domain promotes cell adhesion and inhibits cell migration. The cell line expressing the N-WASP^{ΔVCA} showed a migration pattern slower than both N-WASP^{-/-}(N-WASP) and N-WASP^{-/-}(YFP) cell lines. The VCA domain in N-WASP is important for actin polymerization, which drives the migration of a cell. The expression of the N-WASP^{ΔVCA} mutant inhibits cell migration (Fig. 3.37). The N-WASP^{ΔV} expressing cells displayed slower migration in the absence of the V domain. The absence of the V1 domain of the entire V region (V1V2) may result in inefficient activation of Arp2/3 complex at the VCA region. The N-WASP^{-/-}(N-WASP^{ΔC19}) cells show slower movement and were not efficient in closing the wound. The slower migration indicates that the absence of the A region of N-WASP inhibits cell migration. Hence the absence of the V, C19 domains and the entire VCA region leads to a decrease in cell migration as the

wound is inefficiently closed and comparable to the cell migration characteristics observed in the N-WASP^{+/+} or the N-WASP^{-/-}(N-WASP) cells thus suggesting that the VCA region does not inhibit cell migration and instead.

3.29 Expression of N-WASP^{Y256E} mutant in N-WASP^{-/-} cells does not inhibit cell migration

The N-WASP^{Y256E} phosphomimic mutant, causes N-WASP to localize in the cytoplasm and at the filopodial extensions (Wu et al., 2004). Previous experiments have shown that N-WASP^{-/-}(N-WASP^{Y256E}) cells failed to restore cell-ECM adhesion defect in the N-WASP^{-/-}(YFP) cells (Fig. 3.33). In order to confirm further, a migration assay was done to observe the correlation of the cell adhesion and cell migration. The migration assay showed that the N-WASP^{Y256E} mutant in the N-WASP^{-/-}(N-WASP^{Y256E}) cells does not inhibit cell migration (Fig. 3.37). The cells closed the wound faster in the N-WASP^{-/-}(N-WASP^{Y256E}) cells as compared to the N-WASP^{-/-}(N-WASP) cells ($P < 0.05$). Hence the N-WASP^{-/-}(N-WASP^{Y256E}) cells behave like the N-WASP^{-/-}(YFP) cells. The phosphorylation of N-WASP at the Y256 leads to an open conformation, which promotes its localization to the cytosol thus leading to actin polymerization.

The N-WASP^{-/-}(N-WASP^{Y256F}) mutation on the other hand prevents phosphorylation of the protein and cause the cells to migrate slower in comparison to the N-WASP^{-/-}(YFP) cells, behaving similar to the N-WASP^{-/-}(N-WASP) cells in inhibiting cell migration (Wu et al., 2004).

A.

B.

Figure 3.36: The N-WASP^{-/-}(N-WASP^{ΔWH1}) cells close the wound faster compared to the N-WASP^{-/-}(N-WASP) cells. A. The stable cell lines expressing the different domain deletions of N-WASP (N-WASP^{ΔWH1}, N-WASP^{ΔVCA}, N-WASP^{ΔC19} and N-WASP^{ΔV}) were grown to a 100% confluence. A line was drawn in the middle of the culture with a pipette tip. The cells were then monitored to observe the time taken for the cells to close the wound. B. The scratch widths of the different stable cell lines were measured at the time points 0 and 14 hours. Statistical significance is indicated relative to the respective control cells (N-WASP^{-/-}(N-WASP) and N-WASP^{+/+}). Significance is indicated as stars: **P<0.01, ***P<0.001 (Student's *t*-test).

A.

B.

Figure 3.37: The N-WASP^{-/-}(N-WASP^{Y256E}) cells migrate similar to N-WASP^{-/-} cells. A. The stable cells, N-WASP^{-/-}(N-WASP^{Y256E}) and N-WASP^{-/-}(N-WASP^{Y256F}) were seeded onto wells of a 6 well plate and the cells were grown to a 100% confluence. A line was drawn in the middle of the culture with a pipette tip and monitored to observe the time taken for the cells to close the wound. B. The scratch widths of the different stable cell lines were measured at the time points 0 and 14 hours. Statistical significance is indicated relative to control cells expressing N-WASP^{-/-}(N-WASP). Significance is indicated as stars: * $P < 0.05$ (Student's t -test).

3.30 The redistribution of the vinculin and paxillin results in the altered adhesion and migration characteristics of the stable cells expressing N-WASP mutants

The integrins attach to the ECM and are linked indirectly with the actin filaments through protein complexes of talin-paxillin-vinculin. These protein complexes of the focal adhesion proteins, talin-paxillin-vinculin are involved in stabilizing the focal adhesion structure and relay signals from the extracellular matrix to the nucleus within the cell (Owen et al., 2005).

To further investigate the reason for changes in the cell adhesion and migration characteristics of the cells, immunostaining with anti-paxillin and anti-vinculin was carried out. The N-WASP^{+/+} mouse embryonic fibroblasts showed a higher number of vinculin patches per cell as in comparison to the N-WASP^{-/-} MEFs. On the other hand the paxillin patches per cell were found to be lower in the N-WASP^{+/+} MEF cells as compared to the N-WASP^{-/-} MEF cells (Fig. 3.38). Higher vinculin localization has been observed to promote cell adhesion and cell spreading (Xu et al., 1998). Higher paxillin localization causes a higher turnover of focal adhesion complexes and leads to faster migration (Wade et al., 2002). The higher number of vinculin patches thus can be correlated with the increase in cell-ECM adhesion and spreading as in the N-WASP^{+/+} cells. The higher paxillin patches results in faster migration and hence reduced cell adhesion and spreading as observed in the N-WASP^{-/-} cells. The stable cells N-WASP^{-/-} (YFP) showed comparable number of paxillin and vinculin patches per cell as the N-WASP^{-/-} cells. These above results of the controls (N-WASP^{-/-}, N-WASP^{+/+}, N-WASP^{-/-}(YFP), N-WASP^{-/-}(N-WASP)) correlate well with their adhesion and migration patterns observed earlier.

The N-WASP^{+/+} cells showed a lower number of paxillin patches in comparison to the N-WASP^{-/-} cells thus indicating that N-WASP inhibits paxillin localization (Fig. 3.39). The stable cell line N-WASP^{-/-}(N-WASP^{ΔWH1}) cell line, probed with anti-paxillin showed higher number of patches localizing at the ends of the stress fibres, as compared to the N-WASP^{-/-}(N-WASP) cell line (Fig. 3.38). Hence the above observation suggests that the WH1 domain inhibits paxillin localization. The increase in the paxillin

patches indicates towards an increase in the migration of the cells thus suggesting that the WH1 domain inhibits cell migration. The localization of vinculin patches was observed to be the opposite as the number of patches per cell was higher in the N-WASP^{+/+} (55±1.6) cells as compared to the N-WASP^{-/-} cells (25±1.87) (Fig. 3.39). Hence the N-WASP^{-/-} cells show a defect in the localization of vinculin, which in turn causes the defect in cell-ECM adhesion. The WASP^{-/-}(N-WASP^{ΔWH1}) cell line showed lower number of vinculin patches as compared to the N-WASP^{-/-}(N-WASP) cells however the number of patches per cell was observed to be closer to that in the N-WASP^{-/-}(YFP) cells indicating that the N-WASP^{-/-}(N-WASP^{ΔWH1}) cell line behaves similar to the N-WASP^{-/-}(YFP) cells (Fig. 3.39). The above observation suggests that the N-WASP^{ΔWH1} mutant fails to restore the localization of vinculin patches observed in the N-WASP^{-/-}(YFP) cells.

The number of paxillin patches per cell in the N-WASP^{-/-}(N-WASP^{ΔVCA}) cell line (22±3.34) were lesser in comparison to the N-WASP^{-/-}(YFP) cells hence the VCA domain does not influence the localization of the paxillin patches (36±2) (Fig. 3.38). N-WASP^{-/-}(N-WASP^{ΔVCA}) cell line showed a higher number of vinculin patches in comparison with the N-WASP^{-/-}(YFP) cells hence the expression of the mutant without the VCA domain causes the cells to behave similar to the N-WASP^{-/-}(N-WASP) cells (Fig. 3.39). This suggests that the VCA domain is not required for vinculin localization. The number of paxillin patches in both cell lines, N-WASP^{-/-}(N-WASP^{ΔV}) (20±2) and N-WASP^{-/-}(N-WASP^{ΔC19}) (18±1.5) were found to be the nearly the same as in the N-WASP^{-/-}(N-WASP) cell line (15) (Fig. 3.38). This suggests that the domains, V and A do not inhibit paxillin localization. The N-WASP^{-/-}(N-WASP^{ΔV}) cells (47±3.34) and the N-WASP^{-/-}(N-WASP^{ΔC19}) cells (40±3.76) displayed vinculin patches that were found to be comparable in their number with the number of patches displayed by the N-WASP^{-/-}(N-WASP) cells (55±1.6) (Fig. 3.39). The higher number of vinculin patches per cell observed in comparison to the N-WASP^{-/-}(YFP) suggests that in the absence of the two domains, the stable cell lines N-WASP^{-/-}(N-WASP^{ΔV}) cells and the N-WASP^{-/-}(N-WASP^{ΔC19}) are able to restore the defect in vinculin localization observed in the N-WASP^{-/-} cells. Cell-ECM adhesion was observed to be similar in the N-WASP^{-/-}(N-

WASP^{ΔV}) cells (57%±1.8) and the N-WASP^{-/-}(N-WASP^{ΔC19}) cells (41%±2.1) in comparison to the N-WASP^{-/-}(N-WASP) cells (65%±2.5). Hence the V domain and the A region do not affect cell adhesion as the loss of the domains do not result in a cell adhesion defect.

A.

B.

Figure 3.38: Loss of WH1 shows a redistribution of paxillin patches. A. Immunostaining was carried out as described in Fig. 3.19 with anti-paxillin primary antibody. Bar represents 20μm. D. The number of patches per cell was calculated in 30 cells. Experiments were repeated thrice. Statistical significance is indicated relative to control cells expressing N-WASP^{-/-}(N-WASP). Significance is indicated as stars: ** $P < 0.01$ (Student's t -test).

A.

B.

Figure 3.39: Loss of WH1 shows a redistribution of vinculin patches. A. Immunostaining was carried out as described in Fig. 3.19 with anti-vinculin primary antibody. Bar represents 20μm. D. The number of patches per cell was calculated in 30 cells. Experiments were repeated thrice. Statistical significance is indicated relative to control cells expressing N-WASP^{-/-}(N-WASP). Significance is indicated as stars: ** $P < 0.01$ (Student's *t*-test).

3.31 Phosphorylation at the Tyr256 increases paxillin patches and decreases vinculin patches

The previous experiments of the immunostaining in the cell lines expressing the domain deletions of N-WASP showed that the loss of the WH1 domain results in an increase in the number of paxillin patches and a decrease in the vinculin patches as compared to the N-WASP^{+/+} cells. To further our knowledge about the function of the phosphomutants of N-WASP at the Y256, an immunostaining was performed in the N-WASP^{-/-}(N-WASP^{Y256E}) and the N-WASP^{-/-}(N-WASP^{Y256F}) cell lines. The N-WASP^{-/-}(N-WASP^{Y256E}) cell line showed a restoration in the paxillin focal adhesion localization in comparison to the N-WASP^{-/-}(N-WASP) cell line while the cells expressing N-WASP^{-/-}(N-WASP^{Y256F}) showed lesser number of patches, comparable with that observed in the N-WASP^{-/-}(N-WASP) cells (Fig. 3.40). The N-WASP^{-/-}(N-WASP^{Y256E}) cell line showed lesser number of vinculin patches as compared to the N-WASP^{-/-}(N-WASP) cells however the number of patches per cell was observed to be comparable to that in the N-WASP^{-/-}(YFP) cells indicating that the N-WASP^{-/-}(N-WASP^{Y256E}) cell line behaves similar to the N-WASP^{-/-}(YFP) cells (Fig. 3.41). The above observation suggests that the N-WASP^{-/-}(N-WASP^{Y256E}) cell line fails to restore the localization of vinculin observed in the N-WASP^{-/-}(YFP) cells, which in turn affects cell-ECM adhesion. Thus the mutation causes a defect in vinculin localization.

The number of paxillin patches per cell in the N-WASP^{-/-}(N-WASP^{Y256F}) cell line was comparable to the N-WASP^{-/-}(N-WASP) cells. N-WASP^{-/-}(N-WASP^{Y256F}) cell line showed a higher number of vinculin patches in comparison with the N-WASP^{-/-}(YFP) cells hence the expression of the N-WASP^{Y256F} mutant causes a restoration of vinculin localization (Fig. 3.41). The cell-ECM adhesion was observed to be lower in the N-WASP^{-/-}(N-WASP^{Y256E}) cells, which were observed to display a decrease in the number of vinculin patches (Fig. 3.41 and 3.34). The N-WASP^{-/-}(N-WASP^{Y256F}) cell line showed comparable number of vinculin patches as the N-WASP^{-/-}(N-WASP) cell line. Hence there was no change observed in the cell-ECM adhesion property in the N-WASP^{-/-}(N-WASP^{Y256F}) cell line in comparison with the N-WASP^{-/-}(N-WASP) cell line.

A.

B.

Figure 3.40: N-WASP^{Y256E} mutant causes a redistribution of paxillin patches in the N-WASP^{-/-} cells. A. Immunostaining was carried out as described in Fig. 3.19 with anti-paxillin primary antibody. Bar represents 20μm. D. The number of patches per cell was calculated in 30 cells. Experiments were repeated thrice. Statistical significance is indicated relative to control cells expressing N-WASP^{-/-}(N-WASP). Significance is indicated as stars: ****P*<0.001 (Student's *t*-test).

A.

B.

Figure 3.41: N-WASP^{Y256E} mutant causes a redistribution of vinculin patches in the N-WASP^{-/-} cells. A. Immunostaining was carried out as described in Fig. 3.19 with anti-vinculin primary antibody. Bar represents 20µm. D. The number of patches per cell was calculated in 30 cells. Experiments were repeated thrice. Statistical significance is indicated relative to control cells expressing N-WASP^{-/-} (N-WASP). Significance is indicated as stars: ** $P < 0.01$ (Student's t -test).

Chapter 4

CR16 induces filopodia through IRSp53 independent of N-WASP

4.1 Introduction

The actin binding proteins and the polymerized F-actin, which lends the actin cytoskeleton the function of playing an important role in assembling actin based structures such as endocytosis and cell migration (Pollard, 2007). The above-mentioned actin cytoskeletal structures rely on the force that is generated by actin polymerization, which leads to the formation of filopodia and lamellipodia that are important for cell migration (Pollard, 2002). Cdc42 has been shown to promote the formation of actin based structures; its role particularly in filopodia formation has been extensively studied (Kozma et al., 1995). Several interacting partners of Cdc42 have been identified, including WASP, N-WASP and IRSp53 (Insulin Receptor Substrate p53). N-WASP and WASP are two proteins that have been found to provide a direct link to actin assembly and induce membrane projections such as filopodia in conjunction with Cdc42. It has been shown that N-WASP induces filopodia formation in COS-7; subsequently it was further supported by the direct link of N-WASP with activated form of Cdc42 and Arp2/3 complex, which induced filopodia (Martinez-Quiles et al., 2001; Ward et al., 2004). Contrary to that finding it was found that microinjection of constitutively active Cdc42 can generate filopodia in N-WASP^{-/-} mouse embryonic fibroblasts indicating that N-WASP is dispensable for the induction of filopodia (Lommel et al., 2001). Thus, the loss of N-WASP has led to identification of an alternative pathway to generate filopodia in fibroblast cells. Mammalian verprolins are known to regulate actin cytoskeleton through both a WASP dependent and independent pathway. The role of WIP and WIRE in filopodia has been characterized (Martinez-Quiles et al., 2001; Misra et al., 2010) while the role of CR16 has not been well characterized.

4.2 Conformational Analysis of N-WASP

N-WASP has been proposed to adopt a closed inactive conformation and the closed conformation is stabilized by verprolins. Our laboratory has previously adopted BiFC to show that WASP adopts a closed conformation (Lim et al., 2007). The BiFC (Bi-molecular fluorescence complementation) assay has been adopted to visualize protein interactions or to determine the locations of the protein interactions (Anderie and Schmid, 2007; Hu et al., 2002). This approach is based on the complementation of two halves of a single YFP molecule, which are fused to the two termini of N-WASP. When the two halves are apart there is no fluorescence emission but when the two halves are in close proximity, fluorescence emission is observed. Using this assay, we are able to study the conformational changes of N-WASP (Fig. 4.1).

Structural studies and biochemical studies in published findings have shown that N-WASP and WASP exist in an autoinhibited state, wherein the N-terminus of the protein masks the activity of the C-terminus Arp2/3 complex binding and activating domain (Kim et al., 2000; Rohatgi et al., 2000). Within cells N-WASP exists in a complex with WIP (WASP interacting protein) (Ho et al., 2004). Also, N-WASP has been shown to exist in a complex with CR16 in bovine brain (Ho et al., 2001). In order to investigate the conformational changes observed by the interaction of N-WASP with the vertebrate verprolins, the BiFC assay was performed wherein the HEK 293T cells were transfected with N-WASP sensor (N-WASP tagged with two halves of the YFP molecule to the two termini) alone and N-WASP sensor with one of the verprolins (WIP, WIRE and CR16).

The fluorescence intensity was found to be the highest when N-WASP sensor alone, was transfected as seen from the graphs (Fig. 4.2A and 4.2B). BiFC was analyzed by FACS using a Facscalibur (Becton-Dickinson) using the 488 nm laser for excitation and a 515-545nm bandpass filter for detection.

Histogram represents stimulated cells in the area marked with a line (Fig. 4.2A). This suggests that either N-WASP exists in the auto inhibited state or the existence of other proteins that interact and cause N-WASP to adopt the closed conformation. There was no change observed in the fluorescence intensity

when WIRE was transfected with the N-WASP sensor (Fig. 4.2A and B). The high fluorescence intensity could be due to the closed conformation adopted by N-WASP sensor due to the binding of WIRE or there might other binding partners in the cell that maintain N-WASP in the auto-inhibited conformation. Upon transfecting the N-WASP sensor with WIP or CR16 separately, the fluorescence intensity is comparatively reduced. This indicates that N-WASP adopts an open conformation due to the binding of WIP and CR16. The reduced fluorescence intensity is not due to reduced N-WASP expression in the presence of CR16 (Fig. 4.2C). Hence, on co-transfecting CR16 with the N-WASP sensor, it appears to have yielded a maximum difference in the fluorescence intensity.

Figure 4.1: Schematic representation of BiFC (Bi-molecular fluorescence complementation) (A) Schematic representation of a protein molecule tagged with two halves of a YFP molecule to the two termini. When the molecule adopts a close conformation fluorescence emission occurs and in the open conformation there is no fluorescence emission observed.

A.

B.

C.

Figure 4.2: Regulation of N-WASP conformation by the verprolins A. N-WASP sensor alone and N-WASP sensor with the three verprolins: WIP, WIRE and CR16 were transfected in HEK 293T cells. 24 hours post transfection the cells were analysed in a flow cytometer for their fluorescence intensities. B. The intensities of the cells expressing N-WASP sensor or N-WASP sensor with WIRE, WIP and CR16. Statistical significance is indicated relative to control cells expressing N-WASP sensor alone. Significance is indicated as stars: ** $P < 0.01$ (Student's t -test). C. Transfected cells were lysed 24 hours post transfection and protein estimation was done by Bradford's assay. Equal amount of the protein was loaded and resolved by polyacrylamide gel electrophoresis. The proteins were transferred onto a membrane and probed with anti-N-WASP and anti-GAPDH.

4.3 CR16 interacts with IRSp53

The vertebrate verprolins mediate their effect on the actin polymerisation via the WASP family of proteins (WASP and N-WASP) or independent of the WASP family of proteins (Aspenstrom, 2005). One of the vertebrate verprolins, CR16 exists in two alternatively spliced forms, CR16 and CR16-Exon7. N-WASP interacts with CR16 at the residues encoded by the exon 7. However, the CR16 is the less abundant form and binds G and F actin (Ho et al., 2001). This indicates the possibility for other pathways for CR16 to generate changes in the actin cytoskeleton.

In order to explore the role of CR16 in modulating the actin cytoskeleton in the absence of N-WASP, a yeast two-hybrid screen was employed to identify novel interactors of CR16. The yeast two hybrid screen was done using CR16, as bait. The yeast strains, MATa expressing BD-CR16 (Gal4 DNA Binding Domain) and the (MAT α) harboring AD- library (Gal4 Activation Domain) were mated. Upon mating the selection was carried out for CR16 interacting proteins; based on the ability of the diploid colonies to grow on selective plates lacking histidine. Plasmids from 60 colonies that grew on agar plates devoid of histidine, were isolated and sequenced. The sequence analysis led to the identification of plasmids that had cDNA encoding IRSp53 as an in-frame fusion with Gal4-AD while the remaining had out of frame fusion with other genes. The yeast cells expressing Gal4-BD with Gal4-AD-IRSp53 did not grow on the selective plates (Fig. 4.4A), which indicated that interaction did exist between CR16 and IRSp53. A pull down assay was conducted to confirm the binding of CR16 with IRSp53 by expressing the plasmids, IRSp53-6XHis and CR16-EGFP in HEK 293T cells. The IRSp53-6XHis was brought down from the cell lysate using Ni-agarose beads and the proteins attached to IRSp53 were checked by western blot using anti-His antibody to detect IRSp53 and anti-EGFP antibodies to detect CR16 (Fig. 4.3B). The binding of WIP with IRSp53 was tested using yeast two-hybrid assay and his tag pull down assay but no interaction between WIP and IRSp53 was observed (Fig. 4.3B).

A.

B.

C.

Figure 4.3: CR16 interacts with IRSp53 while WIP does not bind to IRSp53. A. Yeast two hybrid interactions between CR16 (Gal4-BD-CR16) with IRSp53 (Gal4-AD-IRSp53) were detected by growth on medium, which lacked histidine and contained 12.5 mM 3- amino 1,2,4-triazole (3-AT). Photographs of the plates were taken after 3 days. B. IRSp53-His with either CR16-EGFP or WIP-EGFP were transfected into HEK 293T cells. The cells were harvested and the cell lysate obtained was treated with beads and incubated on the rocker for 1-2 hours at 4°C. The beads are then washed in elution buffer at a concentration of 200mM imidazole. After collecting the supernatant, a TCA precipitation of the protein is carried out. 15-20µl of the sample is loaded onto a SDS gel and then selective antibodies are used to probe for the different proteins. C. The following controls- WIP- EGFP, CR16- EGFP and IRSp53-6XHis were transfected separately to check for the binding of sticky proteins to the beads.

4.4 CR16 induces the formation of filopodia along with IRSp53

N-WASP interacts with IRSp53 to generate filopodia in N-WASP^{-/-} cells (Lim et al., 2008). In order to investigate the ability of CR16 and IRSp53 in generating filopodia, the experiments were conducted in the N-WASP^{-/-} cells. The amount of plasmid that was transfected was standardized in all the experiments, thus a total of 3.9 µg of plasmid DNA was transfected in all the experiments. 1.3 µg of each plasmid was used for transfections and the empty vector was used to top up to a total of 3.9 µg of the DNA used for transfection. N-WASP^{-/-} cells were transfected with EGFP, CR16, WIP and IRSp53 separately as controls while, CR16-EGFP and WIP-EGFP and IRSp53 were co-transfected to demonstrate their synergistic role in inducing filopodia. 36 hours post transfection cells were observed for fluorescence. Cells transfected with the controls: EGFP, CR16, IRSp53 resulted in a negligible induction of filopodia while the co-transfection of CR16 and IRSp53 yielded a significantly high number of cells with filopodia (Fig. 4.5). N-WASP^{-/-} cells transfected with EGFP alone generated filopodia in 6.5%±1 of cells with filopodia while 10%±1.5 of cells with CR16 alone, 12%±0.9 of cells with IRSp53 alone and 65%±2 of cells when CR16 and IRSp53 were co-transfected (Fig. 4.6A). Hence IRSp53 when transfected along with CR16, induces a higher number of cells with filopodia indicating it to be a strong inducer of filopodia along with CR16. Transfection of WIP by itself resulted in a small increase in the number of cells with filopodia (EGFP: 6.5% ±1, WIP: 10%±3) but did not observe any further increase in the number of cells with filopodia when transfected with IRSp53 (Fig. 4.5), which is not due to reduced expression of WIP in comparison to WIRE (Fig. 4.6B)

Figure 4.4: IRSp53 and CR16 interaction had a synergistic effect on the generation of filopodia. The N-WASP^{-/-} were transfected with the respective combinations of plasmids expressing the proteins. The images show phenotypes of the N-WASP^{-/-} cells with filopodia upon transfection.

A.

B.

Figure 4.5: The filopodia index shows that CR16 and IRSp53 act in a synergistic manner to induce filopodia.

A. Statistical analysis of observed filopodia was done. 36 hours post transfection cells were checked for the presence of filopodia. The cells were observed under 40X objective and 30 transfected cells were taken from different fields of view. The experiment was performed in triplicates and the mean value is shown. Statistical significance is indicated for filopodia index in cells expressing the CR16+IRSp53 relative to control cells expressing IRSp53 with the control vector. Significance is indicated as stars $*P < 0.05$ (Student's *t*-test). B. A western blot of the transfections of WIP and CR16 was performed. Plasmids expressing WIP-EGFP or CR16-EGFP were co-transfected with plasmid that expressed IRSp53. The western blot was carried out using the cell lysate from the transfected cells and probed with the respective antibodies.

4.5 SH3, I-BAR and the CRIB domains of IRSp53 are crucial for the induction of filopodia along with CR16

To explore the function of various domains of IRSp53 in inducing filopodia with CR16, IRSp53 mutants were co-transfected with CR16. The SH3 domain of IRSp53 is known to interact with CR16 (Lim et al., 2008). The IRSp53^{2A} (F428A, P429A mutations) mutant impairs the binding of proteins to the SH3 domain. The number of cells with filopodia, observed on transfecting the IRSp53^{2A} mutant and CR16 were found to be significantly reduced ($13\% \pm 2.1$) in comparison to when IRSp53 was transfected with CR16 ($65\% \pm 1.8$) (Fig. 4.7 and 4.8A). The I-BAR domain of IRSp53 has been reported to bundle F-actin. The IRSp53^{4A} (4 lysine residues at the amino acid positions 142, 143, 146, and 147 in the I-BAR domain which forms the actin binding site) mutant reduces the actin bundling activity (Millard et al., 2005). The number of cells with filopodia ($27\% \pm 5$) generated by this mutant, IRSp53^{4A} when transfected with CR16 was significantly low in comparison to the wild type counterpart (Fig. 4.8A).

The I-267N (isoleucine to asparagine mutation at the amino acid position 267 in the CRIB domain of IRSp53) mutant impairs the binding to Cdc42 (Govind et al., 2001). IRSp53^{I267N} was transfected along with CR16, which yielded low induction of filopodia of $27\% \pm 5$ indicating the role of the CRIB domain of IRSp53 interaction with Cdc42 in the generation of filopodia. The reduced number of cells with filopodia with the different mutants is not due to the low expression of the mutants in comparison to the wild type IRSp53 expression (Fig. 4.8B).

Figure 4.6: SH3, IMD and CRIB domains of IRSp53 are critical for the induction of filopodia in conjunction with CR16. Fluorescence images of the N-WASP^{-/-} cells display filopodia on transfecting CR16 with the respective IRSp53 mutants.

A.

B.

Figure 4.7: SH3, IMD and CRIB domains of IRSp53 induced filopodia in conjunction with CR16 in N-WASP^{-/-} cells. (A) The N-WASP^{-/-} cells were co-transfected with CR16 and IRSp53 or its mutant: IRSp53^{2A}, IRSp53^{4A}, IRSp53^{I267N}. The cells were observed under 40X oil lens and the filopodia index was calculated using the metaview software. 30 transfected cells were selected at different fields. The experiment was performed in triplicates and the mean value is shown. Statistical significance is indicated for the filopodia index of cells expressing CR16+IRSp53 with either of the three mutants relative to cells expressing CR16+IRSp53. Significance is indicated as stars *** $P < 0.001$ (Student's *t*-test). (B) The transfected cells were lysed and the protein concentration was measured by Bradford's assay. Equal concentrations of the cell extract were loaded and resolved by polyacrylamide gel electrophoresis.

4.6 N-WASP negatively regulates CR16 induced filopodia

N-WASP induces filopodia in conjunction with IRSp53. However, the study did not characterize the role of verprolins in N-WASP/IRSp53 induced filopodia (Lim et al., 2008). In this study mouse embryonic fibroblasts were transfected with different combination of the plasmids to determine if the mammalian verprolin, CR16 is regulated by N-WASP. On co-transfection of CR16 and IRSp53, cells were observed to generate $65\% \pm 3.6$ (Fig. 4.3) whereas the co-transfection of IRSp53 with N-WASP generates a higher filopodia index of $70\% \pm 2.6$. However when CR16 is expressed along with IRSp53 and N-WASP, filopodia index is found to unexpectedly reduce to a $50\% \pm 1.7$. This suggests that CR16 is involved in negatively regulating N-WASP. To understand the role of CR16 regulation by N-WASP, cells were transfected with CR16-EGFP, IRSp53 and N-WASP¹⁻²⁷¹, which led to a further reduction of the filopodia index to $30\% \pm 2.5$. The plasmid that expressed N-WASP¹⁻²⁷¹ contains the WH1 domain and the CRIB region, which binds CR16 and IRSp53, respectively. The reduction in the filopodia index observed in the cells transfected with CR16+IRSp53+N-WASP¹⁻²⁷¹ (Fig. 4.4) confirms that N-WASP interaction with CR16 is negatively regulating CR16 in the generation of filopodia along with IRSp53. The further reduction observed in this case is due to the absence of the VCA domain of N-WASP which is a potent activator of the Arp2/3 complex. A western blot was performed to determine if the proteins were expressed efficiently (Fig. 4.4). Hence, N-WASP plays a role in regulating CR16 in filopodia generation in conjunction with IRSp53.

A.

B.

C.

Figure 4.8: N-WASP negatively regulates CR16/IRSp53 induced filopodia. The N-WASP^{-/-} mouse embryonic fibroblasts were transfected with plasmids expressing the respective combinations (CR16+IRSp53+N-WASP¹⁻²⁷¹, CR16+IRSp53+N-WASP, IRSp53+N-WASP and CR16+IRSp53). A. The images show phenotypes of the cells exhibiting filopodia on expressing the combination of proteins expressed. B. Statistical analysis of observed filopodia was done. 36 hours post transfection cells were checked for the presence of filopodia. The cells were observed under 40X objective and 30 transfected cells were taken from different fields of view. The experiment was performed in triplicates and the mean value is shown. Statistical significance is indicated for the filopodia index of cells expressing CR16+IRSp53+N-WASP¹⁻²⁷¹ relative to cells expressing CR16+IRSp53+N-WASP. Significance is indicated as stars * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's t -test). C. Expression levels of the respective proteins was analyzed by an immunoblot. The western blot was carried out using the cell lysate from the transfected cells and probed with the respective antibodies.

4.7 Both the SH3 domain and the CRIB domain of IRSp53 are critical for CR16-IRSp53 interaction

The three domains of IRSp53 were found to be essential for the induction of filopodia along with CR16 (Section 4.6). While IRSp53+CR16 induced filopodia in 65%±2 of the cells, the combination of IRSp53^{2A} + CR16 led to the induction of filopodia in 10%±3 of the transfected cells (Fig. 4.8A) which is

similar to the observed filopodia index as in the CR16 transfected cells. To determine the domain of IRSp53 responsible for the CR16-IRSp53 interaction pull down assay was done using the HEK 293T cells that were transfected with CR16 and IRSp53 or IRSp53 mutants. The cells were harvested 36 hours post transfection, lysed and a pull down assay was carried out. Pull down assays with IRSp53^{2A} showed that mutation in the SH3 domain abolished the interaction with CR16 (Fig. 4.9A). CR16 did not bring down IRSp53^{267N} even though the CRIB domain is responsible for facilitating the binding with Cdc42. Thus it is possible that the CR16-IRSp53 interaction is dependent on Cdc42, suggesting that Cdc42 leads to a change in the conformation of IRSp53 to promote the interaction with CR16. The IMD domain is not critical for CR16-IRSp53 interaction.

A.

B.

Figure 4.9: CR16-IRSp53 interaction requires a functional SH3 and CRIB domain. HEK 293T cells Plasmids expressing CR16-6XHis in conjunction with either IRSp53 or one of the IRSp53 mutants (IRSp53^{4A}, IRSp53^{2A} or IRSp53^{267N}). Cell lysis was carried out 36 hours post transfection and the His-tagged protein was pulled down using Ni-NTA agarose beads. The proteins bound to the beads were resolved using SDS-PAGE, transferred to Nitrocellulose membrane and immunoblotted with anti-His (CR16) and anti-IRSp53 (a-IRSp53). **B.** The following controls- IRSp53, IRSp53^{4A}, IRSp53^{2A}, IRSp53^{267N} and CR16-6XHis were transfected separately to check for the binding of sticky proteins to the beads.

4.8 Cdc42 regulates filopodia formation by CR16/IRSp53 complex

The important players involved in regulating actin dynamics to produce filopodia and lamellipodia are the Rho GTPases (e.g. Cdc42, Rac1, and RhoA). Cdc42 is known to be a major regulator of filopodia formation in mammalian cells and is known to be an IRSp53 effector (Lim et al., 2008).

N-WASP^{-/-} cells were transfected with plasmids expressing CR16+ IRSp53 in addition with plasmids expressing Cdc42 and or its mutants (Cdc42^{G12V}, Cdc42^{T17N}) to investigate the ability of Cdc42 to induce the generation of filopodia. The cells expressing Cdc42 showed an increase in the filopodia generation along with IRSp53 and CR16 (Fig. 4.10). N-WASP^{-/-} cells transfected with plasmid that expressed Cdc42 along with CR16 and IRSp53 with filopodia was 72%±3 in comparison to that of 65%±2 with CR16 and IRSp53 (Fig. 4.11A). On transfecting with the dominant active form, Cdc42^{G12V} along with CR16 and IRSp53 showed a small increase in the number of cells with filopodia (80%±2.3) as in comparison to the cells expressing the wild type. When the cells were transfected with the plasmid that expressed dominant negative form of Cdc42^{T17N}, number of cells with filopodia was reduced to 29%±0.5. The changes in the

number of cells with filopodia observed on transfecting with the dominant active and negative forms is not due to the variations in the expression levels of the mutants (Cdc42^{G12V} and Cdc42^{T17N}) (Fig 4.11B).

CR16-G+IRSp53+

Figure 4.10: Cdc42 enhances the generation of filopodia induced by CR16 and IRSp53. N-WASP^{-/-} cells were transfected with CR16-EGFP, IRSp53 in addition with plasmid that expressed either Cdc42 or Cdc42 mutant (G12V or T17N). Cells were observed for the presence of filopodia 36 hours post transfection.

4.9 Cdc42 is critical for CR16/IRSp53 interaction

In order to investigate the hypothesis that Cdc42 may promote CR16-IRSp53 interaction, HEK 293T cells were transfected with plasmids; CR16-EGFP and IRSp53-6XHis in addition with either Cdc42^{G12V} or Cdc42^{T17N} expressing plasmid. Pull down assay was done with the HEK 293T cells 36 hours post transfection. The cell lysates were isolated using IRSp53-6XHis and analyzed for the presence of CR16-EGFP and Cdc42. IRSp53-6XHis brought down CR16 in the presence of Cdc42^{G12V} but was inefficient in bringing down CR16 in the presence of Cdc42^{T17N} (Fig. 4.12A). CR16-IRSp53 interaction was tested in Cdc42 knockdown cells (Fig. 4.12B). The knocked down expression of Cdc42 in cells expressing

CR16-EGFP and IRSp53-6XHis significantly reduced CR16-IRSp53 interaction. This suggests that IRSp53 binds to CR16 at the SH3 domain, which requires activated Cdc42 to the CRIB domain of IRSp53.

A.

B.

Figure 4.11: The filopodia index shows an increase in the number of cells with filopodia on transfecting Cdc42^{G12V} with CR16+IRSp53. A. N-WASP^{-/-} cells were transfected with CR16-EGFP, IRSp53 in addition with plasmid that expressed either Cdc42 or Cdc42 mutant (G12V or T17N). Cells were observed for the presence of filopodia 36 hours post transfection. Filopodia index was calculated using the metaview software. 30 transfected cells were selected at different fields. The experiment was performed in triplicates and the mean value of the number of cells with filopodia is shown. Statistical significance is indicated for filopodia index of cells expressing CR16+IRSp53+Cdc42 relative to control cells expressing CR16+IRSp53+Cdc42^{T17N}. Significance is indicated as stars: ***P* < 0.01 (Student's *t*-test). B. Western blot of the lysates extracted from cells that were transfected with CR16+IRSp53 and Cdc42 or its mutants (Cdc42^{G12V} and Cdc42^{T17N}) was performed. Antibodies, anti-Cdc42 and anti-IRSp53 were used to detect the expression levels of the respective proteins.

Figure 4.12: Cdc42 regulates CR16-IRSp53 interaction. A. IRSp53-6XHis and CR16-EGFP were transfected along with Cdc42 mutants (Cdc42^{G12V} or Cdc42^{T17N}) in N-WASP^{-/-} cells. B. Plasmids expressing IRSp53-6XHis and CR16-EGFP along with control shRNA vector or plasmid that expressed Cdc42 specific shRNA were transfected in N-WASP^{-/-} cells. The western blot was carried out using the cell lysate from the transfected cells and probed with the respective antibodies. The His-tagged protein was brought down using Ni-NTA agarose beads. The proteins bound to the beads were resolved using SDS-PAGE, and transferred to nitrocellulose membrane and immunoblotted with anti-His (IRSp53), anti-GFP (CR16) and anti-Cdc42. C. Expression of Cdc42 was determined using the Cdc42 antibody.

4.10 IRSp53 depletion reduces induction of filipodia

The previous sections showed that CR16 and IRSp53 interact to generate filopodia synergistically (Section 4.6). Also, Cdc42 was identified as the critical regulator of the CR16-IRSp53 interaction (Section 4.8 and 4.9). To investigate the role of endogenous IRSp53 in inducing filopodia by CR16 and Cdc42, expression of IRSp53 was knocked down by using mouse specific shRNA in N-WASP^{-/-} cells expressing CR16-EGFP and Cdc42^{G12V} and the filopodia index was calculated (Fig. 4.13A and B). Knocking down the endogenous IRSp53 expression reduced the filopodia index from 30%±1 to 8%±0.4. This suggests that the endogenous IRSp53 is responsible for the the induction of filopodia in N-WASP^{-/-} cells transfected with CR16+Cdc42^{G12V}. The western blot analysis shows the decreased expression of IRSp53 upon using an IRSp53 shRNA and a densitometry analysis of the western blot was done where

the knocked down levels of IRSp53 expression is normalized against GAPDH is observed (Fig. 4.13 C and D).

Figure 4.13: IRSp53 knockdown leads to a reduction in the the induction of filopodia. N-WASP^{-/-} mouse embryonic fibroblasts were transfected with CR16-EGFP and IRSp53 in the presence of plasmid that expressed Cdc42-shRNA. A. Transfected cells were analyzed for the presence of filopodia under 40X objective, 36 hours post transfection. Bar represents 20µm. B. The filopodia index was calculated using the metaview software. 30 transfected cells were selected at different fields of view. The experiment was performed in triplicates and the mean value of the number of cells with filopodia is shown. Statistical significance is indicated for filopodia index in cells expressing CR16+ IRSp53+Cdc42 shRNA relative to control cells expressing CR16+IRSp53 with the empty vector. Significance is indicated as stars: **P*<0.05, ***P*<0.01 (Student's *t*-test). C. Western blot analyses of IRSp53 knock down was performed. The western blot was carried out using the cell lysate from the transfected cells and probed with the respective antibodies. D. Densitometry quantification shows the level of Cdc42 expression normalized against GAPDH in cells expressing CR16-EGFP +IRSp53+vector control in comparison to the CR16-EGFP +IRSp53+Cdc42 shRNA.

4.11 Cdc42 depletion reduces the induction of filopodia

It has been shown that Cdc42^{G12V} enhances the induction of filopodia by CR16 and IRSp53. However the role of endogenous Cdc42 in the induction of filopodia along with CR16 has not been observed. In order to investigate the role of endogenous Cdc42 in inducing filopodia, a shRNA targeting Cdc42 was generated. N-WASP^{-/-} cells were seeded onto coverslips 24 hours before transfection. Using the Cdc42-shRNA knockdown of Cdc42 expression was done in N-WASP^{-/-} cells transfected with IRSp53+CR16 and this led to a significant reduction in filopodia index in comparison to cells transfected with empty vector (Vector 70%±1.6, Cdc42 shRNA 12%±0.43) (Fig. 4.14 A and B). The above result suggests that Cdc42 acts as an important regulator of the induction of filopodia by the CR16-IRSp53 complex. The knockdown of Cdc42 expression was analyzed by western blot. A densitometry analysis of the western blot was done where the knocked down levels of Cdc42 expression normalized against GAPDH is observed (Fig. 4.14C and D).

A.

CR16 + IRSp53

B.

C.

CR16 + IRSp53+

D.

Figure 4.14: Cdc42 is essential for the induction of filopodia by CR16. A. N-WASP^{-/-} mouse embryonic fibroblasts were transfected with CR16-EGFP and Cdc42^{G12V} in the presence of plasmid that expressed Cdc42 specific shRNA. Transfected cells were analyzed for the presence of filopodia under 40X objective, 36 hours post transfection. Bar represents 20µm. B. The filopodia index was calculated using the metaview software. 30 transfected cells were selected at different fields of view. The experiment was performed in triplicates and the mean value of the number of cells with filopodia is shown. Statistical significance is indicated for filopodia index in cells expressing CR16+Cdc42^{G12V}+IRSp53 shRNA relative to control cells expressing CR16+Cdc42^{G12V} with the empty vector. Significance is indicated as stars: **P*<0.05, ***P*<0.01 (Student's *t*-test). C. Western blot analyses of IRSp53 knock down was performed. The total cell lysate from the transfected cells were analyzed by an immunoblot with the respective antibodies. D. Densitometry quantification shows the level of IRSp53 expression normalized against GAPDH in cells expressing CR16-EGFP+Cdc42^{G12V}+vector control in comparison to the CR16-EGFP+Cdc42^{G12V}+IRSp53 shRNA.

4.12 Rac1 acts as an inducer in the generation of lamellipodia along with CR16 and IRSp53

IRSp53 interacts with activated Rac1 at the N-terminal Rac1 binding domain (RCB) (Miki et al., 2000; Choi et al., 2005). Rac1 binds to the partial-CRIB motif, which is weak as compared to Cdc42 (Lim et al., 2008). N-WASP^{-/-} cells were transfected with the Rac1 and its mutants (Rac1^{G12V}, Rac1^{T17N}) along with CR16 and IRSp53 to generate lamellipodia (Fig. 4.15). On transfecting Rac1 with CR16 and IRSp53, 20%±0.6 of the cells were observed with lamellipodia. While transfecting the cells with the dominant active form, Rac1^{G12V} yielded a high number of cells with lamellipodia (45%±1). On transfecting the cells with Rac1^{T17N} a reduction in the number of cells with lamellipodia (5%±0.3) was observed due to poor binding of the dominant active mutant to the IRSp53 (Fig. 4.16A). The different indices observed are not due to reduced expressions of the different forms of the proteins (Fig. 4.16B).

CR16-EGFP+
IRSp53

Figure 4.15: CR16 and Rac1 induce the generation of lamellipodia. Transfections of CR16+IRSp53 with Rac1 (WT or T17N or G12V) in N-WASP^{-/-} cells were analyzed for the presence of lamellipodia 36 hours post transfection.

Figure 4.16: Increase in the number of cells with lamellipodia on transfecting Rac1 with CR16 and IRSp53. A. Cells were checked for the presence of lamellipodia 36 hours post transfection. The cells were observed under 40X objective by counting 30 transfected cells taken from different fields of view. The experiment was performed in triplicates and the mean value is shown. Statistical significance is indicated for filopodia index in cells expressing CR16+IRSp53+Rac1^{G12V} or Rac1^{T17N} relative to control cells expressing CR16+IRSp53+Rac1. Significance is indicated as stars: * $P<0.05$, ** $P<0.01$, *** $P<0.001$ (Student's *t*-test). B A western blot of the transfections of CR16+IRSp53 with Rac1 (WT or T17N or G12V) was performed. The western blot was carried out using the cell lysate from the transfected cells and probed with the respective antibodies.

4.13 The Verprolin domain of CR16 is critical for CR16-IRSp53 interaction and the induction of filopodia

The mammalian verprolins consist of a verprolin homology domain (V) at the N-terminus and a WASP binding domain (WBD) at the C-terminus (Aspenstrom, 2005). The WBD domain of the verprolins is responsible for facilitating interaction with N-WASP, the V domain binds G actin. The binding of the G actin at the V domain is important for the function of verprolins (Aspenstrom, 2005). To investigate the role of the V domain in inducing filopodia, the plasmid that expressed CR16-EGFP or CR16 Δ V-EGFP was transfected along with IRSp53. The CR16 Δ V (10% $\pm$ 0.8) showed a decrease in the induction of filopodia as compared to CR16 (65% $\pm$ 2) when transfected in conjunction with IRSp53 (Fig. 4.17 A and B). Thus N-WASP^{-/-} cells expressing CR16 Δ V+IRSp53 had a showed a similar the induction of filopodia as N-WASP^{-/-} cells expressing IRSp53 by itself (Fig. 4.17A). This decrease in the filopodia index in the presence of CR16 Δ V+IRSp53 is not due to a lower expression expression of CR16 Δ V or IRSp53 (Fig. 4.17C).

A.

B.

C.

D.

Figure 4.17: V domain of CR16 is required for CR16-IRSp53 interaction A. N-WASP^{-/-} cells were transfected with combination of plasmids (CR16-EGFP+ IRSp53 or CR16ΔV-EGFP+IRSp53 or CR16-EGFP+ IRSp53+ Cdc42^{G12V} or CR16ΔV-EGFP+ IRSp53+ Cdc42^{G12V}). Cells were observed for the presence of filopodia 36 hours post transfection. Bar represents 20μm. B. The cells were observed under 40X objective and 30 transfected cells were taken from different fields of view. The experiment was performed thrice and the mean value is shown. Statistical significance is indicated relative to their respective controls (CR16+IRSp53 and CR16+IRSp53+Cdc42^{G12V}). Significance is indicated as stars: **P*<0.05, ***P*<0.01, ****P*<0.001 (Student's *t*-test). C. Plasmid that expressed IRSp53-6XHis was transfected with either CR16-EGFP or CR16DV-EGFP into HEK 293T cells. Cell lysis was carried out 36 hours post transfection and the IRSp53-6XHis was brought down using Ni-NTA agarose beads. The proteins attached to the beads and the proteins present in the flow through fractions were analyzed by an immunoblot using anti-EGFP and anti-6XHIS antibodies. D. The following controls- IRSP53-6XHis, CR16ΔV-EGFP and CR16-EGFP were transfected and checked for the binding of sticky proteins to the beads.

Chapter 5

Discussion

5.1 Actin cytoskeleton and cancer

Actin reorganization plays an essential role in many essential cellular processes in several aspects of cell behavior. The changes in the actin organization are a response to both extra and intra cellular spatial stimuli, which involve several pathways, which eventually activate the actin nucleators. Among the known nucleators Arp2/3 complex comprising of 7 subunits is the most well known and extensively studied nucleator, which advances actin polymerization upon activation. The actin cytoskeleton is comprised of polymerized F-actin, actin nucleating and binding proteins that are essential for maintaining cell shape, the motility of the cells, cell-ECM adhesion, cell-cell adhesion and endocytosis. The actin nucleators and actin are also regulated by the Rho family GTPases through the WASP/WAVE family of proteins which control the kind of actin cytoskeletal processes that are generated which include the: filopodia-thin long extensions, lamellipodia-broad sheet like structures and stress fibres-bundles of actin filaments (reviewed by Chesarone and Goode, 2009; Reviewed by Faix and Weber, 2013). The actin cytoskeleton is a dynamic structure undergoing constant remodeling. Several nucleation promoting factors have been shown to be involved in tumor invasion and metastasis. Nucleation promoting protein cortactin has been implicated in cancers, the expression was altered in the head and neck carcinomas and colorectal cancer (Rothschild, 2006; Hofman, 2008). WAVE 1 and WAVE 3 were reported to promote invasiveness and were down-regulated in prostate cancers. WAVE1 and WAVE3 suppress lung metastasis of breast cancers in mice (Alaoui et al., 2007; Fernando et al., 2010). Knocking down the expression of WAVE2 led to reduced metastasis of melanoma cells (Kurisu et al., 2005). The expression of MENA showed an increase in, tumor cells that were invasive (Wang et al., 2004; Wang et al., 2007). The Rho GTPase Rac1 is found to be important for the development of skin tumors *in vivo* (Wang et al., 2010). Thus actin cytoskeleton regulation play both tumor suppressor role as well as metastasis promoter functions.

5.2 N-WASP and cancer

N-WASP is a key regulator of Arp2/3 complex mediated actin polymerization driving membrane projections (Reviewed in Millard et al., 2004). These N-WASP induced membrane projections play a critical role in cell-ECM adhesion, cell-cell adhesion and cell migration. Alterations in all these processes have been implicated in cancer progression to metastasis (Okegawa et al., 2002). The N-WASP mediated cell adhesion and migration has been characterized previously in Mouse embryonic fibroblasts however the function of N-WASP in cell adhesion and migration in epithelial cells and skin carcinomas has not been well characterized.

Although cellular function of N-WASP in cancer progression has been suggested its exact function during the various stages of cancer has not been deciphered. In MEFs, loss of N-WASP gene leads to enhanced cell migration and reduced cell-matrix adhesion indicating that N-WASP performs a significant role in cell-matrix adhesion thus it may have a role in metastasis when reduced cell-ECM adhesion maybe enhancing cell migration, away from the primary tumor site (Misra et al., 2007). In human breast cancer tissues, N-WASP expression was down regulated (Martin et al., 2008) and N-WASP overexpression in MDA-MB-231 an invasive breast cancer cell line led to reduced motility and invasion. Immunohistochemistry in esophageal tumor samples showed that N-WASP is overexpressed as compared to the normal esophageal samples (Wang et al., 2010). The expression level of N-WASP is reduced in colon tumor tissues as compared to normal tissues (Martin et al., 2012). Sublines of human colon cancer HRT-18 were generated with the N-WASP expression knocked down and with N-WASP overexpressed. The sublines with N-WASP overexpressed showed marked reduction in cell-ECM adhesion, motility and invasiveness. The cell line with knocked down expression of N-WASP showed an increase in cell-ECM adhesion, motility and invasiveness (Martin et al., 2012). Taken together results from mouse embryonic fibroblasts and MDA-MB-231 suggest that N-WASP may play a critical role in tumor cell migration and adhesion.

Several reports suggest that N-WASP localizes at the invadopodia. Protrusions formed through the complex rearrangement of the actin cytoskeleton, which contain proteolytic enzymes that degrade the extra cellular matrix (ECM) to allow for the invasion of malignant cells through ECM (Linder et al., 2007). Several cytoskeletal-associated proteins such as N-WASP, Cdc42 localize during the early stage of invadopodia development (Yamaguchi et al., 2005). Activation of N-WASP through Cdc42 has been found to be critical for invadopodia formation. Furthermore, localization of N-WASP was observed in conjunction with activation of Cdc42 and Rac1 in breast cancer cells (Sturge et al., 2001).

5.3 N-WASP expression in SCC patient samples

N-WASP expression was assayed by qPCR in a total of 33 skin cancer samples obtained from National Skin Centre, Singapore. The qPCR results of the 33 samples showed that the N-WASP expression is lower in tumor cells as compared to their paired perilesional controls. The decreased N-WASP expression in the patient SCC samples suggests a possible correlation between N-WASP expression and tumorigenicity. N-WASP expression has been shown to be decreased in the breast cancer patient samples (Martin et al., 2008). The human colorectal cancer patient samples also showed lower transcript levels of N-WASP (Martin et al., 2012). So far there is one published report that shows that N-WASP expression is increased in the hepatocellular carcinoma patient samples (Jin et al., 2013). The above-mentioned reports showing a decrease in N-WASP expression and the observed reduced N-WASP expression in the SCC patient samples indicates that N-WASP probably plays a role as a tumor suppressor and can be used as a marker for prognosis.

5.4 N-WASP expression in cancer cell lines

The SCC patient samples were provided in paraffin embedded form thus could not be used to study cellular defects. In order to characterize the function of N-WASP in skin cancer progression, three cell lines were selected to study the N-WASP expression- A5-RT3, II-4, and HSC-5, which were compared with HaCaT (Section 3.1.1). The expression level of N-WASP in the aggressively metastatic cell line, A5-RT3 and II-4 were observed to be higher in comparison with HaCaT. On the other hand, the N-WASP expression was found to be reduced in the HSC-5 cell line, which is a non-metastatic, tumorigenic cell line when compared with HaCaT cells. Lastly the expression level in II-4 cell line was found to be higher than that observed in the HaCaT cells. The observed differences in the expression level observed in the tumorigenic, metastatic and tumorigenic, non-metastatic cell lines suggest different levels of N-WASP expression in different stages of cancer progression. The decreased N-WASP expression in HSC-5 cells correlate with the similarly decreased expression pattern in the patient SCC samples.

5.5 Cell adhesion and spreading in N-WASP depleted cells

Cell migration and cell adhesion are highly synchronized processes that are carried out through tight regulation of actin polymerization and cell-ECM adhesion (Yamazaki et al., 2005). A cell adhesion assay of all the four epithelial cell lines used in this study was done. As seen N-WASP expression was observed to be higher in the A5-RT3 cells in comparison to the HaCaT cells (Section 3.2). Knocking down the N-WASP expression in HaCaT cells led to a reduction in cell-ECM adhesion and spreading on fibronectin (Sections 3.6 and 3.7).

The A5-RT3 cells and the HSC-5 cells showed reduced cell-ECM binding as compared to the control cells, HaCaT. The II-4 cells showed similar cell-ECM binding as compared to HaCaT. HaCaT^{N-WASP KD} and A5-RT3^{N-WASP KD} showed a reduction in the cell-ECM adhesion when in comparison to their

controls, HaCaT^{CTR} and A5-RT3^{CTR} respectively. After cell adhesion to the matrix, cells spread and acquire flattened morphology. The HaCaT^{N-WASP KD} cells and the A5-RT3^{N-WASP KD} cells also showed reduced spreading with a rounded morphology as compared to the HaCaT^{CTR} cells and the A5-RT3^{CTR} cells which showed a more flattened morphology within 30 minutes of seeding (Sections 3.18 and 3.19). In this study the N-WASP^{-/-} mouse embryonic fibroblasts showed reduced spreading and cell-ECM adhesion on surface coated with fibronectin as compared to the N-WASP^{+/+} mouse embryonic fibroblasts (Sections 3.24-3.25). Hence N-WASP is found to play a critical role in cell-ECM adhesion and migration, in both fibroblasts and in epithelial cells. It was previously reported that N-WASP promotes cell-ECM adhesion as deletion of the N-WASP gene leads to loss of cell-ECM adhesion and enhanced cell migration (Misra et al., 2007). Defined cell spreading requires reorganization of the actin cytoskeleton as well as proper interaction of integrins with ECM. Cell spreading and adhesion are tightly linked and depend on focal adhesion and integrity of actin cytoskeleton.

N-WASP was shown to be critical in the establishment of the germ layers. This was determined through a study that generated a N-WASP knockout mouse, which was found to be embryonic lethal, N-WASP^{-/-} embryos exhibited reduced growth and abnormalities in extra-embryonal mesoderm differentiation and neurulation. For proper neurulation, regulation of cell adhesion and cytoskeletal dynamics is required and in this case lack of N-WASP was identified to be the cause (Snapper et al., 2001; Lommel et al. 2001).

Vinculin is a focal adhesion protein and has been reported to play an important role in cell-ECM adhesion. Loss of expression of cytoskeletal and focal adhesion proteins leads to disruption in cell-ECM adhesion and migration (Xu et al., 1998). HaCaT^{CTR}, A5-RT3^{CTR} and N-WASP^{+/+} mouse embryonic fibroblasts have increased number of vinculin patches in comparison to the cells with reduced N-WASP expression, HaCaT^{N-WASP KD}, A5-RT3^{N-WASP KD}, N-WASP^{-/-} mouse embryonic fibroblasts respectively. In previous reports, vinculin knockout (vinculin^{-/-}) cells have been reported to display rapid turnover of focal adhesions, which enhanced cell migration (Xu et al., 1998). Also, overexpression of vinculin promotes the increase in size and number of focal adhesions and inhibits cell migration (Fernandez et al.,

1992). The reduced cell adhesion and spreading observed in the HaCaT^{N-WASP KD}, A5-RT3^{N-WASP KD} and N-WASP^{-/-} mouse embryonic fibroblasts are due to changes in expression and recruitment of vinculin to focal adhesion sites.

5.6 Cell migration in epithelial cells with reduced expression of N-WASP

Protrusion at the leading edge, adhesion to the substratum, translocation of the cell body and retraction through the dissolution of older adhesions are the major events in cell migration (Rafelski and Theriot 2004). Thus for cell migration a continuous reorganization of the adhesion complex takes place. Cell migration is the early event in the gastrulation during the establishment of cell layers in embryo that is why it is a critical process throughout the development (Sugihara et al., 1998; Chen et al., 2000). Knockout studies in mouse showed that N-WASP knockout embryos were able to enter gastrulation but unable to finish neurulation and inversion of three germ layers. These abnormalities indicate defects in cell migration or in cell polarization during the formation of three germ layers in N-WASP deleted embryos (Lommel et al., 2001). The previous reports above and the findings in our study show that in addition to N-WASP playing a critical role in cell-ECM adhesion and spreading, it also plays an important part in cell migration (Sections 3.8, 3.14, 3.20 and 3.28-3.29)

Migratory behavior of epithelial cells was compared through a scratch assay. The HaCaT^{N-WASP KD} and A5-RT3^{N-WASP KD} cells showed an increase in cell migration as compared to HaCaT^{CTR} and A5-RT3^{CTR} cells (Sections 3.8 and 3.20). The conclusion drawn from the observed result above is that knockdown of N-WASP led to increased motility of the cells. Decrease in cell migration as a resultant of depletion of N-WASP has also been reported in the N-WASP^{-/-} cells (Misra et al., 2007).

The HaCaT and A5-RT3 cells displayed lesser number of paxillin patches as compared to the HaCaT^{N-WASP KD} and A5-RT3^{N-WASP KD} cells, respectively. These results suggest that N-WASP deficiency leads to

increased paxillin localization. Conversely it was seen that increased N-WASP expression in HSC-5 cells caused a decrease in the number of paxillin patches in comparison to the HSC-5^{CTR} cells. Paxillin knockout fibroblasts exhibited delayed turnover of focal adhesions, which caused reduced integrin mediated cell migration (Webb et al., 2004). The increased motility of the cells observed in the HaCaT^{N-WASP KD}, A5-RT3^{N-WASP KD} and N-WASP^{-/-} mouse embryonic fibroblasts probably due to changes in paxillin expression and recruitment to focal adhesion sites.

5.7 Overexpression of N-WASP in HSC-5^{N-WASP} cells

HSC-5 cell line derived from Human SCC patient was found to have reduced N-WASP expression and reduced cell-ECM adhesion. N-WASP expression in HSC-5 cells increased cell adhesion and spreading on fibronectin. The HSC-5^{N-WASP} cells with a higher N-WASP expression showed an increased spreading and binding in comparison to the HSC-5^{CTR} cells (Sections 3.12 and 3.13). On the other hand on increasing the N-WASP expression in HSC-5 cells, the overexpression led to reduced motility of the cells. The HSC-5^{N-WASP} cells displayed increased number of vinculin patches and decreased number of paxillin patches. The increase in cell-ECM adhesion and decrease in cell migration is explained by the increased vinculin patches and decreased paxillin patches as compared to their controls, respectively.

The lower expression level of N-WASP as observed in a non-metastatic cell line, HSC-5 might indicate its role in uncontrolled proliferation to form a mass of cells. An experiment conducted by a colleague in our laboratory showed decreased proliferation in HSC-5^{N-WASP} cells (unpublished finding). While in a metastatic cell line, A5-RT3 the expression level of N-WASP is found to be higher and this increased expression level of N-WASP suggests a role for N-WASP in the metastasis of a malignant mass formed. Expression level of N-WASP was found to be decreased in human colorectal tumors in comparison to their paired controls (Martin et al., 2012). N-WASP expression was seen to be higher in the later stages (TNM2) in comparison to early stages (TNM1) of tumors. A comparison of N-WASP expression was

also shown by the same group demonstrating that N-WASP expression was higher in metastatic liver sites in comparison to the colon tumors in their early stages (Yanagawa et al., 2001). Hence based on the above observations of results in this thesis and previous published findings it can be proposed that N-WASP plays different roles based on the stage of cancer progression.

5.8 WH1 domain of N-WASP is required for cell-ECM adhesion

To investigate the N-WASP domains responsible for the regulation of cell adhesion and migration N-WASP and its mutants (N-WASP^{ΔVCA}, N-WASP^{ΔWH1}, N-WASP^{ΔC19}, N-WASP^{ΔV}, N-WASP^{Y256E} and N-WASP^{Y256F}) were expressed in N-WASP^{-/-} cells to generate stable cell lines (Sections 3.23 and 3.24).

The N-WASP^{-/-}(N-WASP^{ΔWH1}) cell line displayed reduced cell-ECM adhesion and spreading in comparison with N-WASP^{-/-}(N-WASP) cell line suggesting that the WH1 domain is critical for cell-ECM adhesion. The N-WASP^{+/+} and N-WASP^{-/-} cells displayed the vinculin patches that numbered: 55±1.6 and 25±1.87, respectively. The N-WASP^{-/-}(N-WASP) (44±1) and the N-WASP^{-/-}(YFP) cells (30±2.66) also showed similar number of vinculin patches when compared to the N-WASP^{+/+}(55±1.6) and N-WASP^{-/-} cells (25±1.87), respectively. N-WASP^{-/-}(N-WASP^{ΔWH1}) (34±1.67) cells displayed vinculin patches that were comparable in their number to the N-WASP^{-/-}(YFP) (38±1) cells. Higher vinculin localization has been observed to promote cell adhesion and cell spreading (Xu et al., 1998). Thus the WH1 domain of N-WASP promotes the localization of vinculin patches, which explains the decreased cell adhesion observed in the N-WASP^{-/-}(N-WASP^{ΔWH1}) cells. Thus based on the number of vinculin patches per cell observed, the loss of the WH1 domain caused for a reduction in the localization of the number of vinculin patches in comparison to the number observed in the N-WASP^{-/-}(N-WASP) cells. Thus indicating that the WH1 domain is important for facilitating the localization of vinculin patches which affects cell adhesion.

The N-WASP^{+/+} cells (12±1.21) had reduced number of paxillin patches in comparison to the N-WASP^{-/-} cells (36±2). The controls N-WASP^{-/-}(N-WASP) (15±2.22) and N-WASP^{-/-}(YFP) (38±1) show similar number of paxillin patches to the N-WASP^{+/+} and the N-WASP^{-/-} cells, respectively. The N-WASP^{-/-}(N-WASP^{ΔWH1}) showed better migration as compared to the N-WASP^{-/-}(N-WASP) cells. The N-WASP^{-/-}(N-WASP^{ΔWH1}) cell line exhibits cell migration rate similar to the N-WASP^{-/-}(YFP) cells (Sections 3.27 and 3.28). N-WASP inhibits cell migration as N-WASP^{-/-} cells migrate more efficiently than N-WASP^{+/+}. The N-WASP^{-/-}(N-WASP^{ΔWH1}) cell line did not show a cell migration defect by scratch assay, hence this suggests that the WH1 domain plays an inhibitory role in cell migration. Higher paxillin localization causes a higher turnover of focal complexes, which leads to faster migration (Wade et al., 2002). The N-WASP^{-/-}(N-WASP^{ΔWH1}) cells display paxillin patches similar to the N-WASP^{-/-}(YFP) cells. Hence the N-WASP^{-/-}(N-WASP^{ΔWH1}) cells behave similar to the N-WASP^{-/-}(YFP) cells in migrating efficiently, which suggests that the WH1 domain inhibits paxillin localization and the WH1 domain is not important for cell migration.

Further support for the function of WH1 domain of N-WASP in cell-ECM adhesion and spreading was provided by published data which suggests that hnRNPK, a member of heterogeneous nuclear ribonucleoprotein complex, regulates cell spreading through interacting with WH1 domain of N-WASP (Yoo, et al., 2006). Furthermore it has been shown that interaction of N-WASP with hnRNPK is required for initial stage of cell spreading. It has also been reported that the WH1 domain can promote actin polymerization independent of the VCA domain and was found to accentuate the Arp2/3 complex activation in the presence of the VCA domain. The WH1 domain interacts with the Arp2/3 complex, the mammalian verprolins and the PIP₂, which might be collectively contributing to the actin polymerization activity of the domain independent of the VCA domain (Suetsugu et al., 2001). Reduced adhesion to fibronectin as well as delayed spreading of N-WASP^{-/-} mouse embryonic fibroblasts on fibronectin coated surface is in agreement with the finding that N-WASP regulates the spreading initiation center during the early stages of cell spreading by direct association with hnRNPK (Yoo, et. al., 2006).

5.9 Role of the VCA domain, the V domain, the C domain and the A domain

The N-WASP^{-/-}(N-WASP^{ΔVCA}) cell line did not show a cell adhesion defect in comparison to the N-WASP^{-/-}(YFP) cells and instead showed cell adhesion characteristics similar to N-WASP^{-/-}(N-WASP) cells suggesting that the VCA domain of N-WASP is not critical for cell adhesion. N-WASP^{ΔVCA} (50±1.33), N-WASP^{ΔV} (47±3.34) and N-WASP^{ΔC19} cells (40±3.76) which showed comparable number of vinculin patches per cell to the number of vinculin patches observed in the N-WASP^{-/-}(N-WASP) cells (44±1). The deletion of the VCA domain, V domain and the C19 region didn't lead to any change in the number of vinculin patches observed in comparison to the N-WASP^{-/-}(N-WASP) cells hence indicating that these domains are not involved in mediating changes in the localization of vinculin which in turn leads to no observed cell adhesion defect.

The N-WASP^{-/-}(N-WASP^{ΔC19}) and the N-WASP^{-/-}(N-WASP^{ΔV}) cell lines were found show a migration rate similar to the N-WASP^{-/-}(N-WASP) cells. N-WASP^{-/-}(N-WASP^{ΔVCA}) (22±3.34), N-WASP^{-/-}(N-WASP^{ΔV}) (20±2) and N-WASP^{-/-}(N-WASP^{ΔC19}) cells (18±1.5) had reduced localization of paxillin as the number of patches are found to be low and comparable to that displayed by the N-WASP^{-/-}(N-WASP) cells (15±2.22). Hence the VCA, V and C19 domains do not inhibit cell migration and paxillin localization. The reduced paxillin localization observed in the three cell lines, N-WASP^{-/-}(N-WASP^{ΔVCA}) (22±3.34), N-WASP^{-/-}(N-WASP^{ΔV}) (20±2) and N-WASP^{-/-}(N-WASP^{ΔC19}) cells (18±1.5) as compared to the N-WASP^{-/-}(YFP) (38±1) cells could be due to the lack of the domains resulting in loss of interaction with binding partners that mediate localization of paxillin. Hence a loss of the VCA domain caused the reduction in the localization of the paxillin patches, which may have led to the observed inhibition of cell migration. Hence the V, C19 and the VCA domains were found to be important in mediating the paxillin focal adhesion localization.

5.10 Regulation of N-WASP by phosphorylation

Amongst the two point mutants (N-WASP^{Y256E} and N-WASP^{Y256F}), expression of N-WASP^{Y256E} mutant in N-WASP^{-/-} cells failed to restore the cell-ECM adhesion defect as observed in the N-WASP^{-/-}(N-WASP^{Y256E}) cell line. The phospho-mimicking mutant N-WASP^{Y256E} has been shown to mimic phosphorylation of N-WASP at the Y256 site hence lending a negative charge to the site, which disrupts the auto-inhibited structure of N-WASP leading to an open conformation of N-WASP (Kim et al., 2000). This open conformation allows for the binding of the SH2 domain containing proteins at the Y256 site, the verprolins at the WH1 domain and the actin monomers at the V domain. The N-WASP^{Y256F} maintains N-WASP in the closed conformation preventing the binding of activators and hence actin polymerization. The N-WASP^{-/-}(N-WASP^{Y256E}) (30±2.5) cells displayed lesser number of vinculin patches that were found to be similar to the number observed in the N-WASP^{-/-}(YFP) cells (30±2.66). While the N-WASP^{Y256F} in the N-WASP^{-/-}(N-WASP^{Y256F}) cells did not inhibit the vinculin localization as compared to the N-WASP^{-/-}(N-WASP) cells. The phospho-mimicking mutant Y256E does inhibit the localization of vinculin patches, which explains for the decreased cell adhesion observed in the N-WASP^{-/-}(N-WASP^{Y256E}) cells. Due to the open conformation of N-WASP^{Y256E} there are interactions with other proteins that are now possible due to the unmasked domains which may lead to an inhibition of vinculin localization which results in reduced cell-ECM adhesion.

The N-WASP^{-/-}(N-WASP^{Y256E}) cell line did not inhibit cell migration as compared to the N-WASP^{-/-}(N-WASP) cells (Sections 3.27 and 3.28). The N-WASP^{Y256E} expressed in the N-WASP^{-/-} cells leads to an open conformation of N-WASP. The open conformation of N-WASP causes SH2 domain containing proteins to now bind at the Y256 site, the verprolins bind to the WH1 domain and the VCA domain is now exposed. In contrast, the N-WASP^{Y256F} expressed in the N-WASP^{-/-} cells leads to a closed conformation, which prevents the SH2 domain containing proteins from binding to the Y256 site and inhibition of cell migration is observed. The N-WASP^{-/-}(N-WASP^{Y256F}) showed an inhibition of the localization of paxillin as the number of patches was found to be low and comparable to that displayed by

the N-WASP^{-/-}(N-WASP) cells (15±2.22). In contrast, the N-WASP^{-/-}(N-WASP^{Y256E}) cells displayed number of paxillin patches comparable to the N-WASP^{-/-}(YFP) (38±1) cells. The expression of the N-WASP^{Y256E} mutant disrupts the auto-inhibited structure of N-WASP hence allowing for SH3 domain binding proteins to bind to the site leading to stabilized Arp2/3 complex activation (Kim et al., 2000). The N-WASP^{Y256E} in does not inhibit paxillin localization, the higher number of patches observed promote turnover of focal complexes and hence cell migration.

5.11 Knockdown and overexpression of N-WASP leads to a redistribution of vinculin and paxillin patches in the metastatic and non-tumorigenic epithelial cells

Knockout studies on paxillin suggest that paxillin^{-/-} fibroblast have delayed turnover of focal adhesions resulting in diminished integrin mediated cell migration (Webb et al., 2004). In contrast, vinculin^{-/-} cells displayed rapid turnover of focal adhesions, which in turn enhanced cell migration (Coll et al., 1995; Xu et al., 1998). Vinculin act as a negative regulator of cell migration because vinculin null cells have reduced number of FA and also display high migration in wound healing and transwell assays. Conversely, overexpression of vinculin enhances the size and number of FA and reduces cell migration (Rodriguez et al., 1993).

Knockout studies on vinculin and paxillin are consistent with the results presented in this study. The HaCaT^{N-WASP KD} cells displayed significantly lower number of vinculin patches in comparison to the HaCaT^{CTR} cells (Section 3.8). The A5-RT3^{N-WASP KD} cells also showed a reduced number of vinculin patches in comparison to its control, A5-RT3^{CTR} (Section 3.20). The N-WASP^{-/-} mouse embryonic fibroblasts with no N-WASP expression had significantly lesser number of vinculin patches as compared to N-WASP^{+/+} cells (Section 3.29). The reduced vinculin patches explain the decreased cell-ECM adhesion on fibronectin observed in the N-WASP deficient cells (HaCaT^{N-WASP KD}, A5-RT3^{N-WASP KD}, HSC-5^{CTR}) and in the complete absence of N-WASP observed in the N-WASP^{-/-} mouse embryonic

fibroblasts in comparison to their controls with N-WASP expression (HaCaT^{CTR}, A5-RT3^{CTR}, HSC-5^{N-WASP} and N-WASP^{+/+} MEFs).

Difference was also observed in the paxillin localization in the HaCaT^{N-WASP KD} cells as compared to the HaCaT^{CTR} cells as the former showed greater number of patches. A5-RT3^{N-WASP KD} also displayed greater number of patches in comparison to the A5-RT3^{CTR} cells thus suggesting that deficiency of N-WASP leads to a decrease in the number of paxillin patches. N-WASP^{-/-} mouse embryonic fibroblasts showed more paxillin patches in comparison to the N-WASP^{+/+} MEFs. Overexpression of N-WASP in HSC-5 cells shows a decrease in the number of paxillin patches in comparison to the HSC-5^{CTR} cells. Thus the above results suggest that N-WASP inhibits paxillin localization and that the enhanced migration observed in the wound-healing assay is due to deregulation of paxillin dynamics.

The N-WASP^{+/+} mouse embryonic fibroblasts were observed to show a greater number of vinculin patches in comparison with the N-WASP^{-/-} MEFs. The expression of the mutants, N-WASP^{ΔWH1} and the N-WASP^{Y256E} in the N-WASP^{-/-} failed to restore the cell adhesion and cell-spreading defect observed in the N-WASP^{-/-} cells. When probed for vinculin, the cells also displayed reduced number of vinculin patches per cell in the N-WASP^{-/-}(N-WASP^{ΔWH1}) and the N-WASP^{-/-}(N-WASP^{Y256E}) cell lines as compared to the N-WASP^{-/-}(N-WASP) cells. In addition to the decrease in the number of vinculin patches, an increase in the number of paxillin patches is observed, which, explains the increase in cell migration. The reduced number of vinculin patches thus may account for the reduced cell-ECM adhesion. The N-WASP^{-/-}(N-WASP^{ΔVCA}) cells displayed lesser number of paxillin patches and higher number of vinculin patches which were found to be comparable with that observed in the N-WASP^{+/+} cells. The N-WASP^{-/-}(N-WASP^{ΔV}) and N-WASP^{-/-}(N-WASP^{ΔC19}) cell lines were stained for vinculin and paxillin patches. The results observed showed no changes in the number of vinculin and paxillin patches in comparison with the N-WASP^{+/+} cell line. N-WASP hence regulates the focal adhesion turnover through changes in the localization of vinculin and paxillin.

During membrane protrusion, the Arp2/3 complex binds transiently to vinculin in response to PI3K and Rac1 (DeMali et al., 2002). Another study suggests that in breast cancer cells N-WASP interacts with integrin $\beta 1$ subunit (Sturge et al., 2002). More importantly $\alpha 5 \beta 1$ supports adhesion of migrating cells to fibronectin and also critically involved in the assembly of focal adhesions (Zamir et al., 2000). Thus several experiments in this study suggest an interesting possibility of a direct correlation between integrin signaling and regulation of focal adhesion dynamics mediated by integrin $\beta 1$ subunit, N-WASP, Arp2/3 complex and vinculin. N-WASP may regulate integrin avidity and affinity through the recruitment of vinculin to the cytoplasmic domain of $\beta 1$ integrin via Arp2/3 complex dependent actin polymerization. The data shows that loss of N-WASP results in reduced number of vinculin patches and decreased clustering of the integrins, which results in decreased cell adhesion. Thus N-WASP is required for cell adhesion probably because it is involved in integrin clustering and assembly of vinculin at focal adhesions. These results are in accordance with the observation that WASP is important for adhesion of T-cells to ICAM-1 (Zhang et al., 2006).

5.12 N-WASP knockdown results in reduced cell-cell contact

Cell migration is essential for both normal development as well as metastasis (Thiery, 2002). Metastasis is a process with a multitude of steps, involving the destruction of basement membrane and the invasion of cells into a primary site, intravasation and survival in the circulatory system, cell migration to other sites and survival plus cell proliferation at the secondary site (Pantel and Brakenhoff, 2004). Most of the human cancers that originate from epithelial undergo epithelial to mesenchymal transitions (EMT) before metastasis (Thiery, 2002). A large number of cancer cells die due to their inability to adapt; the cells undergo adaptations, which include changes in the signaling cascade and gene expression, which promote evasion of apoptosis and establishment of metastases (Zhou et al., 2006). The HaCaT^{N-WASP KD} and A5-RT3^{N-WASP KD} cells showed reduced localization of E-cadherin at the junctions as compared to the HaCaT

and the A5-RT3 cells due to reduced expression of E-cadherin as a result of the knockdown of N-WASP in epithelial cells. In addition the cell lines, HaCaT^{N-WASP KD} and A5-RT3^{N-WASP KD} showed reduced expression of E-cadherin by western blot (Sections 3.18 and 3.23). The localization of E-cadherin at the junctions and the expression of E-cadherin were found to show an increase on overexpressing N-WASP in HSC-5 cells (Section 3.29). This indicates that N-WASP acts as a metastatic suppressor.

5.13 The induction of filopodia independent of WASP family of proteins

N-WASP has been proposed to generate filopodia formation as microinjection of N-WASP in COS7 cells enhanced filopodia formation (Mikki et al. 1998). Microinjection of polyclonal antibodies against N-WASP inhibited the induction of filopodia further confirming a role for N-WASP in the induction of filopodia (Martinez Quiles et al., 2001). Subsequently, Lommel et al (2001) found that microinjection of constitutively active Cdc42 can generate filopodia in N-WASP deleted MEFs. This suggests an intriguing possibility that in the absence of N-WASP, an alternative pathway could lead to the induction of filopodia in fibroblast cells.

One of the major proteins involved in contributing to actin polymerization include the formins, in particular mDia proteins (Mellor, 2010; Campellone and Welch, 2010). VASP, Mena and Evl (known as Ena/VASP proteins) have been reported to localize at the tips of filopodial extensions (Bear and Gertler, 2009). The I-BAR domain containing proteins such as IRSp53 are capable of generating filopodia along with N-WASP or Mena (Kim et al., 2008; Chesarone and Goode, 2009). Myosin-X is another protein that facilitates the generation of filopodia by driving the delivery of actin monomers to promote filament elongation of filopodial tips. The other mechanism by which Myosin-X promotes motor function could induce actin filament convergence at the leading edge to initiate filopodium extension (Tokuo et al., 2007). One of the extensively studied Rho GTPases is Cdc42, which induces

filopodia through each of these three proteins-IRSp53, mDia2 and N-WASP. In addition to Cdc42, RhoF/Rif generate filopodia via mDia2 in neurons (Hotulainen et al., 2009).

5.14 CR16 enhances the induction of filopodia by IRSp53

The WASP family of proteins (WASP, N-WASP, WAVE1, WAVE2 and WAVE3) influence the actin cytoskeleton by of the Arp2/3 complex activation through the VCA domain, which is located at the C-terminal of these proteins (Takenawa et al., 2007). Cdc42 is required for the activation of Arp2/3 complex by N-WASP, which leads to the generation of filopodia (Miki et al., 1998; Svitkina et al., 2003). N-WASP binds to the verprolin family of proteins (WIP, WIRE and CR16) and it was postulated that WIP maintains N-WASP in the folded conformation (Martinez-Quiles et al., 2001). In cultured fibroblasts overexpression of WIRE itself is sufficient to induce the formation of thick actin fibers (Kato et al., 2005) as well as to induce thick bundles of actin filaments as well as ruffles like projections at the membrane periphery (Aspenstrom, 2004; Kato et al., 2002). Despite the similarity of domain structure between CR16 and WIP, WIP was not found to bind IRSp53 indicating differences in the way the individual verprolins influence the actin cytoskeleton. Studies on WIP indicate that induction of filopodia by WIP is N-WASP dependent (Martinez-Quiles et al., 2001). Moreover, in PDGF treated NIH 3T3 cells WIP overexpression enhanced ruffle formation and loss of WIP reduces ruffle formation in response to PDGF treatment (Anton et al., 2003; Kinley et al., 2003) These observations clearly suggest that WIP and CR16 behave differently to regulate actin organization. These studies raise the possibility that CR16 could generate filopodia through different complex while WIP generates filopodia in conjunction with N-WASP. Little is known about regulatory proteins that interact with CR16 to mediate remodeling of the actin cytoskeleton. In order to characterize the functions of CR16 independent of N-WASP, interactors of CR16 were screened and IRSp53 was identified as a novel CR16 interacting protein. CR16 interacts with IRSp53 to generate filopodia in N-WASP^{-/-} MEF cells while WIP and IRSp53 failed to generate filopodia

in conjunction, which suggests that the complex of CR16-IRSp53 generate filopodia independent of N-WASP. WIP and WIRE have been reported to generate filopodia in conjunction with N-WASP (Martinez-Quiles et al., 2001; Aspenstrom et al., 2002). It was shown that N-WASP induced filopodia via IRSp53 (Lim et al., 2008) while WIRE can generate filopodia in addition with IRSp53 in a manner independent of N-WASP (Misra et al., 2010). SH3 domain of IRSp53 was shown to interact with N-WASP directly and IRSp53 failed to generate filopodia in N-WASP^{-/-} mouse embryonic fibroblasts suggesting that IRSp53 could generate filopodia in conjunction with N-WASP (Lim et al., 2008). It has been shown that CR16 and WIP interact with N-WASP and N-WASP in turn interacts with IRSp53 (Lim et al., 2008), however an interaction between CR16 and N-WASP is not essential for the induction of filopodia by CR16 in N-WASP^{-/-} cells (Fig. 4.4). Hence, it is possible that CR16-IRSp53 induce the generation of filopodia in a manner independent of N-WASP, while WIP induces filopodia by binding to N-WASP (Lim et al., 2008; Martinez-Quiles et al., 2001). It has not been determined if WIP forms a WIP/ N-WASP/IRSp53 complex to generate filopodia.

5.15 The I-BAR domain of IRSp53 is important for filopodia dynamics

The three IRSp53 domains (I-BAR, CRIB, SH3) have been implicated in the induction of filopodia (Govind et al., 2001; Krugmann et al., 2001; Millard et al., 2005; Misra et al., 2010). BAR domain containing proteins such as Toca-1, CIP4, FBP17 couples membrane curvature to actin dynamics through proteins such as N-WASP to perform endocytosis and membrane tubulation. Toca-1, CIP4 and FBP17 share similar domains with IRSp53 such as SH3 domain and Cdc42 binding site (Ho et al., 2004). These proteins contain a Cdc42 binding domain, which are a Cdc42 binding site adjacent to BAR domain and a SH3 binding domain. Toca-1 binds with Cdc42 as well as with N-WASP and induces filopodia in a Cdc42 dependent manner (Bu et al., 2010; Ho et al., 2004). Thus these proteins could have unique ability to couple the membrane curvature with actin polymerization under the control of Cdc42. The positive

curvature formation which is required for vesicle budding into the cell is induced by the F-BAR domain while the negative curvatures required for outward membrane protrusions and F-actin bundling is generated by the I-BAR domain (Lee et al., 2007; Millard et al., 2005). Structural and functional studies on I-BAR domain of IRSp53 revealed that it generates membrane protrusions rather than membrane invagination. In previous reports it was suggested that I-BAR domain of IRSp53 induced filopodia in COS7 cells (Millard et al., 2005).

5.16 Interaction between SH3 domain of IRSp53 and Verprolin homology domain of CR16 is critical for the induction of filopodia

Proline-rich proteins interact with SH3 domain containing proteins to mediate various actin-based structures such as endocytosis and actin remodeling. The SH3 domain of IRSp53 is critical for the induction of filopodia and was shown to interact with N-WASP, WAVE2, Mena/VASP, Eps8 and mDia (Krugmann et al., 2001). While I-BAR domain of IRSp53 binds to the membrane and actin filaments, SH3 domain acts as an adaptor that binds with the protein complex to bring them to the plasma membrane. SH3 domain of IRSp53 binds with proline-rich stretch of Mena/VASP and synergistically promotes filopodia formation. Similarly, SH3 domain of IRSp53 has also been shown to interact with proline rich region of N-WASP and generate filopodia (Lim et al., 2008). Localization of IRSp53 at the filopodia tip could be permissible for further elongation. Involvement of IRSp53 SH3 domain in multiple signaling pathways suggests that CR16, WIRE, Mena, N-WASP, WAVE and/or mDia could compete for the binding with IRSp53. While the SH3 domain of IRSp53 is involved in protein-protein interactions, it also contributes in induction of filopodia in conjunction with CR16 in a manner independent of N-WASP. In the previous studies it was found that IRSp53 function depends on SH3 domain as IRSp53^{2A} mutation reduced binding with N-WASP and Mena. Thus to investigate the role of SH3 domain of IRSp53, IRSp53^{2A} mutation was made and compared with the phenotype of wild-type IRSp53. It was

found that 73% cells induced filopodia in wild-type IRSp53 transfected cells while IRSp53^{2A} induced filopodia only in 10% cells, which is in accordance with the pull down assay with IRSp53^{2A} showed that mutation in SH3 domain caused the interaction with CR16 to be abolished. These results are in corroboration with previous findings where mutation in SH3 domain of IRSp53 caused the interaction with N-WASP, Mena and WIRE to be abolished hence confirmed that SH3 domain of IRSp53 performs a significant role in the induction of filopodia. The binding of IRSp53 to the V domain will bring the actin-binding motif in V domain to the plasma membrane.

All the verprolins contain Verprolin homology domain (V) at N-terminus and a WBD (WASP binding domain) at C-terminus (Kato et al., 2002; Anton et al., 2006). While WBD domain is required for interaction with WASP family of proteins, the V domain mediates interaction with G-actin, which is critical for the function of verprolins. V domain is rich in proline residues, which may promote the interaction with IRSp53. Thus it was hypothesized that V domain of CR16 could mediate interaction with SH3 domain of IRSp53. The results suggest that deletion of V domain of CR16 leads to loss of interaction with IRSp53. Thus, to analyze further whether deletion of V domain could affect the ability of CR16-IRSp53 to generate filopodia independent of N-WASP, V domain was deleted in CR16 and the induction of filopodia was analyzed. Results suggest that deletion of V domain from CR16 significantly reduced ability of CR16-IRSp53 complex to generate filopodia in N-WASP^{-/-} cells suggesting that V domain of CR16 is crucial for filopodia formation in conjunction with IRSp53, suggesting that the SH3 domain of IRSp53 binds with the V domain of CR16 and this interaction is crucial for filopodia formation. Taken together with the data on CR16 Δ V, these results suggest that the SH3 domain of IRSp53 binds with the V domain of CR16 and is required for CR16-IRSp53 mediated filopodia formation in a manner independent of N-WASP.

5.17 Cdc42 is critical for CR16-IRSp53 complex formation and filopodia formation.

The CRIB domain mutant of IRSp53 led to reduced the induction of filopodia by CR16-IRSp53 which is in accordance with the published data (Lim et al., 2008, Misra et al., 2010) and it also led to decreased CR16-IRSp53 interaction. This indicates that the binding of Cdc42 to the CRIB domain is crucial for facilitating the CR16-IRSp53 interaction. This was shown in the pull down assay experiment where IRSp53 was found to interact with CR16 in the presence of Cdc42^{G12V} but not in the presence of Cdc42^{T17N} (Section 4.6). Cdc42^{G12V} is a constitutively active mutant in the GTP bound state and the Cdc42^{T17N} is a dominant-negative mutant, which is in a GDP bound state. Likewise on knocking down the Cdc42 expression using a shRNA, there was no CR16-IRSp53 interaction observed (Section 4.8). This indicates that binding of Cdc42 to the CRIB domain of IRSp53 is important for the change in conformation of IRSp53, which promotes the binding of IRSp53 to the V domain of CR16. It has been previously reported that CR16 is involved in inducing filopodia in N-WASP depleted cells unlike WIP which requires N-WASP to generate filopodia (Martinez-Quiles et al., 2001). All the three domains of IRSp53 have been reported to be important in inducing filopodia through N-WASP (Lim et al., 2008) and these domains of IRSp53 are also crucial for generating filopodia conjunction CR16 and IRSp53. The interaction between CR16 and IRSp53 is found to be via the SH3 domain of IRSp53 and the V domain present in the CR16. This interaction is important as it leads to the generation of filopodia. This CR16-IRSp53 interaction is facilitated and regulated by the binding of Cdc42 to IRSp53 at the CRIB domain.

Conclusion

The ubiquitously expressed N-WASP is involved in integrating signal from cell surface to the actin cytoskeleton. The results from this thesis suggest that N-WASP may have dual roles during cancer. N-WASP may have dual effects on cancer progression. During the early phase of oncogenesis, N-WASP reduces cell migration, increases proliferation and possibly helps to maintain cell-cell adhesion and thus suppresses metastasis. However, at the later stage of cancer progression, cells undergo EMT, thus cancer cells become highly invasive where the N-WASP expression is enhanced. During metastasis, N-WASP may promote invasion by promoting invadopodia formation and act as a metastasis promoter. Thus, N-WASP may act as either tumor suppressor or enhancer depending on the type of cancer and its pathological stage. In this thesis the function of N-WASP in non-tumorigenic HaCaT, tumorigenic and metastatic A5-RT3 and tumorigenic and non-metastatic HSC-5 was studied. HaCaT^{N-WASP KD} and A5-RT3^{N-WASP KD} cell lines were used to study changes in cell-ECM adhesion and migration characteristics and compared to their control cell lines (HaCaT^{CTR} and A5-RT3^{CTR}). The assays showed that N-WASP is required for cell adhesion and knockdown of the expression of the protein leads to a cell-ECM adhesion defect (Fig. 6.1). Overexpression of N-WASP in HSC-5 cells leads to increased cell-ECM binding and spreading. The knockdown of N-WASP in HaCaT leads to a reduction in the E-cadherin expression hence promoting EMT. However, to further validate role of N-WASP as a tumor suppressor or enhancer, further characterization of N-WASP in the different skin malignancies and in the different stages of cancer is needed. In addition an *in vivo* study of the role of N-WASP would provide a confirmation of the changes in the N-WASP expression in the different stages of cancer.

In this study, function of N-WASP domains was also studied in detail by using N-WASP knockout fibroblast cells. Using N-WASP^{-/-} and N-WASP^{+/+} MEFs, it was demonstrated that the N-WASP performs a significant role in fibroblast cell adhesion and migration. Loss of N-WASP gene leads to higher cell migration and reduced cell adhesion to fibronectin. Moreover, N-WASP may affect vinculin and paxillin patch assembly. In addition the different domains of N-WASP were studied for their role on

cell adhesion and migration. The adhesion and migration assays as well as immunostainings of paxillin and vinculin showed that the WH1 domain is critical for cell-ECM adhesion. The WH1 domain probably inhibits cell migration by promoting cell-ECM adhesion. The expressed phosphomimic mutant N-WASP^{Y256E} was not able to rescue the cell adhesion defect of N-WASP^{-/-} cells suggesting that phosphorylating N-WASP promotes cell migration by reducing cell-ECM adhesion (Fig. 6.2, 6.3 and 6.4).

N-WASP is known to generate filopodia formation however it is not clear whether N-WASP alone is sufficient to generate filopodia or it works in conjunction with verprolin family members. Thus in order to characterize verprolin family member CR16, a yeast two hybrid assay was carried out and IRSp53 was identified as a CR16 interacting protein. Expression of IRSp53 with CR16 in N-WASP^{-/-} mouse embryonic fibroblast cells (MEFs) induced filopodia. Induction of filopodia generation requires CR16-IRSp53 interaction as mutation in the SH3 domain of IRSp53 abolishes CR16-IRSp53 interaction and hence the ability to induce filopodia. Likewise, the Verprolin (V)-domain of CR16 is critical for IRSp53-CR16 interaction and for filopodia formation. Cdc42 regulates the interaction between CR16 and IRSp53 as the mutation, which caused the induction of filopodia by the CR16-IRSp53 to be abolished also caused for the Cdc42- IRSp53 binding to be abolished, as shown by pull down assay. The similar role of WIRE has been reported where WIRE-IRSp53 interaction is regulated by cdc42 and the complex is localized at the plasma membrane to induce filopodia (Misra et al., 2010). The expression of CR16-IRSp53 along with Cdc42^{G12V} (active mutant) led to a significant increase in the induction of filopodia. Thus the results show that Cdc42 regulates the activity of IRSp53 by controlling the IRSp53-CR16 interaction in generating filopodia (Fig. 6.5).

A.

B.

Figure 6.1: N-WASP inhibits cell migration and promotes cell adhesion. A. Knockdown of N-WASP expression leads to increase in cell migration and decrease in cell-ECM adhesion. B. Loss of N-WASP in fibroblasts leads to increase in cell migration and decrease in cell-ECM adhesion.

A.

B.

C.

D.

Figure 6.2: Schematic representation of the function of the N-WASP domains critical for cell adhesion and migration. A. N-WASP expressed in N-WASP^{-/-} cells rescues cell adhesion defect but inhibits cell migration. B. YFP expressed in the N-WASP^{-/-} cells leads to a cell adhesion defect but does not inhibit cell migration. C. Expression of N-WASP^{ΔWH1} in the N-WASP^{-/-} cells restores the cell adhesion defect but does not inhibit cell migration. Hence the WH1 domain plays an inhibitory role in cell migration. D. Expression of N-WASP^{ΔVCA} in the N-WASP^{-/-} cells restores the cell adhesion defect and it does not inhibit cell migration. Hence the VCA domain does not inhibit cell migration.

E.

F.

Figure 6.3: Schematic representation of the function of the N-WASP domains critical for cell adhesion and migration. E. N-WASP^{ΔC19} expression in N-WASP^{-/-} cells restores cell adhesion and does not inhibit cell migration. F. N-WASP^{ΔV} expression in N-WASP^{-/-} cells does not restore cell adhesion defect and does not inhibit cell migration.

G.

H.

Figure 6.4: Schematic representation of the function of the N-WASP domains critical for cell adhesion and migration. E. N-WASP^{ΔC19} expression in N-WASP^{-/-} cells restores cell adhesion and does not inhibit cell migration. F. N-WASP^{ΔV} expression in N-WASP^{-/-} cells does not restore cell adhesion defect and does not inhibit cell migration. G. Cells expressing the mutant, N-WASP^{Y256E} lead to an open conformation of N-WASP, which leads to cell adhesion defect and no cell migration defect. H. The cells expressing the N-WASP^{Y256F} mutant maintains N-WASP in a closed conformation, which leads to no cell adhesion defect but leads to a cell migration defect.

Figure 6.5: Model of CR16-IRSp53 mediated filopodia formation. 1. Activated Cdc42 recruits IRSp53 to the plasma membrane. Usually IRSp53 remains in close conformation where the SH3 domain is masked 2. Binding of activated Cdc42 with CRIB domain may induce conformational change in IRSp53. 3. Due to the conformational change induced by activated Cdc42, SH3 domain may be exposed to bind with CR16 or WIRE. Cdc42 along with CR16 or WIRE recruits IRSp53 to the plasma membrane and induces filopodia.

Future directions

Expression of N-WASP was found to be down regulated in all the 33 human squamous cell carcinoma (SCC) samples analyzed. Expression of N-WASP was also found to vary in different epithelial cell lines representing the different stages of cancer. As N-WASP expression was found to be higher in the liver metastases of breast cancers (Martin et al., 2012). Also, expression of N-WASP was higher in the later stages (TNM2) of colon cancer as compared to early stages (TNM1) (Yanagawa et al., 2001). Hence to better understand the role of N-WASP at different stages of tumor progression, patient samples obtained from a single patient at progressive stages of cancer should be checked for expression of N-WASP

A mouse model with SCC could be developed to study the expression of N-WASP and function at different stages of tumor progression to obtain a more comprehensive picture of the role of N-WASP in carcinoma development and progression. Epithelial cell lines with over-expression of N-WASP and knockdown of N-WASP could be used to inject into nude mice to check for development of tumors and metastases of skin, which would confirm the invasive potential of the cells. These models could then be studied over a period of time to observe for the development of the tumor.

In order to determine the role of N-WASP expression in metastasis or in the development of invasiveness, the expression of mesenchymal markers should be checked in cells expressing reduced expression of N-WASP and cells overexpressing N-WASP. Decreased expression of the proteins such as Cytokeratin, Occludin, mucin-1 and E-cadherin while increased expression of vimentin, N-cadherin, vitronectin, thrombospondin and cadherin 11 are associated with the EMT process.

A number of signaling pathways (Wnt signaling, Smad signaling, FAK signaling and TGF- β pathways), have been implicated in skin cancers. Assessing the expression of these genes by qPCR in the tumorigenic epithelial cell lines with altered expression of N-WASP will lead to better understanding of the changes in signaling pathways critical for carcinoma development and progression to metastatic cancers.

It has been seen that N-WASP knockdown in epithelial cell lines lead to reduced cell migration. However cells in vivo behave differently due to the difference between the 3D microenvironment in the animal compared to the artificial 2D environment in tissue culture flask. Hence a 3D cell migration assay of the N-WASP knocked down cells would be closer to the real scenario.

Angiogenesis plays an important role in the growth of tumors as the newly formed blood vessels brings nutrients for the growth of tumors. Role of N-WASP in angiogenesis has not been studied. The nucleation promoting factor, WASP and actin modulatory protein, filaminA have been implicated in promoting angiogenesis through endothelial cell migration (Verissimo et al., 2009). Hence the role of N-WASP in endothelial cell migration can be studied to further our understanding of the role of N-WASP in later stages of tumor progression. Mice models with late stages of SCC can be investigated for angiogenesis markers such as vascular endothelial growth factor-A (VEGFA), placental growth factor (PIGF) and angiopoietin-1 (ANG1) (Hattori et al., 2001; Hattori et al., 2002).

The domain deletions and point mutations in N-WASP when expressed in the N-WASP^{-/-} cells led to the identification of the WH1 domain as being important in cell adhesion. These domain deletions and point mutations in N-WASP can be used to identify the role of the domain in promoting tumorigenicity or suppressing tumor formation, which could be of therapeutic potential. Invasiveness of the epithelial cell lines with reduced and increased expression of N-WASP can be further characterized for invadopodia formation. In addition the deletion and point mutation constructs of N-WASP can be expressed in these epithelial cell lines to further study the role of the domains in promoting invasiveness through invadopodia formation.

The phosphorylation of N-WASP at Tyr256 is mediated by FAK (Wu et al., 2004). The FAK pathway through N-WASP has been implicated in the development of tumorigenesis (Lefever et al., 2009). In this study it was found that N-WASP^{-/-}(N-WASP^{Y256E}) cells had poor cell-ECM adhesion compared to N-WASP^{-/-}(N-WASP) cells and migrated faster than N-WASP^{-/-}(N-WASP). The phosphomimicking mutant

lends a negative charge to the Y256 site upon phosphorylation, which leads to an open conformation of N-WASP. This open conformation allows for the binding of other SH2 domain containing proteins at the Y256 site, actin monomers and hence the Arp2/3 complex activation by the now unmasked VCA domain and the verprolins at the WH1 domain. However which of the three domains lead to increased cell migration is not determined. Hence mutants of N-WASP, N-WASP^{Y256EΔWH1} and N-WASP^{Y256EΔVCA}, N-WASP^{Y256FΔWH1} and N-WASP^{Y256FΔVCA} can be expressed along with the controls, N-WASP^{-/-}(N-WASP^{Y256E}), N-WASP^{-/-}(N-WASP^{Y256F}), N-WASP^{-/-}(N-WASP), N-WASP^{-/-}(N-WASP^{YFP}) compared to identify the domain facilitating the cell migration in this case.

N-WASP is an actin-associated protein which regulates the actin cytoskeleton by regulating actin nucleation activity of the Arp2/3 complex and regulates transcription by RNA polymerase II (Wu et al., 2006). Both its role in regulating actin cytoskeleton and transcription is likely to affect cancer progression at various stages of cancer. Thus it is critical to decipher the role of N-WASP in the various stages of cancer to develop effective therapeutic strategies.

References

- Adams, C.L., Y.T. Chen, S.J. Smith, and W.J. Nelson.** 1998. Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *The Journal of cell biology*. 142:1105-1119.
- Adams, C.L., W.J. Nelson, and S.J. Smith.** 1996. Quantitative analysis of cadherin-catenin-actin reorganization during development of cell-cell adhesion. *The Journal of cell biology*. 135:1899-1911.
- Aitken, J., J. Welch, D. Duffy, A. Milligan, A. Green, N. Martin, and N. Hayward.** 1999. CDKN2A variants in a population-based sample of Queensland families with melanoma. *Journal of the National Cancer Institute*. 91:446-452.
- Alexandrova AY, Arnold K, Schaub S, Vasiliev JM, Meister JJ, Bershadsky AD, Verkhovsky AB.** 2008. Comparative dynamics of retrograde actin flow and focal adhesions: Formation of nascent adhesions triggers transition from fast to slow flow. *PLoS One* 3: 3234.
- Chesarone, M., and B.L. Goode.** 2009. Actin nucleation and elongation factors: mechanisms and interplay. *Curr. Opin. Cell Biol.* 21:28-37.
- Aronheim, A., Y.C. Broder, A. Cohen, A. Fritsch, B. Belisle, and A. Abo.** 1998. Chp, a homologue of the GTPase Cdc42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton. *Current Biology*. 8:1125-1128.
- Alonso, L., and E. Fuchs.** 2003. Stem cells of the skin epithelium. *Proceedings of the National Academy of Sciences of the United States of America*. 100 Suppl 1:11830-11835.
- Aspenstrom, P., A. Ruusala, and D. Pacholsky.** 2007. Taking Rho GTPases to the next level: the cellular functions of atypical Rho GTPases. *Experimental cell research*. 313:3673-3679.
- Ayala, I., M. Baldassarre, G. Caldieri, and R. Buccione.** 2006. Invadopodia: a guided tour. *European journal of cell biology*. 85:159-164.
- Ayollo, D.V., I.Y. Zhitnyak, J.M. Vasiliev, and N.A. Gloushankova.** 2009. Rearrangements of the actin cytoskeleton and E-cadherin-based adherens junctions caused by neoplastic transformation x

- Azuma, K., M. Tanaka, T. Uekita, S. Inoue, J. Yokota, Y. Ouchi, and R. Sakai.** 2005. Tyrosine phosphorylation of paxillin affects the metastatic potential of human osteosarcoma. *Oncogene*. 24:4754-4764.
- Balda, M.S., and K. Matter.** 1998. Tight junctions. *Journal of cell science*. 111 (Pt 5):541-547.
- Balda, M.S., and K. Matter.** 2009. Tight junctions and the regulation of gene expression. *Biochimica et biophysica acta*. 1788:761-767.
- Baum, B., and M. Georgiou.** 2011. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *Journal of Cell Biology*. 192:907-917.
- Baum, B., J. Settleman, and M.P. Quinlan.** 2008. Transitions between epithelial and mesenchymal states in development and disease. *Seminars in cell & developmental biology*. 19:294-308.
- Beckerle, M.C., K. Burridge, G.N. DeMartino, and D.E. Croall.** 1987. Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell*. 51:569-577.
- Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M.M. Mareel, and W. Birchmeier.** 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *The Journal of cell biology*. 120:757-766.
- Benitah, S.A., P.F. Valeron, L. van Aelst, C.J. Marshall, and J.C. Lacal.** 2004. Rho GTPases in human cancer: an unresolved link to upstream and downstream transcriptional regulation. *Biochimica Et Biophysica Acta-Reviews on Cancer*. 1705:121-132.
- Bershadsky, A.** 2004. Magic touch: how does cell-cell adhesion trigger actin assembly? *Trends in cell biology*. 14:589-593.
- Benninger, Y., T. Thurnherr, J.A. Pereira, S. Krause, X. Wu, A. Chrostek-Grashoff, D. Herzog, K.A. Nave, R.J. Franklin, D. Meijer, C. Brakebusch, U. Suter, and J.B. Relvas.** 2007. Essential and distinct roles for cdc42 and rac1 in the regulation of Schwann cell biology during peripheral nervous system development. *The Journal of cell biology*. 177:1051-1061.

- Birchmeier, W., and J. Behrens.** 1994. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochimica et biophysica acta*. 1198:11-26.
- Bowden, E.T., M. Barth, D. Thomas, R.I. Glazer, and S.C. Mueller.** 1999. An invasion-related complex of cortactin, paxillin and PKC mu associates with invadopodia at sites of extracellular matrix degradation. *Oncogene*. 18:4440-4449.
- Bowden, E.T., E. Onikoyi, R. Slack, A. Myoui, T. Yoneda, K.M. Yamada, and S.C. Mueller.** 2006. Co-localization of cortactin and phosphotyrosine identifies active invadopodia in human breast cancer cells. *Experimental cell research*. 312:1240-1253.
- Burridge, K., and M. Chrzanowska-Wodnicka.** 1996. Focal adhesions, contractility, and signaling. *Annual review of cell and developmental biology*. 12:463-518.
- Burridge, K., K. Fath, T. Kelly, G. Nuckolls, and C. Turner.** 1988. Focal Adhesions - Transmembrane Junctions between the Extracellular-Matrix and the Cytoskeleton. *Annual Review of Cell Biology*. 4:487-525.
- Bandarchi, B., L. Ma, R. Navab, A. Seth, and G. Rasty.** 2010. From melanocyte to metastatic malignant melanoma. *Dermatology research and practice*. 2010.
- Bataille, V.** 2000. Genetics of familial and sporadic melanoma. *Clinical and experimental dermatology*. 25:464-470.
- Bataille, V.** 2003. Genetic epidemiology of melanoma. *European journal of cancer*. 39:1341-1347.
- Bellovin, D.I., K.J. Simpson, T. Danilov, E. Maynard, D.L. Rimm, P. Oettgen, and A.M. Mercurio.** 2006. Reciprocal regulation of RhoA and RhoC characterizes the EMT and identifies RhoC as a prognostic marker of colon carcinoma. *Oncogene*. 25:6959-6967.
- Bis, S., and H. Tsao.** 2013. Melanoma genetics: the other side. *Clinics in dermatology*. 31:148-155.
- Braga, V.M., and A.S. Yap.** 2005. The challenges of abundance: epithelial junctions and small GTPase signalling. *Current opinion in cell biology*. 17:466-474.

- Brash, D.E., J.A. Rudolph, J.A. Simon, A. Lin, G.J. McKenna, H.P. Baden, A.J. Halperin, and J. Ponten.** 1991. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proceedings of the National Academy of Sciences of the United States of America*. 88:10124-10128.
- Carlier, M.F.** 1998. Control of actin dynamics. *Current opinion in cell biology*. 10:45-51.
- Caron, E.** 2003. Regulation by phosphorylation. Yet another twist in the WASP story. *Developmental Cell*. 4:772-773.
- Chamaraux, F., S. Fache, F. Bruckert, and B. Fourcade.** 2005. Kinetics of cell spreading. *Physical review letters*. 94:158102.
- Chen, W.T.** 1989. Proteolytic Activity of Specialized Surface Protrusions Formed at Rosette Contact Sites of Transformed-Cells. *Journal of Experimental Zoology*. 251:167-185.
- Cuvelier, D., M. Thery, Y.S. Chu, S. Dufour, J.P. Thiery, M. Bornens, P. Nassoy, and L. Mahadevan.** 2007. The universal dynamics of cell spreading. *Current biology : CB*. 17:694-699.
- De La Cruz, E.M., E.M. Ostap, R.A. Brundage, K.S. Reddy, H.L. Sweeney, and D. Safer.** 2000. Thymosin-beta(4) changes the conformation and dynamics of actin monomers. *Biophysical journal*. 78:2516-2527.
- Derry, J.M., H.D. Ochs, and U. Francke.** 1994. Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell*. 78:635-644.
- Disanza, A., A. Steffen, M. Hertzog, E. Frittoli, K. Rottner, and G. Scita.** 2005. Actin polymerization machinery: the finish line of signaling networks, the starting point of cellular movement. *Cellular and molecular life sciences : CMLS*. 62:955-970.
- Dorsky, R.I., R.T. Moon, and D.W. Raible.** 1998. Control of neural crest cell fate by the Wnt signalling pathway. *Nature*. 396:370-373.

- Dunn, K.J., B.O. Williams, Y. Li, and W.J. Pavan.** 2000. Neural crest-directed gene transfer demonstrates Wnt1 role in melanocyte expansion and differentiation during mouse development. *Proceedings of the National Academy of Sciences of the United States of America*. 97:10050-10055.
- Einspahr, J.G., D.S. Alberts, J.A. Warneke, P. Bozzo, J. Basye, T.M. Grogan, M.A. Nelson, and G.T. Bowden.** 1999. Relationship of p53 mutations to epidermal cell proliferation and apoptosis in human UV-induced skin carcinogenesis. *Neoplasia*. 1:468-475.
- Ezzell, R.M., W.H. Goldman, N. Wang, N. Parashurama, and D.E. Ingber.** 1997. Vinculin promotes cell spreading by mechanically coupling integrins to the cytoskeleton. *Experimental cell research*. 231:14-26.
- Firat-Karalar, E.N., and M.D. Welch.** 2011. New mechanisms and functions of actin nucleation. *Current opinion in cell biology*. 23:4-13.
- Fletcher, D.A., and R.D. Mullins.** 2010. Cell mechanics and the cytoskeleton. *Nature*. 463:485-492.
- Fristrom, D.** 1988. The cellular basis of epithelial morphogenesis. A review. *Tissue & cell*. 20:645-690.
- Fuchs, E., and D.W. Cleveland.** 1998. A structural scaffolding of intermediate filaments in health and disease. *Science*. 279:514-519.
- Gouw, L.G., N.S. Reading, S.D. Jenson, M.S. Lim, and K.S.J. Elenitoba-Johnson.** 2005. Expression of the Rho-family GTPase gene RHOF in lymphocyte subsets and malignant lymphomas. *British Journal of Haematology*. 129:531-533.
- Garrod, D.R.** 1993. Desmosomes and hemidesmosomes. *Current opinion in cell biology*. 5:30-40.
- Geiger, B., and D. Ginsberg.** 1991. The cytoplasmic domain of adherens-type junctions. *Cell motility and the cytoskeleton*. 20:1-6.
- Goldman, W.H., and D.E. Ingber.** 2002. Intact vinculin protein is required for control of cell shape, cell mechanics, and rac-dependent lamellipodia formation. *Biochemical and biophysical research communications*. 290:749-755.

- Goley, E.D., and M.D. Welch.** 2006. The ARP2/3 complex: an actin nucleator comes of age. *Nature reviews. Molecular cell biology*. 7:713-726.
- Gumbiner, B., B. Stevenson, and A. Grimaldi.** 1988a. The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *The Journal of cell biology*. 107:1575-1587.
- Gumbiner, B., B. Stevenson, and A. Grimaldi.** 1988b. The Role of the Cell-Adhesion Molecule Uvomorulin in the Formation and Maintenance of the Epithelial Junctional Complex. *Journal of Cell Biology*. 107:1575-1587.
- Gumbiner, B.M.** 1996. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell*. 84:345-357.
- Hajra, K.M., and E.R. Fearon.** 2002. Cadherin and catenin alterations in human cancer. *Genes, chromosomes & cancer*. 34:255-268.
- Hanahan, D., and R.A. Weinberg.** 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646-674.
- Han, S., H.T. Zhang, Z. Wang, Y. Xie, R. Tang, Y. Mao, and Y. Li.** 2006. DNA repair gene XRCC3 polymorphisms and cancer risk: a meta-analysis of 48 case-control studies. *European journal of human genetics : EJHG*. 14:1136-1144.
- Ho, H.Y., R. Rohatgi, A.M. Lebensohn, M. Le, J. Li, S.P. Gygi, and M.W. Kirschner.** 2004. Toca-1 mediates Cdc42-dependent actin nucleation by activating the N-WASP-WIP complex. *Cell*. 118:203-216.
- Ho, H.Y.H., R. Rohatgi, L. Ma, and M.W. Kirschner.** 2001. CR16 forms a complex with N-WASP in brain and is a novel member of a conserved proline-rich actin-binding protein family. *Proceedings of the National Academy of Sciences of the United States of America*. 98:11306-11311.
- Humphreys, T.R.** 2001. Skin cancer: recognition and management. *Clinical cornerstone*. 4:23-32.

Huttenlocher, A., and A.R. Horwitz. 2011. Integrins in cell migration. *Cold Spring Harbor perspectives in biology*. 3:a005074.

Horwitz, A.R., and J.T. Parsons. 1999. Cell migration--movin' on. *Science*. 286:1102-1103.

Huttenlocher, A., and A.R. Horwitz. 2011. Integrins in cell migration. *Cold Spring Harbor perspectives in biology*. 3:a005074.

Hynes, R.O. 2002. Integrins: Bidirectional, allosteric signaling machines. *Cell*. 110:673-87

Jaffe, A.B., and A. Hall. 2005. Rho GTPases: biochemistry and biology. *Annual review of cell and developmental biology*. 21:247-269.

Jaken, S., K. Leach, and T. Klauck. 1989. Association of type 3 protein kinase C with focal contacts in rat embryo fibroblasts. *The Journal of cell biology*. 109:697-704.

Janes, S.M., and F.M. Watt. 2006. New roles for integrins in squamous-cell carcinoma. *Nature reviews. Cancer*. 6:175-183.

Jaffe, A.B., and A. Hall. 2005. Rho GTPases: biochemistry and biology. *Annual review of cell and developmental biology*. 21:247-269.

Jones, M.B., H. Krutzsch, H.J. Shu, Y.M. Zhao, L.A. Liotta, E.C. Kohn, and E.F. Petricoin. 2002. Proteomic analysis and identification of new biomarkers and therapeutic targets for invasive ovarian cancer. *Proteomics*. 2:76-84.

Proteomics. 2:76-84.

Kamai, T., T. Yamanishi, H. Shirataki, K. Takagi, H. Asami, Y. Ito, and K.I. Yoshida. 2004. Overexpression of RhoA, Rac1, and Cdc42 GTPases is associated with progression in testicular cancer. *Clinical Cancer Research*. 10:4799-4805.

Ko, J.M., and D.E. Fisher. 2011. A new era: melanoma genetics and therapeutics. *The Journal of pathology*. 223:241-250.

Kolev, V., A. Mandinova, J. Guinea-Viniegra, B. Hu, K. Lefort, C. Lambertini, V. Neel, R. Dummer, E.F. Wagner, and G.P. Dotto. 2008. EGFR signalling as a negative regulator of Notch1

gene transcription and function in proliferating keratinocytes and cancer. *Nature cell biology*. 10:902-911.

Kaiser, H.W., W. Ness, I. Jungblut, R.A. Briggaman, H.W. Kreysel, and E.J. O'Keefe. 1993. Adherens junctions: demonstration in human epidermis. *The Journal of investigative dermatology*. 100:180-185.

Kaplan, K.B., K.B. Bibbins, J.R. Swedlow, M. Arnaud, D.O. Morgan, and H.E. Varmus. 1994. Association of the Amino-Terminal Half of C-Src with Focal Adhesions Alters Their Properties and Is Regulated by Phosphorylation of Tyrosine-527. *Embo Journal*. 13:4745-4756.

Kelly, A.E., H. Kranitz, V. Dotsch, and R.D. Mullins. 2006. Actin binding to the central domain of WASP/Scar proteins plays a critical role in the activation of the Arp2/3 complex. *The Journal of biological chemistry*. 281:10589-10597.

Kelly, T., S.C. Mueller, Y. Yeh, and W.T. Chen. 1994. Invadopodia promote proteolysis of a wide variety of extracellular matrix proteins. *Journal of cellular physiology*. 158:299-308.

Kim, A.S., L.T. Kakalis, N. Abdul-Manan, G.A. Liu, and M.K. Rosen. 2000. Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. *Nature*. 404:151-158.

Kim, S.A., C.Y. Tai, L.P. Mok, E.A. Mosser, and E.M. Schuman. 2011. Calcium-dependent dynamics of cadherin interactions at cell-cell junctions. *Proceedings of the National Academy of Sciences of the United States of America*. 108:9857-9862.

Labouesse, M. 2004. Epithelium-mesenchyme: a balancing act of RhoGAP and RhoGEF. *Current biology : CB*. 14:R508-510.

Kowalski, J.R., C. Egile, S. Gil, S.B. Snapper, R. Li, and S.M. Thomas. 2005. Cortactin regulates cell migration through activation of N-WASP. *Journal of cell science*. 118:79-87.

Kumar, N.M., and N.B. Gilula. 1996. The gap junction communication channel. *Cell*. 84:381-388.

Lampugnani, M.G., A. Zanetti, F. Breviario, G. Balconi, F. Orsenigo, M. Corada, R. Spagnuolo, M. Betson, V. Braga, and E. Dejana. 2002. VE-cadherin regulates endothelial actin activating Rac and increasing membrane association of Tiam. *Molecular biology of the cell*. 13:1175-1189.

Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell*. 84:359-369.

Lefever, T., E. Pedersen, A. Basse, R. Paus, F. Quondamatteo, A.C. Stanley, L. Langbein, X.W. Wu, J. Wehland, S. Lommel, and C. Brakebusch. 2010. N-WASP is a novel regulator of hair-follicle cycling that controls antiproliferative TGF beta pathways. *Journal of cell science*. 123:128-140.

Li, M., and D.S. Sakaguchi. 2002. Expression patterns of focal adhesion associated proteins in the developing retina. *Dev Dyn*. 225:544-553.

Lim, K.B., W. Bu, W.I. Goh, E. Koh, S.H. Ong, T. Pawson, T. Sudhaharan, and S. Ahmed. 2008. The Cdc42 effector IRSp53 generates filopodia by coupling membrane protrusion with actin dynamics. *The Journal of biological chemistry*. 283:20454-20472.

Lorenz, M., H. Yamaguchi, Y.R. Wang, R.H. Singer, and J. Condeelis. 2004. Imaging sites of N-WASP activity in lamellipodia and invadopodia of carcinoma cells. *Current Biology*. 14:697-703.

Lyubimova, A., J.J. Garber, G. Upadhyay, A. Sharov, F. Anastasoie, V. Yajnik, G. Cotsarelis, G.P. Dotto, V. Botchkarev, and S.B. Snapper. 2010. Neural Wiskott-Aldrich syndrome protein modulates Wnt signaling and is required for hair follicle cycling in mice. *Journal of Clinical Investigation*. 120:446-456.

Mackinnon, A.C., M. Tretiakova, L. Henderson, R.G. Mehta, B.C. Yan, L. Joseph, T. Krausz, A.N. Husain, M.E. Reid, and R. Salgia. 2011. Paxillin expression and amplification in early lung lesions of high-risk patients, lung adenocarcinoma and metastatic disease. *Journal of clinical pathology*. 64:16-24.

Martin TA, Toms AM, Davies LM, Cheng S, Jiang WG. 2012. The clinical and biological implications of N-WASP expression in human colorectal cancer. *Transl Gastrointest Cancer*. 1:10-20.

- Mariotti, A., P.A. Kedeshian, M. Dans, A.M. Curatola, L. Gagnoux-Palacios, and F.G. Giancotti.** 2001. EGF-R signaling through Fyn kinase disrupts the function of integrin $\alpha 6 \beta 4$ at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. *The Journal of cell biology*. 155:447-458.
- Martin, T.A., G. Pereira, G. Watkins, R.E. Mansel, and W.G. Jiang.** 2008. N-WASP is a putative tumour suppressor in breast cancer cells, *in vitro* and *in vivo*, and is associated with clinical outcome in patients with breast cancer. *Clinical & Experimental Metastasis*. 25:97-108.
- Martinez-Quiles, N., R. Rohatgi, I.M. Anton, M. Medina, S.P. Saville, H. Miki, H. Yamaguchi, T. Takenawa, J.H. Hartwig, R.S. Geha, and N. Ramesh.** 2001. WIP regulates N-WASP-mediated actin polymerization and filopodium formation. *Nature cell biology*. 3:484-491.
- Merajver, S.D., and S.Z. Usmani.** 2005. Multifaceted role of Rho proteins in angiogenesis. *Journal of mammary gland biology and neoplasia*. 10:291-298.
- Miki, H., T. Sasaki, Y. Takai, and T. Takenawa.** 1998. Induction of filopodium formation by a WASP-related actin-depolymerizing protein N-WASP. *Nature*. 391:93-96.
- Miki, H., and T. Takenawa.** 1998. Direct binding of the verprolin-homology domain in N-WASP to actin is essential for cytoskeletal reorganization. *Biochemical and biophysical research communications*. 243:73-78.
- Misra, A., R.P. Lim, Z. Wu, and T. Thanabalu.** 2007. N-WASP plays a critical role in fibroblast adhesion and spreading. *Biochemical and biophysical research communications*. 364:908-912.
- Misra, A., R. Rajmohan, R.N. Pei, Z. Lim, S. Bhattacharyya, and T. Thanabalu.** 2010. The mammalian Verprolin, WIRE induces filopodia independent of N-WASP through IRSp53. *Experimental cell research*. 316:2810-2824.
- Mueller, S.C., and W.T. Chen.** 1991. Cellular Invasion into Matrix Beads - Localization of Beta-1 Integrins and Fibronectin to the Invadopodia. *Journal of cell science*. 99:213-225.

- Nakagawa, H., H. Miki, M. Nozumi, T. Takenawa, S. Miyamoto, J. Wehland, and J.V. Small.** 2003. IRSp53 is colocalised with WAVE2 at the tips of protruding lamellipodia and filopodia independently of Mena. *Journal of cell science*. 116:2577-2583.
- Nelson, W.J.** 2008. Regulation of cell-cell adhesion by the cadherin-catenin complex. *Biochemical Society Transactions*. 36:149-155.
- Nagore, E., J. Climent, M.D. Planelles, E. Ledesma, F. Rubio-Moscardo, J.M. Fortea, and V. Oliver.** 2000. Analysis of the CDKN2A and CDK4 genes and HLA-DR and HLA-DQ alleles in two Spanish familial melanoma kindreds. *Acta dermato-venereologica*. 80:440-442.
- Oleksy, A., L. Opalinski, U. Derewenda, Z.S. Derewenda, and J. Otlewski.** 2006. The molecular basis of RhoA specificity in the guanine nucleotide exchange factor PDZ-RhoGEF. *The Journal of biological chemistry*. 281:32891-32897.
- Nurnberg, A., T. Kitzing, and R. Grosse.** 2011. Nucleating actin for invasion. *Nature reviews. Cancer*. 11:177-187.
- Okada, T., L. You, and F.G. Giancotti.** 2007. Shedding light on Merlin's wizardry. *Trends in cell biology*. 17:222-229.
- Onder, T.T., P.B. Gupta, S.A. Mani, J. Yang, E.S. Lander, and R.A. Weinberg.** 2008. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer research*. 68:3645-3654.
- Ozawa, M., M. Ringwald, and R. Kemler.** 1990. Uvomorulin Catenin Complex-Formation Is Regulated by a Specific Domain in the Cytoplasmic Region of the Cell-Adhesion Molecule. *Proceedings of the National Academy of Sciences of the United States of America*. 87:4246-4250.
- Pantaloni, D., C. Le Clainche, and M.F. Carlier.** 2001. Mechanism of actin-based motility. *Science*. 292:1502-1506.
- Parsons, J.T., A.R. Horwitz, and M.A. Schwartz.** 2010. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nature reviews. Molecular cell biology*. 11:633-643.

- Peng, X., L.E. Cuff, C.D. Lawton, and K.A. DeMali.** 2010. Vinculin regulates cell-surface E-cadherin expression by binding to beta-catenin. *Journal of cell science*. 123:567-577.
- Petrie, R.J., A.D. Doyle, and K.M. Yamada.** 2009. Random versus directionally persistent cell migration. *Nature reviews. Molecular cell biology*. 10:538-549.
- Puius, Y.A., N.M. Mahoney, and S.C. Almo.** 1998. The modular structure of actin-regulatory proteins. *Current opinion in cell biology*. 10:23-34.
- Pille, J.Y., C. Denoyelle, J. Varet, J.R. Bertrand, J. Soria, P. Opolon, H. Lu, L.L. Pritchard, J.P. Vannier, C. Malvy, C. Soria, and H. Li.** 2005. Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. *Molecular Therapy*. 11:267-274.
- Pollard, T.D., and G.G. Borisy.** 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 112:453-465.
- Ridley, A.J.** 2006. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends in cell biology*. 16:522-529.
- Remold-O'Donnell, E., F.S. Rosen, and D.M. Kenney.** 1996. Defects in Wiskott-Aldrich syndrome blood cells. *Blood*. 87:2621-2631.
- Reynolds, A.B., J. Daniel, P.D. McCrea, M.J. Wheelock, J. Wu, and Z. Zhang.** 1994. Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Molecular and cellular biology*. 14:8333-8342.
- Ridley, A.J., and A. Hall.** 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell*. 70:389-399.
- Rimm, D.L., E.R. Koslov, P. Kebriaei, C.D. Cianci, and J.S. Morrow.** 1995. Alpha(1)(E)-Catenin Is an Actin-Binding and Actin-Bundling Protein Mediating the Attachment of F-Actin to the Membrane Adhesion Complex. *Proceedings of the National Academy of Sciences of the United States of America*. 92:8813-8817.

- Rohatgi, R., L. Ma, H. Miki, M. Lopez, T. Kirchhausen, T. Takenawa, and M.W. Kirschner.** 1999. The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell*. 97:221-231.
- Schaller, M.D.** 2001. Paxillin: a focal adhesion-associated adaptor protein. *Oncogene*. 20:6459-6472.
- Schwartz, M.A., M.D. Schaller, and M.H. Ginsberg.** 1995. Integrins: emerging paradigms of signal transduction. *Annual review of cell and developmental biology*. 11:549-599.
- Scita, G., S. Confalonieri, P. Lappalainen, and S. Suetsugu.** 2008. IRSp53: crossing the road of membrane and actin dynamics in the formation of membrane protrusions. *Trends in cell biology*. 18:52-60.
- Small, J.V., K. Rottner, and I. Kaverina.** 1999. Functional design in the actin cytoskeleton. *Current opinion in cell biology*. 11:54-60.
- Suetsugu, S., Y. Banzai, M. Kato, K. Fukami, Y. Kataoka, Y. Takai, N. Yoshida, and T. Takenawa.** 2007. Male-specific sterility caused by the loss of CR16. *Genes to cells : devoted to molecular & cellular mechanisms*. 12:721-733.
- Aitken, J., J. Welch, D. Duffy, A. Milligan, A. Green, N. Martin, and N. Hayward.** 1999. CDKN2A variants in a population-based sample of Queensland families with melanoma. *Journal of the National Cancer Institute*. 91:446-452.
- Aronheim, A., Y.C. Broder, A. Cohen, A. Fritsch, B. Belisle, and A. Abo.** 1998. Chp, a homologue of the GTPase Cdc42Hs, activates the JNK pathway and is implicated in reorganizing the actin cytoskeleton. *Current Biology*. 8:1125-1128.
- Bandarchi, B., L. Ma, R. Navab, A. Seth, and G. Rasty.** 2010. From melanocyte to metastatic malignant melanoma. *Dermatology research and practice*. 2010.
- Bataille, V.** 2000. Genetics of familial and sporadic melanoma. *Clinical and experimental dermatology*. 25:464-470.
- Bataille, V.** 2003. Genetic epidemiology of melanoma. *European journal of cancer*. 39:1341-1347.

- Bellovin, D.I., K.J. Simpson, T. Danilov, E. Maynard, D.L. Rimm, P. Oettgen, and A.M. Mercurio.** 2006. Reciprocal regulation of RhoA and RhoC characterizes the EMT and identifies RhoC as a prognostic marker of colon carcinoma. *Oncogene*. 25:6959-6967.
- Bis, S., and H. Tsao.** 2013. Melanoma genetics: the other side. *Clinics in dermatology*. 31:148-155.
- Braga, V.M., and A.S. Yap.** 2005. The challenges of abundance: epithelial junctions and small GTPase signalling. *Current opinion in cell biology*. 17:466-474.
- Brash, D.E., J.A. Rudolph, J.A. Simon, A. Lin, G.J. McKenna, H.P. Baden, A.J. Halperin, and J. Ponten.** 1991. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proceedings of the National Academy of Sciences of the United States of America*. 88:10124-10128.
- Choi CK, Vicente-Manzanares M, Zareno J, Whitmore LA, Mogilner A, Horwitz AR.** 2008. Actin and α -actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat Cell Biol* 10: 1039–1050.
- Cory, G. O. and Ridley, A. J.** 2002. Cell motility: braking WAVES. *Nature* **418**, 732-733.
- Dorsky, R.I., R.T. Moon, and D.W. Raible.** 1998. Control of neural crest cell fate by the Wnt signalling pathway. *Nature*. 396:370-373.
- Dunn, K.J., B.O. Williams, Y. Li, and W.J. Pavan.** 2000. Neural crest-directed gene transfer demonstrates Wnt1 role in melanocyte expansion and differentiation during mouse development. *Proceedings of the National Academy of Sciences of the United States of America*. 97:10050-10055.
- Einspahr, J.G., D.S. Alberts, J.A. Warneke, P. Bozzo, J. Basye, T.M. Grogan, M.A. Nelson, and G.T. Bowden.** 1999. Relationship of p53 mutations to epidermal cell proliferation and apoptosis in human UV-induced skin carcinogenesis. *Neoplasia*. 1:468-475.
- Etienne-Manneville and Hall, 2002 S.** 2002. Rho GTPases in cell biology. *Nature*, 420. pp. 629-635

- Gouw, L.G., N.S. Reading, S.D. Jenson, M.S. Lim, and K.S.J. Elenitoba-Johnson.** 2005. Expression of the Rho-family GTPase gene RHOF in lymphocyte subsets and malignant lymphomas. *British Journal of Haematology*. 129:531-533.
- Han, S., H.T. Zhang, Z. Wang, Y. Xie, R. Tang, Y. Mao, and Y. Li.** 2006. DNA repair gene XRCC3 polymorphisms and cancer risk: a meta-analysis of 48 case-control studies. *European journal of human genetics : EJHG*. 14:1136-1144.
- Ho, H.Y., Rohatgi, R., Ma, L., and Kirschner, M.W.** 2001. CR16 forms a complex with N-WASP in brain and is a novel member of a conserved proline-rich actin-binding protein family. *Proc Natl Acad Sci USA* 98: 11306–11311.
- Iglesias, P. A., and Devreotes, P. N.** 2008. Navigating through models of chemotaxis. *Curr. Opin. Cell Biol.* 20, 35–40.
- Jin KM, Lu M, Liu FF, Gu J, Du XJ, Xing BC.** 2001. N-WASP is highly expressed in hepatocellular carcinoma and associated with poor prognosis. *Neoplasia*. 5: 395–401.
- Jaffe, A.B., and A. Hall.** 2005. Rho GTPases: biochemistry and biology. *Annual review of cell and developmental biology*. 21:247-269.
- Jones, M.B., H. Krutzsch, H.J. Shu, Y.M. Zhao, L.A. Liotta, E.C. Kohn, and E.F. Petricoin.** 2002. Proteomic analysis and identification of new biomarkers and therapeutic targets for invasive ovarian cancer. *Proteomics*. 2:76-84.
- Kamai, T., T. Yamanishi, H. Shirataki, K. Takagi, H. Asami, Y. Ito, and K.I. Yoshida.** 2004. Overexpression of RhoA, Rac1, and Cdc42 GTPases is associated with progression in testicular cancer. *Clinical Cancer Research*. 10:4799-4805.
- Kato, M., Miki, H., Kurita, S., Endo, T., Nakagawa, H., Miyamoto, S., and Takenawa, T.** 2002. WICH, a novel verprolin homology domain-containing protein that functions cooperatively with N-WASP in actin-microspike formation. *Biochem Biophys Res Commun* 291: 41–47.

Ko, J.M., and D.E. Fisher. 2011. A new era: melanoma genetics and therapeutics. *The Journal of pathology*. 223:241-250.

Kolev, V., A. Mandinova, J. Guinea-Viniegra, B. Hu, K. Lefort, C. Lambertini, V. Neel, R. Dummer, E.F. Wagner, and G.P. Dotto. 2008. EGFR signalling as a negative regulator of Notch1 gene transcription and function in proliferating keratinocytes and cancer. *Nature cell biology*. 10:902-911.

Labouesse, M. 2004. Epithelium-mesenchyme: a balancing act of RhoGAP and RhoGEF. *Current biology : CB*. 14:R508-510.

Munn, A.L., Stevenson, B.J., Geli, M.I., and Riezman, H. 1995. end5, end6, and end7: mutations that cause actin delocalization and block the internalization step of endocytosis in *Saccharomyces cerevisiae*. *Mol. Biol. Cell*. 6: 1721–1742.

Nagore, E., J. Climent, M.D. Planelles, E. Ledesma, F. Rubio-Moscardo, J.M. Fortea, and V. Oliver. 2000. Analysis of the CDKN2A and CDK4 genes and HLA-DR and HLA-DQ alleles in two Spanish familial melanoma kindreds. *Acta dermato-venereologica*. 80:440-442.

Nurnberg, A., T. Kitzing, and R. Grosse. 2011. Nucleating actin for invasion. *Nature reviews. Cancer*. 11:177-187.

Nordlund, J.J., Boissy, R.E., Hearing, V.J., King, R.A., Ortonne, J.-P. 1998. The Pigmentary System - Physiology and Pathophysiology, Oxford University Press, Oxford.

Oleksy, A., L. Opalinski, U. Derewenda, Z.S. Derewenda, and J. Otlewski. 2006. The molecular basis of RhoA specificity in the guanine nucleotide exchange factor PDZ-RhoGEF. *The Journal of biological chemistry*. 281:32891-32897.

Pille, J.Y., C. Denoyelle, J. Varet, J.R. Bertrand, J. Soria, P. Opolon, H. Lu, L.L. Pritchard, J.P. Vannier, C. Malvy, C. Soria, and H. Li. 2005. Anti-RhoA and anti-RhoC siRNAs inhibit the proliferation and invasiveness of MDA-MB-231 breast cancer cells in vitro and in vivo. *Molecular Therapy*. 11:267-274.

- Pollard, T.D., and G.G. Borisy.** 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 112:453-465.
- Ridley, A.J.** 2006. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends in cell biology*. 16:522-529.
- Salh, B., A. Marotta, R. Wagey, M. Sayed, and S. Pelech.** 2002. Dysregulation of phosphatidylinositol 3-kinase and downstream effectors in human breast cancer. *International Journal of Cancer*. 98:148-154.
- Sandilands, E., S. Akbarzadeh, A. Vecchione, D.G. McEwan, M.C. Frame, and J.K. Heath.** 2007. Src kinase modulates the activation, transport and signalling dynamics of fibroblast growth factor receptors. *EMBO reports*. 8:1162-1169.
- Simpson, K.J., A.S. Dugan, and A.M. Mercurio.** 2004. Functional analysis of the contribution of RhoA and RhoC GTPases to invasive breast carcinoma. *Cancer research*. 64:8694-8701.
- Smalley, K.S.** 2003. A pivotal role for ERK in the oncogenic behaviour of malignant melanoma? *International journal of cancer. Journal international du cancer*. 104:527-532.
- Swaney K.F., Huang C.H., Devreotes P.N.** 2010. Eukaryotic chemotaxis: a network of signaling pathways controls motility, directional sensing, and polarity. *Annu Rev Biophys*. 39:265–289. doi: 10.1146/annurev.biophys.093008.131228.
- Rodriguez, O.C. (2003) Conserved microtubule-actin interactions in cell movement and morphogenesis. *Nat. Cell Biol.* 5, 599-609
- Small, J. V., Geiger, B., Kaverina, I. and Bershadsky, A.** 2002. How do microtubules guide migrating cells? *Nat Rev Mol Cell Biol* 3, 957–964.
- Thanabalu, T., and A.L. Munn.** 2001. Functions of Vrp1p in cytokinesis and actin patches are distinct and neither requires a WH2/V domain. *The EMBO journal*. 20:6979-6989.

- Tsao, H., M.C. Mihm, Jr., and C. Sheehan.** 2003. PTEN expression in normal skin, acquired melanocytic nevi, and cutaneous melanoma. *Journal of the American Academy of Dermatology*. 49:865-872.
- Takeichi, M.** 1995. Morphogenetic Roles of Classic Cadherins. *Current opinion in cell biology*. 7:619-627.
- Thoreson, M.A., P.Z. Anastasiadis, J.M. Daniel, R.C. Ireton, M.J. Wheelock, K.R. Johnson, D.K. Hummingbird, and A.B. Reynolds.** 2000. Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. *The Journal of cell biology*. 148:189-202.
- Torres, E., and M.K. Rosen.** 2003. Contingent phosphorylation/dephosphorylation provides a mechanism of molecular memory in WASP. *Molecular cell*. 11:1215-1227.
- Tsukita, S., M. Furuse, and M. Itoh.** 1999. Structural and signalling molecules come together at tight junctions. *Current opinion in cell biology*. 11:628-633.
- Uong, A., and L.I. Zon.** 2010. Melanocytes in development and cancer. *Journal of cellular physiology*. 222:38-41.
- Vaezi, A., C. Bauer, V. Vasioukhin, and E. Fuchs.** 2002. Actin cable dynamics and Rho/Rock orchestrate a polarized cytoskeletal architecture in the early steps of assembling a stratified epithelium. *Developmental Cell*. 3:367-381.
- Vasioukhin, V., C. Bauer, M. Yin, and E. Fuchs.** 2000. Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell*. 100:209-219.
- Vega, F.M., and A.J. Ridley.** 2008. Rho GTPases in cancer cell biology. *FEBS letters*. 582:2093-2101.
- Volberg, T., B. Geiger, R. Dror, and Y. Zick.** 1991. Modulation of Intercellular Adherens-Type Junctions and Tyrosine Phosphorylation of Their Components in Rsv-Transformed Cultured Chick Lens Cells. *Cell Regulation*. 2:105-120.

Vera DesMarais,¹ Hideki Yamaguchi, Lilian Soon, Ghassan Mouneimne, Corina Sarmiento,¹ Robert Eddy,¹ and John Condeelis. 2009. N-WASP and cortactin are involved in invadopodium-dependent chemotaxis to EGF in breast tumor cells. *Cell Motil Cytoskeleton*. 6:303-316

Volberg, T., Y. Zick, R. Dror, I. Sabanay, C. Gilon, A. Levitzki, and B. Geiger. 1992. The effect of tyrosine-specific protein phosphorylation on the assembly of adherens-type junctions. *The EMBO journal*. 11:1733-1742.

Wade, R., J. Bohl, and S. Vande Pol. 2002. Paxillin null embryonic stem cells are impaired in cell spreading and tyrosine phosphorylation of focal adhesion kinase. *Oncogene*. 21:96-107.

Wang, W.S., H.J. Zhong, D.W. Xiao, X. Huang, L.D. Liao, Z.F. Xie, X.E. Xu, Z.Y. Shen, L.Y. Xu, and E.M. Li. 2010. The expression of CFL1 and N-WASP in esophageal squamous cell carcinoma and its correlation with clinicopathological features. *Diseases of the Esophagus*. 23:512-521.

Watt, F.M. 2002. Role of integrins in regulating epidermal adhesion, growth and differentiation. *The EMBO journal*. 21:3919-3926.

Welch, M. D. and Mullins, R. D. 2002. Cellular control of actin nucleation. *Annu. Rev. Cell Dev. Biol.* 18, 247-288.

Wheelock, M.J., and P.J. Jensen. 1992. Regulation of Keratinocyte Intercellular Junction Organization and Epidermal Morphogenesis by E-Cadherin. *Journal of Cell Biology*. 117:415-425.

Wodarz, A., and R. Nusse. 1998. Mechanisms of Wnt signaling in development. *Annual review of cell and developmental biology*. 14:59-88.

Wu, X., S. Suetsugu, L.A. Cooper, T. Takenawa, and J.L. Guan. 2004. Focal adhesion kinase regulation of N-WASP subcellular localization and function. *The Journal of biological chemistry*. 279:9565-9576.

Wu X, Yoo Y, Okuhama NN, Tucker PW, Liu G, Guan JL. 2006. Regulation of RNA-polymerase-II-dependent transcription by N-WASP and its nuclear-binding partners *Nat Cell Biol*. 8:756

- Xiang, W., Z. Wen, W. Pang, L. Hu, C. Xiong, and Y. Zhang.** 2011. CR16 forms a complex with N-WASP in human testes. *Cell and tissue research*. 344:519-526.
- Xu, W., H. Baribault, and E.D. Adamson.** 1998a. Vinculin knockout results in heart and brain defects during embryonic development. *Development*. 125:327-337.
- Xu, W.M., H. Baribault, and E.D. Adamson.** 1998b. Vinculin knockout results in heart and brain defects during embryonic development. *Development*. 125:327-337.
- Yamada, K.M., and B. Geiger.** 1997. Molecular interactions in cell-ECM adhesion complexes. *Current opinion in cell biology*. 9:76-85.
- Yamada, K.M., and S. Miyamoto.** 1995. Integrin transmembrane signaling and cytoskeletal control. *Current opinion in cell biology*. 7:681-689.
- Yamaguchi, H., M. Lorenz, S. Kempiak, C. Sarmiento, S. Coniglio, M. Symons, J. Segall, R. Eddy, H. Miki, T. Takenawa, and J. Condeelis.** 2005. Molecular mechanisms of invadopodium formation: the role of the N-WASP-Arp2/3 complex pathway and cofilin. *Journal of Cell Biology*. 168:441-452.
- Yamazaki, D., T. Oikawa, and T. Takenawa.** 2007. Rac-WAVE-mediated actin reorganization is required for organization and maintenance of cell-cell adhesion. *Journal of cell science*. 120:86-100.
- Yap, A.S., W.M. Brieher, and B.M. Gumbiner.** 1997. Molecular and functional analysis of cadherin-based adherens junctions. *Annual review of cell and developmental biology*. 13:119-146.
- Yap, A.S., C.M. Niessen, and B.M. Gumbiner.** 1998. The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *The Journal of cell biology*. 141:779-789.
- Yilmaz, M., and G. Christofori.** 2010. Mechanisms of motility in metastasizing cells. *Molecular cancer research : MCR*. 8:629-642.
- Yonemura, S., Y. Wada, T. Watanabe, A. Nagafuchi, and M. Shibata.** 2010. alpha-Catenin as a tension transducer that induces adherens junction development. *Nature cell biology*. 12:533-U535.

Zamir, E., and B. Geiger. 2001. Molecular complexity and dynamics of cell-matrix adhesions. *Journal of cell science*. 114:3583-3590.

Zhao, L., W. Li, C. Marshall, T. Griffin, M. Hanson, R. Hick, T. Dentchev, E. Williams, A. Werth, C. Miller, H. Bashir, W. Pear, and J.T. Seykora. 2009. Srcasm inhibits Fyn-induced cutaneous carcinogenesis with modulation of Notch1 and p53. *Cancer research*. 69:9439-9447.

Wang S, Huang C. 2012. N-WASP, a colorectal cancer suppressor? *Transl Gastrointest Cancer*. 2224-4778.2011.12.04