Characterization of molecular differences between WASP and N-WASP and characterization of mutations causing Wiskott-Aldrich syndrome

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UNIVERSITY

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SCHOOL OF BIOLOGICAL SCIENCES

**A thesis submitted to Nanyang Technological University in
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2013

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Abbreviations

APC	Antigen presenting cells
AR	Acidic region
Arp2/3	Actin related protein complex
AbI	Abelson tyrosine kinase
BiFC	Bi-molecular fluorescence complementation
Btk	Brutonagammaglobulinemia tyrosine kinase
BSA	Bovine Serum Albumin
BR	Basic region
BCR	B-cell receptor
Cdc42	Cell division cycle 42
cDNA	Complementary DNA
CH	Cofilin homology
CR16	Glucocorticoid-regulated gene product
CRIB	Cdc42/Rac-interactive binding region
DMSO	Dimethylsulfoxide
DTT	1,4 Dithiothreitol
DCs	Dendritic cells
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphate
ECM	Extracellular Matrix
EBV	Epstein-Barr Virus
Ena/Mena	Enabled/Mammalian enabled
E.coli	Escherichia coli
FBS	Foetal Bovine Serum
F-actin	Filamentous actin
FGR	Gardner-Rasheed feline sarcoma viral (v-fgr) oncogene
Fyn	Proto-oncogene tyrosine-protein kinase Fyn
FACS	Fluorescence Activated Cell Sorting
GBD	GTPase Binding domain
GFP	Green fluorescent protein
Grb2	Growth Factor Receptor-Bound Protein 2

G-actin	Globular-actin
GST	Glutathion-S-Transferase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Hck	Hematopoietic cell kinase
HLA	Human leukocyte antigen
I30	Internal 30 amino acid
IRSp53	Insulin Receptor Substrate p53
IL-2	Interleukin-2
JMY	Junction-mediating and regulatory protein
Lck	Lymphocyte-specific protein tyrosine kinase
LB	Luria-Bertani
LiAc	Lithium acetate
Ltk	Leukocyte receptor tyrosine kinase
MEFs	Mouse Embryonic Fibroblasts
MCS	Multiple Cloning Site
Nck	Nonbinding non-catalytic region of tyrosine kinase
N-WASP	Neural-WASP
NLS	Nuclear localisation signal
NPFs	Nucleation promoting factors
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
PMSF	Phenylmethylsulfonyl fluoride
PRR	Proline rich region
PIK	phosphoinositide-3-kinase
PEG	Polyethylene glycol
PIP2	Phosphatidylinositol (4,4) Bisphosphate
PSTPIP1	Phosphatase Interacting Protein 1
Rac1	Ras-related C3 botulinum toxin substrate 1
RNAi	Retrovirus vector mediated
RPMI-1640	Rosewell Park Memorial Institute medium-1640
RT-PCR	Real-time PCR
RNA	Ribonucleic Acid
SDF-1 α	Stromal Cell Derived Factor-1 α

SD	Synthetic Defined
SDM	Site Directed Mutagenesis
SDS-PAGE	Sodium Dodecyl Sulfate polyacrylamide gel electrophoresis
SFM	Serum Free Medium
SCAR	Suppressor of cAMP receptor
SHD	SCAR homology domain
SH2	Src Homology (domain) 2
SH3	Src Homology (domain) 3
SLP-76	Src homology 2 (SH2) domain-containing leukocyte protein of 76 kDa
TCR	T cell receptor
TEMED	N,N,N,N,-tetramethylethylenediamine
TAE	Tris-acetate-EDTA
TBS	Tris Buffered Saline
Toca1	Transducer of Cdc42-dependent actin assembly
V	Verprolin homology domain
VASP	Vasodilator-stimulated phosphoprotein
VH	Verprolin homology
WAS	Wiskott Aldrich Syndrome
WASP	Wiskott Aldrich Syndrome protein
WAVE	WASP-family verprolin homologous proteins
WASH	WASP and SCAR homologue
WHAMM	WASP homologue associated with actin, membranes and microtubules
WBD	WASP binding domain
WH1/EVH1/PH	WASP homology domain 1/ Ena-VASP homology domain 1 / Pleckstrin homology
WH1	WASP-homology 1
WH2	WASP-homology 2
WIP	WASP-interacting protein
WIRE	WIP related
XLN	X-linked severe congenital neutropenia
XLT	X-linked thrombocytopenia
YFP	Yellow fluorescent protein
ZAP-70	Zeta-chain-associated protein kinase 70

Publications

A. Publications in this study

1. **Jain N**, Thanabalu T. Identification and characterization of unique region of WASP in Jurkat T-cells chemotaxis. (*Manuscript under preparation*).
2. **Jain N**, Thanabalu T. Molecular characterization of WASP missense mutation P373S, its role in T-cell activation and chemotaxis towards SDF-1 α . (*Manuscript under preparation*)

B. Publications in other studies

1. Misra A, George B, Rajmohan R, **Jain N**, Wong MH, Kambadur R, Thanabalu T. Insulin receptor substrate protein 53kDa (IRSp53) is a negative regulator of myogenic differentiation. Int J Biochem Cell Biol. 2012 Jun;44(6): 928-41. 2012 Mar 24.

C. Conference attended

Presented a poster (Poster number: B109) of my Ph.D project entitled ***“Identification and characterization of unique region of WASP in Jurkat T-cells chemotaxis”*** in 51th annual meeting of American Society for Cell biology (ASCB) held at Denver, Colorado, USA; 3-7th December 2011.

Abstract

Wiskott-aldrich Syndrome is caused by mutation in gene encodes WASP. WASP and its homologue N-WASP regulate actin cytoskeleton reorganization, share more than 50% sequence identity. Knocking down of WASP expression impaired Jurkat T-cells chemotaxis towards SDF-1 α and increased adhesion to fibronectin, which was rescued by exogenous expression of WASP (WASP_R: shRNA resistant) but not by N-WASP. Moreover, WASP is indispensable for formation of anti-CD3/anti-CD28 stimulated membrane projections. However, N-WASP rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells suggesting both overlapping and unique functions of WASP and N-WASP in T-cells. WASP has a unique 30 amino acid (I30) region (a.a.158-187) between its WH1 domain and basic region which is absent in N-WASP. The I30 region of WASP interacts with eight (Fyn, Toca1, Nck, Grb2, FGR, Src, Lyn and Hck) out of 17 known WASP binding proteins. The I30 region of WASP is required for Jurkat T-cells chemotaxis and IL-2 gene transcription, which are the two independent activities of WASP. These defects observed in WASP ^{Δ I30} expressing WASP^{KD} Jurkat T-cells could be due to a partially open conformation of WASP ^{Δ I30}, resulting increase Hck mediated Tyr²⁹¹ phosphorylation. Insertion of I30 region in N-WASP (N-WASP-I30) rescued all the impairments of WASP^{KD} Jurkat T-cells and mediates its localization to TCR activation sites. Thus, suggesting that I30 region of WASP is one of the important determinants of functional differences between WASP and N-WASP.

Two point mutations in the PRR domain of WASP (S339Y/P373S) have been reported to occur together in WAS patients. These mutations were

characterized either individually (WASP^{S339Y}, WASP^{P373S}) or together (WASP^{SP/YS}) for their effects in Jurkat T-cell functions. WASP^{PRR} mutants did not rescue the defective chemotaxis of WASP^{KD} Jurkat T-cells. Unlike WASP_R^{S339Y}, cells expressing WASP_R^{P373S} or WASP_R^{SP/YS} did not rescue the IL-2 gene transcription defect and adhesion to fibronectin suggesting that point mutation P373S is sufficient to inhibit WASP mediated Jurkat T-cell activation. Moreover, a diffused pattern of localization of WASP^{PRR} mutants at TCR activation sites was observed suggesting that WASP^{PRR} mutants abolish WASP function in TCR mediated signalling. Thus, both mutations individually cause some defects but together have additive effects on WASP mediated T-cell functions resulting in WAS. Most of the WAS associated missense mutations occur in the WH1 domain of WASP. Twenty five of these missense mutations were analysed for their interaction with CIB1 (WASP^{WH1} domain interacting protein). Six of these mutations (G40V, T45M, L46P, A47D, P58L, G70W) abolished WASP-CIB1 interaction but not WASP-WIP interaction. Four mutations (G40V, T45M, P58L, G70W) expressed poorly in WASP^{KD} Jurkat T-cells suggesting a possible cause of disease. The remaining two mutants (L46P, A47D) expressed well but did not rescue the chemotaxis defect of WASP^{KD} Jurkat T-cell and negatively regulate spreading of cells on anti-CD3/anti-CD28 coated coverslip. WASP^{KD} Jurkat T-cells expressing WASP^{L46P} expressing cells were defective in T-cell activation. Taken together, this study has led to the elucidation of the molecular difference between WASP and N-WASP and characterised the molecular defect of some of the WASP mutations causes WAS.

1. Introduction

The cell is the smallest autonomously functional unit of all living organisms. Cells can be categorized into eukaryotic cells and prokaryotic cells. Prokaryotes are unicellular organisms like bacteria while eukaryotes are either unicellular organism like yeast or multicellular organism such as mammals. Despite the evolutionary differences between them, eukaryotic and prokaryotic cells have certain similarities, both types of cells are membrane bound, contain DNA as the genetic material and synthesize proteins by utilizing ribosomes. All the cells maintain their correct shape, interact with their environment and have ability to migrate from one place to another place. All these spatial and mechanical functions depend to a higher degree in eukaryotes and to a lesser extent in prokaryotes, on a network of proteins filaments collectively called as cytoskeleton. The entire focus of this dissertation would be on the proteins associated with eukaryotic cytoskeleton, particularly the actin filaments.

The **cytoskeleton** is a cellular skeleton contained within a cell's cytoplasm that assists in communication of the cell with external environment, maintain cell shape and regulate cell motility. The eukaryotic cytoskeleton is an organized network of three primary cytoskeletal filaments: microtubules, intermediate filaments and actin filaments. These structures are made up of smaller sub-units that can form linear or branched filaments. There are several accessory proteins that associate with these filaments such as nucleation-promoting factors, capping proteins, depolymerizing factors, severing factors, cross-linkers and filaments stabilizing proteins [74]. The three cytoskeletal filaments along with their regulatory proteins make a dynamic network that can

assemble, disassemble and maintain the integrity of intracellular components [34].

Microtubules are the least flexible among the three types of cytoskeletal filaments [74]. They are hollow tubes of 25 nm in diameter formed from 13 laterally associated protofilaments. Each protofilament is an assembly of α and β -tubulin heterodimers. Microtubules are organised by microtubule-organising centers (MTOCs) and are associated with motor proteins such as dyneins/kinesins which regulates long-range intracellular transport, mitosis and cell movement [91,108,227].

Intermediate filaments are around 8-11 nm in diameter and are made up of tetrameric subunits formed by monomers with a central α -helical domain. Intermediate filaments are stable, durable and prominent in the cells subjected to mechanical stress. In vertebrates, intermediate filament presence and its compositions is not only species dependent, but it also varies with tissue and cell type. The common intermediate filaments are nuclear lamins that give structural support to the nuclear envelope; keratins are mainly found in epithelial cells; vimentins and desmins which provide mechanical strength to muscle cells [220].

Actin filaments or microfilaments are the thinnest filaments (7 nm in diameter) of the cytoskeleton. Microfilaments are linear polymers of actin subunits. Actin filaments assist in cell movement, cell division, cell shape change, mechanical support, transport of intracellular materials and maintain cell-cell junctions. Actin is present in globular-actin (G-actin) and the

filamentous actin (F-actin) states which is formed through the self assembly of G-actin. Actin polymerization involves three distinct steps. First step is the aggregate formation of three actin monomers (nucleation) which is a rate limiting step. The second step is the elongation step where actin monomers are added reversibly to both ends of the filament. The final step is the steady state or equilibrium phase in which the rate at which subunits are added is balanced by rate at which subunits are dissociated from the filament. The actin nucleus formation is energetically unfavourable [194]. This barrier is overcome by actin nucleators such as Arp2/3 (Actin-related protein) complex. It is a 220 kDa complex that is made up of seven proteins: Arp2, Arp3 and subunits ARPC1-5 (Actin-related protein complex 1-5). Arp2 and Arp3 are structurally similar to monomeric actin. The complex binds at a 70 degree angle to the side of existing filaments and initiates growth of a new filament forming a branched actin network. Nucleation activity of Arp2/3 complex is low by itself [147]. Interaction of nucleation promoting factors (NPFs) with the Arp2/3 complex enhances its activity. These proteins induce conformational changes in Arp2/3 complex, bringing Arp2 and Arp3 subunits in close proximity, which mimics an actin dimer. Actin monomers are then recruited to the complex by the NPFs [51] resulting in enhanced actin polymerization. Members of NPFs include Wiskott Aldrich Syndrome protein (WASP) family of proteins which includes WASP, neural-WASP (N-WASP), WASP family verprolin homologue (WAVE/SCAR) 1-3 and WASP and SCAR homologue (WASH). Once the actin nucleus is formed, actin monomers are added to its barbed (+) and pointed (-) ends rapidly leading to accelerated elongation of actin filament. Elongation rate depends on the concentration of actin monomers available. The

barbed end of the filament grows faster than pointed end. During elongation step, ATP-bound G-actin is preferentially added to the barbed end. ATP is hydrolysed after the G-actin molecule is incorporated into an actin filament and is lost through the pointed (-) end in the ADP-bound state. The result of this is the growth of F-actin at the barbed end and shortening at the pointed end [122]. As the F-actin filament grows, the concentration of G-actin decreases until they reach equilibrium state where there is no net increase in filament length. The dynamic nature of actin polymerization drives many cellular processes such as cell motility, cell division and cell shape change. In response to any external stimuli, pools of actin monomers are assembled and polymerized to actin filaments at specific subcellular locations. Actin polymerization at the cell membrane provides force for formation of membrane projections which are critical for cell migration [121]. For immune cells, migration is an important step in detection and destruction of foreign pathogens.

1.1 Wiskott Aldrich Syndrome (WAS)

Wiskott Aldrich Syndrome (WAS) is a rare X-linked recessive disorder which is characterised by eczema, thrombocytopenia (low platelet count, small size platelets), recurrent bacterial or viral infections, autoimmune disorders and malignancies [161,209]. It was first described in two brothers with chronic bloody diarrhoea [237], X-linked pattern of inheritance and platelet abnormalities were deciphered later [3]. The gene affected in Wiskott Aldrich Syndrome was identified by positional cloning and was mapped to chromosome region Xp11.23 [67]. The disease results from the mutations in a 502 amino acid protein known as Wiskott Aldrich Syndrome protein (WASP),

which plays a key role in actin cytoskeleton reorganization [77]. WAS occurs at a frequency of 10 in 1 million [174,188]. Though 90% of the affected patients are males, female carriers of the defective WASP gene are also at risk due to the non random inactivation of the functional WASP allele [12]. In 2007, 220 new cases of WAS were reported in European Society for immunodeficiency disease (ESID) and found to be approximately 3% of total congenital immunodeficiencies reported (ESID 2008). A large number of different types of mutations have been reported to occur on WASP gene which affects its expression and give rise to variable clinical phenotypes which includes: classical Wiskott-Aldrich Syndrome (WAS), X-linked thrombocytopenia (XLT) and X-linked severe congenital neutropenia (XLN). In case of classical Wiskott Aldrich syndrome, WASP expression is absent [95] and most of the patients are succumbed to death by the age of 10 years due to internal haemorrhages or recurrent bacterial or viral infections [213]. Severe eczema is a frequent manifestation of classical WAS during childhood. WASP gene sequence analysis is an essential and confirmatory diagnostic step for WAS. XLT (a milder form of WAS) is a result of missense mutations which cause reduced expression of defective WASP. Thrombocytopenia with small sized platelets but without immunodeficiency and eczema are major characteristics of XLT patients [157]. XLN is caused by missense mutations (L270P, S272P, I294T) in the GTPase binding domain (GBD) of WASP which destroy the auto-inhibitory conformation of WASP, resulting in enhanced actin polymerization activity of WASP [5,68]. Chronic neutropenia and monocytopenia are the features of XLN. A simple clinical symptoms scoring system has been adopted to categorize the severity of WAS (Fig. 1.1).

Score	Low platelets	Small Platelets	Eczema	Infection	Malignancy/ Autoimmunity
0.5	Intermittent	Yes	No	No	No
1	Yes	Yes	No	No	No
2	Yes	Yes	Mild	Mild	No
3	Yes	Yes	Mod	Mod	No
4	Yes	Yes	Severe	Severe	No
5	Yes	Yes	Severe	Severe	Yes

	Intermittent X-linked thrombocytopenia
	X-linked thrombocytopenia
	Wiskott Aldrich Syndrome

Figure 1.1 Clinical scoring systems for WAS and XLT [48,103,159,251].

1.2 WASP gene mutations and genotype-phenotype correlations

In one study, more than 141 unique WASP mutations have been reported in a cohort of 227 unrelated WAS/XLT families. The most common are missense mutations, followed by splice-site mutation, short deletions and nonsense mutations. Complex mutations, large deletions and insertions are rare (Table. 1.1). Most of the deletion and insertion mutations resulted in frameshift and premature stop of translation. Most of the point mutations are found in the WASP^{WH1} domain (exons 1-4; region is essential for interaction with WIP) [96]. Some of these WASP^{WH1} missense mutations resulted in reduced WASP expression in T-cells isolated from WAS/XLT patients [103] and/or affected WASP-WIP interaction [181,205]. The second most common WASP gene mutation type observed was splice sites mutations which are found predominantly towards the carboxyl terminus (exons 6-11) of the WASP gene. Complex mutations were rare in WAS patients and involved double missense mutations (S339Y/P373S) (WASPbase), combination of deletions and

insertions, point mutation followed by deletion. The other types of mutations are evenly distributed throughout the gene and at intron-exon junctions.

Table: 1.1 Distribution of mutations in WAS/XLT families [103].

Mutation Type	Families affected (%)
Missense	35.3
Splice site	19.4
Deletion	17.6
Nonsense	15.4
Insertion	7.9
Complex	4.4
Total	100

Five mutational hotspots in WASP gene have been identified and found to occur in more than 20% of WAS/XLT families reported [103]. These are as follow: replacement of arginine at amino acid position 86 with cystine/glycine/histidine (Arg86Cys/Gly/His), replacement of threonine with methionine at position 45 (Tre45Met), IVS6+5g>a (disruption of a variant splice site, resulting in a protein truncation at 190aa) resulted in simultaneously expression of mutated and reduced amount of normal WASP protein expression, 665C>T (the nonsense mutation Arg211X) and IVS8+1g>n (splice site mutation at exon 8, frameshift stop at amino acid 246) [103]. In order to understand genotype-phenotype correlation, WAS patients were divided in two categories: WASP-positive patients in which full-length mutated WASP protein was expressed and WASP-negative patients in which WASP protein expression was absent or truncated WASP protein was expressed [103,161,214]. Patients with reduced expression of full-length WASP protein

often had the XLT phenotype. In contrast, patients with classical WAS did not express WASP or had truncated WASP expression in their lymphocytes ($P < 0.001$) [159,161]. Although, the genotype-phenotype correlation is clear, but it not absolute. Different patients having same mutations in their WASP gene can have different levels of WASP protein expression suggesting that clinical phenotype of WASP mutations is influenced by other factors such as genetic mutations in other locus or environment factors, which could be responsible for the heterogeneity and diversity of defects seen in patients with the disease.

1.3 Treatments for WAS/XLT

Bone marrow transplantation was the only curative therapy in early decades for classical WAS patients, when HLA-identical siblings were available. However, unrelated or partially matched donors provided less satisfactory outcomes due to graft failure, graft versus host disease and severe infections [73]. The first sibling donor bone marrow transplantation for WAS was reported in 1968 [17]. Symptomatic treatment such as splenectomy (surgical removal of patient's spleen) also helped by artificially raising the platelet count in 90% of the patients [117]. However, splenectomy had an increased risk of septicemia. Genetic approaches to correct the autologous hematopoietic stem cells or T-cells offered more specific and less toxic therapeutic options. It has been shown that gene therapy approach such as retroviral mediated WASP gene reconstitution in T-cells from WAS patients improved the T-cell defects and restored the normal functions of T-cell including proliferation, IL-2 production [71]. Furthermore, studies using WASP^{-/-} mice revealed that gene therapy could rescue the functional defects in several immune cell lineages through

safe and long term expression of WASP [29]. Moreover, it has recently been reported that lentiviral vector containing a gammaretrovirus derived promoter provided high level of WASP gene expression in hematopoietic lineages and corrected the WAS phenotype in WASP deficient mice [15]. All these studies suggest that gene therapy can be used for future clinical trials for WAS.

1.4 Wiskott Aldrich Syndrome protein (WASP)

WASP is a member of the WASP/WAVE family of proteins which regulates actin cytoskeleton reorganization [118]. In *S. cerevisiae*, Las17p is the only member [126] whereas in mammals, WASP family of proteins consist of eight members namely: WASP, N-WASP (neural-WASP) [141], SCAR/WAVE (1-3) (Suppressor of cAMP receptor/WASP-family verprolin homologous protein) [208], WASH (WASP and SCAR homologue), WHAMM (WASP homolog-associated protein with actin, membranes and microtubules) and JMY (Junction mediated and regulatory protein) [42,168]. WASP was the founding member of WASP family of proteins [67] whereas WASH, WHAMM and JMY have been discovered more recently [41,127,256]. WASP is a 502 amino acid protein, expression of which is restricted to hematopoietic cells unlike N-WASP and SCAR/WAVE1-3 which expressed ubiquitously [141]. The differences in expression pattern could be because of the different roles played by WASP and N-WASP in cell specific manner. WASP^{KO} mice showed defects in T-cell activation, T-cell migration and dendritic cell functions while N-WASP^{-/-} mice were embryonic lethal at day 11 [129,202]. All these studies indicate that WASP is crucial for hematopoietic cell functions while N-WASP plays a crucial role for many cell types due to its ubiquitous expression

[202,215]. WASP family of proteins (WASP, N-WASP, SCAR/WAVE 1-3, WASH, WHAMM, JMY) possess a similar domain structure (Fig. 1.2). WASP family of proteins possess C-terminal verprolin homology (also known as WASP-homology-2 domain) (WH2 or V), cofilin homology (C) and acidic region (A). Together, these three domains are known as VCA domain in which the V-domain binds to an actin monomer while the CA domain binds to Arp2/3 (Actin related protein) complex. The VCA domain is the functional output domain of WASP family of proteins. Upon assembly of three actin monomers, in which two actin related molecules are from the Arp2/3 complex, actin polymerization can be initiated [131,169,177]. The WASP family of proteins (WASP, N-WASP, WAVE 1-3) also share the basic region (BR), which interacts with phosphoinositides [88]. Besides VCA and BR, WASP family of proteins consist of a proline rich region (PRR) upstream of VCA domain which encompasses several consecutive proline residues and PXXP motifs. PRR of WASP and N-WASP have been reported to bind to the SH3 domain of various tyrosine kinases and adaptor proteins such as Nck, Grb2 [22,96]. In addition to VCA, PRR and BR, WASP and N-WASP possess a central GTPase binding domain (GBD). The GBD of WASP and N-WASP has been reported to bind to Rho family of GTPases such as Cdc42 and mediate their effects on actin cytoskeletal rearrangement [1,211]. At the N-terminus, WASP and N-WASP possess WASP-homology 1 domain (WH1 domain) [40] (Fig. 1.2). The WAVE proteins have a unique domain at their N-terminus known as the SCAR homology domain (SHD).

Figure 1.2: Schematic diagram illustrating the common domains shared by the WASP family of proteins [168]. WH1: WASP homology domain 1, SHD: Scar homology domain, WHD1: WASH homology domain-1, WHD2: WASH homology domain-2, NTD: N-terminal homology domain, CC: Coiled coil domain, BR: Basic region, GBD: GTPase binding domain, V: Verprolin homology domain, C: Cofilin homology domain or connecting region, A: Acidic region.

The WH1 domain of WASP and N-WASP interacts with a specific proline rich motif, which is present in the C-terminal region of WASP interacting protein (WIP) [182,228], WIP related (WIRE) [13] and glucocorticoid-regulated gene product (CR16) [232]. WIP protects WASP from degradation by cytoplasmic proteases [52,64]. The WASP homologue yeast protein Las17p also possess an N-terminus WH1 domain which interacts with the yeast homologue of WIP (Verprolin; Vrp1p) [152,182]. Inside the cell, WIP regulates WASP and N-WASP activity and functions as a regulator of actin polymerization [8-9].

1.5 Physiological role of WASP

WASP is a hematopoietic cells specific protein [172]. In order to study the functional importance of WASP, WASP-deficient mice have been generated by

several groups to study the development of hematopoietic cell lineages and the function of WASP in various immune cells (Fig. 1.3) [35,201,215,248].

1.5.1 Role of WASP in lymphoid development

The development of early progenitors of hematopoietic cell lineages was found to be normal in WASP deficient mice [60,140,201,248]. Furthermore, there were no consistent defects observed in the haematopoiesis in WAS patients. This might be because of presence of other WASP family members (N-WASP, WAVE) that may serve some redundant function with WASP [60,236]. However, development of mature differentiated WASP-deficient hematopoietic cells was impaired as the circulating T-cells numbers was found to be reduced in WASP^{-/-} mice as well as in WAS patients [160,171,201].

Figure 1.3 Role of WASP in immune cells. WASP plays an important role in myeloid and lymphoid immune cells functions such as phagocytosis and podosome formation which require WASP dependent actin polymerization. WASP also behaves as an adaptor protein which assembles signalling complexes downstream of TCR or integrin engagement [215].

Naturally occurring reversion mutations have been reported in 11% of WAS patients who restored the WASP expression to normal level. This high frequency of multiple distinct reversion mutants within a patient indicate that there is a selective advantage for cells that express WASP in humans. However, this is restricted to mature lymphocytes such as T-cells, B-cells and natural killer (NK) cells, but not for myeloid lineages [11,62,230]. Further studies need to be done to investigate the hematopoietic lineages and stages of development in which WASP is crucial for homeostatic regulation.

1.5.2 Role of WASP in the innate immune system

Phagocytosis is a major mechanism used by cells of the innate immune system to remove pathogens in a non-specific manner. Phagocytosis is mediated by formation of phagocytic cups that are enriched in actin [222-223] and formation of such cups requires functional WASP expression. These structures undergo internalisation, allowing ingestion of extracellular particles, forming phagosomes inside the cell [210]. The phagocytic cells of immune system include macrophages, dendritic cells and neutrophils. Impaired phagocytosis has been observed in these WASP-deficient phagocytic cells suggesting the importance of WASP in phagocytosis [124,130,248]. The role of WASP in phagocytosis and phagocytic cup formation was further confirmed by knocking down WASP expression using siRNA [222-223]. Beside defects in phagocytosis, WASP-deficient human or murine macrophages and DCs fail to form podosomes [37,97]. Podosomes are adhesive structures that contain an actin core, surrounded by focal adhesion proteins like vinculin, talin and paxillin [215]. Beside a lack of podosomes formation in WASP-deficient DCs

and macrophages, these cells were also defective in formation of filopodia and sustained lamellipodia at their leading edges, resulted in impaired chemotactic response of these cells towards specific chemo-attractants such as macrophage chemo-attractant CSF-1 [18,37,252]. The chemotactic defect of DCs was confirmed using WASP-deficient mice in which homing of DCs from periphery to the T-cells zones of secondary lymphoid tissues was found to be impaired [32,33,65]. Beside the requirement of actin cytoskeleton remodelling, integrin dependent cell adhesions are also essential for efficient cell migration. A defect in $\beta 2$ integrins clustering has been observed in WASP-deficient DCs and macrophages [36]. Defective $\beta 2$ integrin clustering due to deficiency of WASP in both human and murine neutrophils led to impairment in $\beta 2$ integrin dependent neutrophils functions such as neutrophil activation, degranulation and respiratory burst [247]. The role of WASP in NK cells have been reported previously. NK cells contain proteins such as perforin and granzymes which they use to kill virus infected cells and tumours cells. Defective cytotoxic activity has been observed in WASP deficient NK cells which could be due to impairment in immune synapse formation between NK cells and target T-cells [79,165].

1.5.3 Function of WASP in the adaptive immune system

Both humoral and cell-mediated immunity were found to be defective in WAS patients. WASP-deficient T-cells have abnormal cytoskeleton architecture with fewer microvilli like surface projections compared to WT T-cells [77]. WASP-deficient T-cells had defective chemokine responses [200]. Moreover, a marked reduction in peripheral T-cells number was observed in WAS patients

that could be due to impaired homing of WASP-deficient T-cells to secondary lymphoid tissues [82,171]. WASP deficiency in T-cells also causes impaired actin polymerization and recruitment of other signalling proteins to the immunological synapse upon TCR ligation [70,190]. As a result of defective TCR activation, T-cell proliferation and IL-2 gene transcription were impaired in WASP deficient T-cells [43,143,201] which could be the consequence of immune synapse (IS) disruption as WASP functions as a scaffold protein within a signalling complex [70]. Restoration of WASP expression by using lentiviral or retroviral gene therapy method rescued the defects observed in T-cells from WAS patients [49,229]. WASP-deficient CD4⁺ or CD8⁺ T-cells failed to express Th₁ cytokines after TCR stimulation suggesting that WASP may also have a role in transcriptional regulation of genes in T-cells [146].

Despite the defects observed in humoral immunity of WAS patients, a clear understanding about the role of WASP in B-cell functions is lacking. Although, B-cell development appeared normal in WAS patients, the number of marginal zone B-cells was observed to be less which may be due to abnormal homing of B-cells to or retention of them in the marginal zone [140,234,235]. WASP has been shown to be required for B-cell surface integrins clustering following B-cell receptor (BCR) activation. Therefore, WASP-deficient B-cells failed to form immunological synapse, which compromised cognate B-cell activation [140,234]. B-cells from WAS patients have decreased response to the polysaccharide antigens [160]. Failure to produce a correct immune response against such antigens make WAS patients susceptible to bacterial infection. Moreover, increased levels of IgA, IgE and decreased levels of circulating IgM

immunoglobulins were observed in WAS patients [160,209]. Thus, all these studies signify WASP's functions in B-cell homeostasis as well as development of humoral immunity.

1.6 Regulation of WASP by WASP binding proteins.

WASP is a multi-domain protein, which interacts with numerous cytoplasmic molecules, resulting in successful integration of extracellular signals and Arp2/3 complex activation (Fig. 1.4).

1.6.1 Activation of WASP by Cdc42, PIP2 and Toca1

In resting cells, WASP exists in an auto-inhibited closed conformation which is stabilised by interaction with WIP. This intramolecular closed conformation of WASP is mediated by interaction between its basic region and GBD with VCA domain, thus prevents Arp2/3 complex dependent actin polymerization [111,125]. GTP loaded Cdc42 was the first protein identified that binds to GBD of WASP. Binding of Cdc42 results in WASP activation and actin polymerization via Arp2/3 complex [14,88,111,211]. Cdc42 regulates wide range of cellular processes including cell division, cell-substrate adhesion, filopodia formation, chemotaxis, vesicular transport, microtubule dynamics and gene expression [85,99,100,155]. However, subsequent studies have shown that Cdc42 alone is insufficient for WASP activation [88]. Phosphatidyl inositol 4, 5-bisphosphate (PIP2) alone was reported to activate the actin nucleation activity of purified WASP and addition of Cdc42 further enhanced the actin polymerization activity of WASP [88], suggesting that PIP2 acts co-operatively with Cdc42 to activate WASP. A similar synergism between Cdc42

and PIP2 was observed for N-WASP activation [186]. However whether PIP2 could induce WASP activation has now become controversial. Recently, it has been reported that PIP2 vesicles alone have no effect on WASP activation. Infact, PIP2 has been shown to inhibit Cdc42 mediated WASP activation [217].

Figure 1.4 Schematic diagram showing functional domains of WASP and its conformation in presence of different regulators [96]. In inactive state, VCA region of WASP interacts with GTPase binding domain (GBD) and this close conformation of WASP is stabilized by interaction with WIP. The closed conformation of WASP is disrupted when Cdc42 interacts with GBD. The VCA region is subsequently unmasked and is free to promote actin polymerization by activating the Arp2/3 complex. Tyrosine kinases phosphorylate WASP at residue 291 (Tyr²⁹¹), which enhance the actin polymerization activity of WASP. **WBD:** WASP/N-WASP binding domain, **V:** Verproline homology domain.

Toca1 (Transducer of Cdc42 dependent actin assembly) was identified and characterized as an essential co-factor for Cdc42-induced actin assembly via N-WASP in cell extract system [89]. Toca1 is a member of PCH (Pombe Cdc15 homology) family of proteins which contains N-terminal F-BAR domain that

can induce membrane invagination, a central homology region that interacts with Cdc42 and a C-terminal SH3 domain that binds to N-WASP. Previous report suggests that recombinant N-WASP can be activated by Cdc42 *in vitro*, however inside cells another N-WASP interacting proteins such as WIP regulates the activity of N-WASP. Toca1, together with Cdc42, have been shown to activate N-WASP-WIP/CR16 complex [89]. Similar experiments on WASP have not been performed; however WASP has been reported to interact with other two members of PCH family of protein namely: CIP4 [216] and FBP17 [226]. The PRR of WASP interacts with CIP4 via its SH3 domain which was shown to regulate WASP localization to microtubules [216]. Similarly, FBP17 has been shown to recruit WASP to plasma membrane which is essential for podosomes and phagocytic cups formation in macrophages [226]. Recently, using bimolecular fluorescence complementation assay, it has been shown that Toca1 is able to open up the closed conformation of WASP even in the presence of WIP [125].

1.6.2 Cdc42 independent WASP activation

There are several evidences for Cdc42 mediated WASP and N-WASP activation both *in vitro* and *in vivo* has been reported. However, depending on specific cell types or experimental system, it has also been reported that involvement of Cdc42 in WASP/N-WASP activation can be bypassed. T-cell receptor induced cell proliferation, actin polymerization, and localization of WASP at T-cell: antigen presenting cell (APC) contact site were found to be normal in T-cells expressing GBD deleted WASP [21,27,44]. Furthermore, experiments using WASP mutant (WASP^{H246D}) or N-WASP mutant (N-

WASP^{H208D}) which abolished WASP/N-WASP interaction with Cdc42 displayed normal *in vitro* actin polymerization or *in vivo* actin patches (WASP) and microspike (N-WASP) formation [75,105]. All these studies signify that although Cdc42 functions in transmitting cell surface receptor signals to actin cytoskeleton, there are other signalling networks that activate WASP and promote actin polymerization.

1.6.3 Regulation of WASP by phosphorylation

Phosphorylation of WASP has been reported as another regulatory mechanism of WASP activation. Both tyrosine (Tyr²⁹¹) and serine phosphorylation (Ser⁴⁸³ and Ser⁴⁸⁴) take place at distinct positions on WASP molecule [30,57]. Serine phosphorylation is mediated by casein kinase II which enhances the VCA domain affinity to Arp2/3 complex required for optimal activity of WASP [57]. Serine phosphorylation is constitutive whereas Tyr²⁹¹ phosphorylation is regulated. There are seven tyrosine residues in WASP which can be phosphorylated, however, tyrosine 291 phosphorylation is critical for WASP regulation has been shown in both *in vitro* and *in vivo* assays [16,21,58]. Two families of non-receptor tyrosine kinases, namely the Src family of tyrosine kinases (Fyn, Hck) and Tec family of tyrosine kinases (Itk, Btk) bind to the PRR of WASP and phosphorylate WASP (Tyr²⁹¹) or N-WASP (Tyr²⁵⁶) at their tyrosine residue [16,58]. Phosphorylation of this tyrosine residue has been shown to disrupt the auto-inhibited conformation of WASP and activates WASP *in vitro* and *in vivo* [58,218]. Many groups have studied the importance of WASP tyrosine phosphorylation by using WASP phosphomimetic mutant (WASP^{Y291E}) or non-phosphorylatable mutant (WASP^{Y291F}) of WASP as these

point mutations only affect the charges on the residues without altering binding of other WASP interactors. For example, expression of WASP^{Y291E} mutant in macrophages enhanced filopodia formation [58], accelerated the rate of podosome assembly and F-actin content per podosome area [69]. However, recently it has been shown that WASP^{Y291E} knock-in mice have similar traits as WASP knockout mice and WASP^{Y291F} knockin mice [30]. Also, compared to wild-type WASP, the expression of WASP^{Y291E} was found to be reduced implying that phosphorylation may target WASP for proteasome mediated degradation [30]. The relationship between tyrosine phosphorylation and Cdc42 mediated WASP/N-WASP activation was extensively studied by Torres and Rosen [218-219]. They found that binding of Cdc42 destabilizes the closed conformation of WASP molecule and makes Tyr²⁹¹ accessible to kinases. There are some *in vivo* data supporting these findings where WASP phosphorylation by Lck (Src family of tyrosine kinase) and Btk in mast-cells is enhanced in the presence of constitutively active Cdc42 [81]. Furthermore, WASP^{L270P} mutant (identified in XLN patients) is present in an open conformation, because of which it displayed increase in tyrosine phosphorylation [68, 170]. Phosphorylated WASP can bind to the SH2 domain of Src or Tec family of kinases with considerable affinity, resulting in disruption of GBD interaction with VCA domain [69]. Data from other studies does not support cooperation between Cdc42 and tyrosine kinases in WASP activation. In one study, it has been reported that Cdc42 does not affect Hck mediated WASP activation in COS7 cells [58]. Similarly, Cdc42 expression had no effect on phosphorylation of N-WASP by Fyn in PC12 cells [207]. This could be due to the presence of alternative pathways for WASP activation.

Therefore, further studies need to be done to elucidate the co-operative role of Cdc42 and tyrosine phosphorylation mediated activation of WASP.

1.6.4 Role of adaptor proteins in the activity of WASP

Adaptor proteins containing SH3-SH2 domain are a small group of proteins that are deficient in any intrinsic enzymatic activity themselves, but mediate protein-protein interactions and drive the formation of proteins complexes. These proteins can bind to both polyproline motifs which are generally found in the regulators of cytoskeleton via their SH3 domain and their SH2 domain can interact with phosphorylated tyrosine residues. Structurally, these proteins have one SH2 domain and one or multiple SH3 domains [50]. Nck1 and Grb2 are the first proteins to be reported which interact with polyproline region of WASP [185,196]. Nck1 and Grb2 have both been identified to bind diverse range of effector proteins and are involved in a variety of cellular functions. Recently, it has been reported that Nck1 together with PIP2 but independent of Cdc42 can activate WASP and enhance *in vitro* actin polymerization [167]. In contrast, Grb2 functions with Cdc42 in regulating N-WASP activation [45]. Recently, it has been identified that WASP dimerisation is mediated by multiple SH3 domains of Nck1 [167]. WASP dimer binds to Arp2/3 complex with higher affinity, thus enhancing the actin polymerization activity of WASP. Apart from WASP, Nck also interacts with WIP and p21 activated kinases 1 (PAK1) suggesting its central role in actin dynamics [10,31]. Nck1 also mediates WASP recruitment to the immunological synapses via interacting with adaptor protein Slp-76 [44]. Slp-76 is a scaffold protein which brings Nck-WASP complex in the proximity of Vav1-Cdc42-GTP complex [249]. Another SH3 domain containing protein PST-PIP has been shown to regulate

WASP activity by interacting with regulatory protein PTP-PEST (a tyrosine phosphatase). PST-PIP acts as a scaffold protein which binds to WASP and PTP-PEST. This leads to dephosphorylation of WASP at Tyr²⁹¹, resulting in reduction of actin polymerization activity of WASP [20]. Cortactin is another SH3 domain containing protein which activates WASP and co-localizes with WASP at immunological synapse [135]. Sorting nexin 9 (SNX9) is a peripheral membrane protein which regulate endocytosis [46,238]. WASP has been shown to interact with SNX9 and this complex is involved in clathrin mediated endocytosis of CD28 following TCR activation [19]. Similarly, intersectin 2 (an endocytic adaptor protein) associates with WASP and regulate T-cell antigen receptor endocytosis [139]. Vasodilator-stimulated phosphoprotein (VASP) is another protein implicated in actin dynamics and found to be localized at the site of actin assembly. VASP binds WASP directly via interaction of its VASP-EVH1 domain with the PRR of WASP, thus induces the actin polymerization and membrane protrusion at the leading edge of migrating cell [47]. Together, these studies imply that polyproline region of WASP regulates WASP activity and its localization via interaction with a number of SH3 domain containing adaptor proteins and tyrosine kinases.

1.7 WASP-WH1 domain interacting proteins

1.7.1 The verprolin family of proteins

The human verprolin family of proteins include WIP, WIRE and CR16 [13,106,182]. The verprolin family of proteins regulates actin cytoskeleton reorganization independently of WASP or by regulating WASP family of proteins [8]. WIP is a 503 amino acid residue protein which was first identified

as a WASP binding protein in a yeast two hybrid screen using WASP as bait [182]. The expression of WIP and WIRE is ubiquitous while CR16 expression is restricted to the brain, heart, lungs and colon [13,106,182,232]. WIP is highly expressed in hematopoietic cells and WIP^{-/-} mice have impaired T-cell functions [7,182].

Figure 1.5 Schematic representation of the human verprolin family: WIRE, CR16 and WIP. All the verprolins are characterized by the presence of G-actin binding V domain at N-terminus and WASP binding domain (WBD) at the C-terminus. Verprolin family of proteins also contain PRR which interacts with SH3 domain containing proteins. **WIRE:** WIP related, **CR16:** Glucocorticoid-regulated gene product, **WIP:** WASP interacting protein, **V:** Verprolin homology domain, **WBD:** WASP binding domain.

WIP has a verprolin homology domain (V domain) at its N-terminus which contains a KLKK motif (mediate interaction with actin) and three XPPPPP sequences (mediate interaction with profilin). WIP has multiple proline rich regions which interact with SH3 domain containing proteins such as Nck and Cortactin [10,112]. WIP contains a C-terminal WASP binding domain (WBD) which interacts with the WH1 domain of WASP (Fig. 1.5) [52,246]. WIP has been shown to stabilize actin filament and promote actin bundling [136]. When overexpressed in B-cells, it increased F-actin content as well as promoted subcortical patches formation whereas, WIP null B-cells have profound defect in subcortical patches formation [7,182]. In spite of disrupted actin network in

WIP null B-cells, the WIP null B-cells are able to mount normal antibody responses. However, the functions of WIP null T-cells were found to be significantly impaired. T-cells from WIP-deficient mice were impaired in conjugate formation, proliferation, IL-2 secretion, polarization and chemotactic migration towards SDF-1 α [8,76].

1.7.2 Significance of WASP-WIP interaction

Importance of WIP and WASP interaction is underscored by the fact that approximately eighty percent of missense mutations of WASP in Wiskott Aldrich syndrome patients are identified in WASP^{WH1} domain [96] and some of these mutations abolished WASP-WIP interaction [205]. Therefore, WIP and WASP interaction is critical for WASP functions. It has been reported that WIP stabilizes WASP and prevent its degradation as WIP^{-/-} mice have reduced WASP expression in their T-cells and WASP expression was restored by the reintroduction of WIP expression [64,120,115]. Recently, a WAS patient was reported to have normal WASP mRNA level, but the expression of WASP protein was undetectable. The reason was due to the presence of a nonsense mutation in the *WIPF1* gene. Introduction of a wild-type WIP into the patient's T-cells restored WASP expression [119]. In another study, WIP-derived 41 a.a. long peptide (called as nanoWIP), when introduced in to the B-cells derived from XLT patients with missense mutation in the WIP binding domain of WASP corrected their actin cytoskeleton defects [137]. WIP has also been shown to localize WASP at TCR and promote WASP activation [190].

1.7.3 Calcium and integrin binding protein 1 (CIB1)

Beside WIP, CIB1 has been shown to interact with WH1 domain of WASP [225]. CIB1 was first identified as a binding partner of the cytoplasmic tail of platelet integrin $\alpha\text{IIb}\beta 3$ in a yeast-two hybrid screen [150]. CIB1 (calmyrin, and KIP) contains four EF hand motives, two of which are located in C-terminus half of protein and have been shown to bind calcium with high affinity [78,241]. It has been discovered that the N-terminal region of CIB1 binds directly to the N-terminal region of WASP at residues 1-105 and this WASP-CIB1 complex is essential for both the binding of platelet integrin $\alpha\text{IIb}\beta 3$ to fibrinogen as well as the stimulation of actin polymerisation in the event of platelet aggregation after stimulation [224]. Disruption of WASP-CIB1 complex due to presence of missense mutations in the WASP^{WH1} domain reduced the $\alpha\text{IIb}\beta 3$ -mediated platelet adhesion and aggregation [225], thus, suggesting an explanation for the common symptom of bleeding in WAS patients [224]. CIB1 is widely distributed and its other physiological functions which are independent of platelet-specific integrin have been characterized. CIB1 is known to interact with presenilin-2 [203], Rac3 [83], DNA-dependent protein kinase [239], fibroblast growth factor and serum-inducible kinases [107]. It has been reported that p21 protein-activated kinase 1 (PAK1) and Focal Adhesion Kinase (FAK) are direct binding partners of CIB1 [123,151]. PAK1 is a serine/threonine kinase that was initially identified as a downstream effector of the GTPases Rac and Cdc42 [72,250]. Its amino acid residues 130 to 137 were found to be important for CIB1-PAK1 interaction [123]. One study suggested that PAK1 activates and phosphorylate the targets that are involved in actin cytoskeleton remodelling after its recruitment to the T-cell: APC

contact site during the dynamic process of immunological synapse formation [175]. Role of CIB1 in cell migration has been studied. CIB1 has been shown to interact with FAK and induces migration of CHO cells. Its overexpression in CHO cells, up-regulated focal adhesion complexes formation. Moreover, expression of dominant negative form of FAK (FRNK: FAK-related non kinase) along with CIB1 in CHO cells inhibited CIB1 induced cell migration suggesting that CIB1-FAK interaction is important for platelet spreading through regulation of FAK [151]. Using *Cib1*^{-/-} mice, it has been found that CIB1 functions in facilitating tumour growth and tumour-induced angiogenesis [245]. CIB1 is shown to be essential for spermatogenesis [243]. Later it has been reported that *Cib1*^{-/-} mice did not display any significant defect in platelet function as expression of CIB1 homologue CIB3 was increased in these CIB1^{-/-} megakaryocytes [66]. Recently, using *Cib1*^{-/-} mice, another group has shown that CIB1 regulates megakaryocytes ploidy, adhesion and migration towards SDF-1 α gradient [116]. It has been shown that CIB1-Plk3 interaction [149], is involved in regulation of cell cycle [255]. Plk3 activity was inhibited because of upregulation of CIB1 expression in cancer cells, resulting in abnormal cell cycle regulation in breast cancer cells [149]. Most of the studies on interaction of CIB1 with WASP have been performed in platelets as they are the only cells in which WASP, CIB1 and integrin α IIb β 3 are expressed together. But, CIB1 is widely distributed and may be involved in other regulatory pathways. Therefore, identification of role of WASP-CIB1 interaction in the cells of hematopoietic system will provide a better understanding of WASP-CIB1 regulated pathways.

1.8 Molecular and functional differences between N-WASP and WASP

N-WASP and WASP share more than 50% sequence homology, having similar domain organization, similar binding partners and similar basic functions. In resting cells, both WASP and N-WASP exist in an auto-inhibitory closed conformation [111,186] which prevents the interaction between VCA regions with Arp2/3 complex. N-WASP has two V- domains thus, the C-terminal domain (VVCA) can interact with two actin monomer, resulting in superior actin polymerization activity compared to WASP [142,240,244]. Cdc42 interacts with GBD of WASP and N-WASP and relieve their auto-inhibition [111,178,187] whereas Rac1 (member of Rho family of GTPase) activates WAVE 1-3 [242]. Recently, it has been shown that Rac1 is a more effective activator of N-WASP, while Cdc42 is a more potent activator of WASP [217]. Previously it has been reported that Phosphoinositides 4, 5 biphosphate (PIP2) synergize with the Cdc42 and mediated WASP and N-WASP activation [88,186]. However, Tomasevic et al have shown that PIP2 negatively regulate WASP but not N-WASP activation mediated by Cdc42 [217]. Additionally, Grb2 and Nck1 are found to be more effective activator of N-WASP than WASP [217]. WASP plays a unique role in osteoclasts sealing zone formation which cannot be compensated by N-WASP expression in WASP-deficient osteoclasts [39].

Studies using pathogenic microorganism revealed that WASP family of proteins may not compensate for each other completely. For example: motility of *Shigella* cannot be rescued by expression of WASP in N-WASP-deficient

embryonic stem cell derived fibroblast like cells [202]. However, WASP and N-WASP could compensate for each other's absence for the motility of vaccinia virus and *Mycobacterium marinum* [204]. WASP-deficient macrophages were defective in chemotaxis and unable to form podosomes on their ventral surface [97,223]. Similar to WASP localization in podosomes, N-WASP has been shown to localize in podosome-like structures called invadopodia formed by certain aggressive cancer cells or Src-transformed cells [28,128]. Because WASP and N-WASP are closely related proteins and co-expressed in hematopoietic cells, it can be hypothesised that WASP and N-WASP may be able to compensate for each other functions. Shcherbina et al have shown that early events of platelet activation such as filopodia formation, shape change, actin polymerization were normal in platelets from WAS patients [195]. These early rapid Arp2/3 dependent cytoarchitectural responses in WASP deficient platelets could be mediated by N-WASP. However, late phase events dependent on WASP degradation by calpain were abnormal in WASP deficient platelets [195]. Calpain-mediated WASP degradation was found to be crucial as inhibition of calpain by peptide inhibitors has been shown to reduce podosomes turnover rate, resulted in impairment in motility of dendritic cells [38]. Compared to WASP, N-WASP is insensitive to calpain [195]. N-WASP expression in WASP^{-/-} hematopoietic stem cells partially restored the T-cells defects [114]. T-cell development in WASP and N-WASP double-knockout mice (DKO) was found to be impaired resulting in reduced numbers of T lymphocytes. In contrast, mice that were only deficient in WASP had relatively normal T-cell development suggesting that WASP and N-WASP play overlapping roles in the development of T-cells [60]. It has been reported

that N-WASP can compensate for WASP-deficiency in macrophage functions such as podosome formation, matrix degradation. However, it failed to rescue the defect in Fcγ-R mediated phagocytosis [97,170]. All these studies suggest that WASP and N-WASP have both unique and overlapping roles. Therefore, further studies need to be done in order to identify the molecular and functional differences between WASP and N-WASP.

1.9 Objectives of the project

WASP family of proteins play a crucial role in actin cytoskeleton reorganization. Although WASP and N-WASP are homologues N-WASP can only partially restore the functional defects of WASP-deficient T-cells. Thus, suggesting both unique as well as redundant functions of WASP and N-WASP. It is possible that the activities of WASP and N-WASP may be regulated by different mechanisms. Thus, characterizing the molecular and functional difference between WASP and N-WASP will lead to a better understanding of the molecular defects giving rise to Wiskott-Aldrich Syndrome. Therefore, the aims of this project are:

(1) Identification and characterization of WASP domains which are crucial for its function in hematopoietic cells that causes functional differences between WASP and N-WASP. Using WASP-deficient Jurkat T-cells as a system, WASP mutants and WASP/N-WASP chimeras will be expressed and the molecular and functional differences between WASP and N-WASP will be studied.

(2) The other aim of this study is to characterize WASP^{WH1} domain mutations which cause WAS. The WH1 domain of WASP comprises only 20% of the

protein but 80% of mutations causing WAS/XLT are found in this region. Some of these mutations affect WASP-WIP interaction and/or WASP protein stability. It is possible that besides WIP, the WH1 domain of WASP may interact with unknown proteins which regulate WASP functions. Therefore, by identifying WASP^{WH1} domain interacting proteins (using cDNA library screening) will lead to identification of new mechanism of WASP regulation.

(3) Two missense mutations in the proline rich region of WASP (S339Y/P373S) have been reported to cause WAS. Proline rich region of WASP is important for mediate interactions with SH3 domain containing proteins and regulate WASP activation and localization to the immunological synapse. Therefore, characterizing the molecular defects caused by mutations (WASP^{P373S}, WASP^{S339Y}) at proline rich region of WASP will lead to a better understanding of the regulation of WASP by SH3 domain containing proteins. These WASP^{PRR} mutants will be used to analyse how these mutations affect the conformation of WASP, interaction with SH3 domain containing proteins, Tyr²⁹¹ phosphorylation of WASP and ability to rescue the chemotactic defects of WASP-deficient Jurkat T-cells.

Together, these findings will shed new light on the molecular and functional differences between WASP and N-WASP. Also, characterizing the WASP missense mutations will provide mechanisms of regulation of WASP by WASP interacting proteins as these mutations might affect WASP interactions with known WASP binding partners. The dynamics of WASP and its regulation studied in this project can potentially aid in uncovering new strategies to treat WAS or XLT.

2. Materials and Methods

2.1 Materials

2.1.1 Plasmids

The lists of plasmids used in this study are listed in Appendix 1.

The lists of primers used in this study are listed in Appendix 2.

2.1.2 Commercial Vectors

For DNA subcloning experiments, the vectors pUC19 and pUC18 were used.

For yeast two hybrid assays, vectors pACT2 and pAS2-1 were used for the expression of Gal4p activation domain (Gal4p-AD) and the Gal4p binding domain (Gal4p-BD) fusions respectively. Vectors YEplac112, YEplac181 and YEplac195 which contain different selective markers, were used to express proteins in *Saccharomyces cerevisiae* cells. For expression of proteins in mammalian cells, the vectors pFIV-H1/U6-copGFP and PEPCG were used.

Table 2.1 summarized the commercial vectors used and their applications.

Vector	Resistant gene	Selective marker	Expression host	Used in
pUC18	Amp	-	Bacteria	DNA subcloning
pUC19	Amp	-	Bacteria	DNA subcloning
pACT2	Amp	Leu	Bacteria/Yeast	Yeast two hybrid
pAS2-1	Amp	Trp	Bacteria/Yeast	Yeast two hybrid
YEplac112	Amp	Trp	Bacteria/Yeast	Expression in yeast cells
YEplac181	Amp	Leu	Bacteria/Yeast	Expression in yeast cells
YEplac195	Amp	Ura	Bacteria/Yeast	Expression in yeast cells
pFIV-H1/U6	Amp	-	Bacteria/Mammalian	Expression in mammalian
PEPCG-CopNeo	Kan	-	Bacteria/Mammalian	Expression in mammalian

Table 2.1. List of commercial vectors used.

2.1.3 Bacterial strains

The bacterial strain, *Escherichia coli* (*E. coli*) DH5 α was used for plasmids amplification and DNA subcloning.

2.1.4 Yeast strains

Yeast strain PJ69-4A (*MATa his3 leu2 ura3 trp1 gal 4 Δ gal80 Δ met2::GAL7-lacZ GAL2-ADE2 LYS2::GAL1-HIS3*) is used as described in [101]. Yeast strain IDY166 (*MATa his3 leu2 ura3 trp1 las17 Δ ::URA3*) is used as described in [152].

2.1.5 Mammalian cell lines

HEK293T, Phoenix Amphotropic packaging cell line (for reterovirus production) and Jurkat T-cells (clone E6-1) were purchased from American Type Culture Collection (ATCC, USA).

2.1.6 Mammalian cell lines, culture media and plasticware

Cell lines	Media
HEK293T	Dulbecco's Minimal Eagles medium (DMEM) - Hyclone (Thermo Scientific)
Amphotropic cells	DMEM Medium
Jurkat T-cells (Clone E6-1)	Roswell Park Memorial Institute medium (RPMI-1640) – Hyclone (Thermo Scientific)

Table 2.2 List of mammalian cell lines and the respective media used.

Reagents	Source
Fetal Bovine serum (FBS)	Hyclone (Thermo Scientific)
Penicillin-streptomycin	Hyclone (Thermo Scientific)
SDF-1 α	Sigma (USA) (cat no: 300-28A)
G418	PAA Laboratories (cat no: P02-012)
Anti-Human CD3 clone OKT3	eBioscience (cat no:14-0037-82)
Anti-Human CD28 clone CD28.2	eBioscience (cat no:14-0289-82)
Fibronectin from Human plasma	Sigma

Table 2.3 List of antibiotics and supplements used.

2.1.7 Antibodies

The antibodies used in this study are listed below (Table 2.4 & 2.5) and their respective dilutions used for the Western blot experiments are specified. The secondary antibodies were conjugated to Horseradish peroxidase (HRP).

2.1.7.1 Primary antibodies

Antibody	Source	Dilution for WB	Blocking conditions
Mouse Anti-GFP polyclonal antibody	Clontech Laboratories, Mountain View, CA, USA	1:1000	5% skim milk
Mouse Anti-GAPDH monoclonal antibody	Ambion, Austin, TX, USA (AM4300)	1:10000	5% skim milk
Rabbit Anti-His polyclonal antibody	Delta biolabs, Gilroy, CA, USA (DB063)	1:1000	5% skim milk
Rabbit Anti-WASP monoclonal antibody	Santa Cruz (sc-5300)	1:1000	5% skim milk
Rabbit Anti-N-WASP polyclonal antibody	This study	1:1000	5% skim milk
Mouse Anti-Phosphotyrosine clone-4G10	Upstate biotechnology, Lake placid, NY, USA (05-312)	1:1000	5% BSA
Goat Anti-TOCA-1 (E-17) polyclonal antibody	Santa Cruz (sc-103902)	1:1000	5% skim milk
Rabbit anti-Hexokinase antibody	This study	1:0000	5% skim milk
Mouse anti- β 1 integrin antibody	BD Transduction Laboratories (610468)	1:500	3% skim milk

Table 2.4: Primary antibodies and there dilutions used for Western blot analysis and immune fluorescence.

2.1.7.2 Secondary antibodies:

Antibody	Source	Dilution for WB
Anti-mouse IgG-HRP	Sigma, St. Louis, Mo, USA	1:10000
Anti-rabbit IgG-HRP	Sigma, St. Louis, Mo, USA	1:10000
Donkey anti goat IgG-HRP	Santa Cruz	1:5000

Table 2.5 List of secondary antibodies used in this study.

Alexa Fluor 488 and 594 were purchased from Molecular Probe

2.1.8 Bacterial and yeast culture media

2.1.8.1 Luria-Bertani (LB) broth

1% bacto-tryptone, 0.5% bacto-yeast extract and 0.5% NaCl was dissolved in distilled water and autoclaved.

2.1.8.2 LB agar plates

2% of bacto-agar was added to LB broth and autoclaved. Medium was left at room temperature to cool to below 60°C and 25 ml of medium was poured into each plate and left to solidify.

2.1.8.3 YPUAD medium

2% of bacto-peptone, 1% of bacto-yeast extract, 2% glucose, 0.008% of adenine hemisulphate and 0.004% of uracil were dissolved in distilled water and autoclaved.

2.1.8.4 YPUAD agar plates

2% of bacto-agar was added to YPUAD medium and autoclaved. Medium was left at room temperature to cool to below 60°C and 25 ml of medium was poured into each plate and left to solidify.

2.1.8.5 Synthetic defined (SD) medium

0.67% of Yeast Nitrogen base, 2% of Glucose and the different concentrations of the dropout supplements as described in Table 2.6 were dissolved in distilled water and autoclaved.

Supplements	Amount in dropout powder (g)	Final working concentration (mg/L)
Adenine	2.5	40
L-arginine (HCl)	1.2	20
L-aspartic acid	6.0	100
L-glutamic acid (monosodium salt)	6.0	100
L-histidine	1.2	20
L-isoleucine	1.8	30
L-leucine	3.6	60
L-lysine (mono-HCl)	1.8	30
L-methionine	1.2	20
L-phenylalanine	3.0	50
L-serine	22.5	375
L-threonine	12.0	200
L-tryptophan	2.4	40
L-tyrosine	1.8	30
L-valine	9.0	150
Uracil	1.2	20

Table 2.6 List of supplements used in the SD medium [23].

2.1.8.6 SD agar plates

2% of bacto-agar was added to SD medium and autoclaved. Medium was left at room temperature to cool to below 60°C and 25 ml of medium was poured into each plate and left to solidify. For Yeast Two Hybrid assay, SD agar plates with 2 mM 3-AT but without histidine were used. 3-AT (1M stock concentration dissolved in distilled water) was filter-sterilised and added to SD medium containing 2% bacto-agar without histidine to a final concentration of 2 mM.

2.1.9 Enzymes and kits

The restriction endonucleases obtained from Fermentas (Hanover, MD, USA) or New England Biolabs (NEB) (Ipswich, MA, USA) and T4 DNA ligase and buffer from Fermentas (Hanover, MD, USA) were used for DNA subcloning.

For site directed mutagenesis (SDM) experiments, QuikChange Site-Directed Mutagenesis kit was purchased from Stratagene (La Jolla, CA, USA). For isolation of plasmid DNA from *E. coli* cells, QIAprep Spin Miniprep Kit (Qiagen) (Valencia, CA, USA) was used while for the isolation of RNA from mammalian cells, RNA-Sol from E.Z.N.A DNA/RNA isolation system was used. QIAquick Gel extraction Kit (Qiagen) was used to extract DNA from agarose gels. The Bradford solution (Bio-Rad DC protein assays) used for measuring protein concentration was purchased from Bio-Rad (Hercules, CA, USA). The kits used for developing western blot include Millipore Immobilon (Millipore Corp, Billerica, MA, USA) and Amersham ECL Plus (GE healthcare, Buckinghamshire, UK).

2.1.10 Polymerase chain reaction (PCR)

DNA polymerase KAPA HiFi from KAPA biosystems (Woburn, MA, USA) or DNA polymerase from BIOTOOLS (Madrid, Spain) was used. dNTPs (dTTP, dATP, dGTP and dCTP) mixtures were obtained from Invitrogen (Carlsbad, CA, USA). For Quantitative Real time PCR analysis, Maxima SYBR Green/ROX qPCR master mix was purchased from Fermentas (Thermo scientific).

2.1.11 Chemicals and reagents

2.1.11.1 DNA work

DNA loading ladder and 6X DNA loading buffer were obtained from NEB (Ipswich, MA, USA). Ampicillin and Kanamycin used in selective plates and medium were purchased from Sigma-Aldrich (St. Louis, Mo, USA).

2.1.11.2 Protein work

Phenylmethylsulfonyl fluoride (PMSF), 1, 4-Dithiothreitol (DTT) and protease inhibitors complete-mini protease tablets used in cell lysis were bought from Roche (Indianapolis, IN, USA). Protein molecular weight marker was purchased from Bio-Rad Laboratories (Hercules, CA, USA) and nitrocellulose membrane used for western blot was obtained from Bio-Rad (Hercules, CA, USA). The rest of the chemicals for protein work were purchased from Sigma Aldrich (St. Louis, Mo, USA).

2.1.12 General buffers and solutions

2.1.12.1 DNA subcloning experiments

2.1.12.1.1 1X Tris-acetate-EDTA (TAE)

40 mM Tris-acetate (pH 8.3), 1.0 mM EDTA. The 1X TAE buffer was used in both making agarose gel and as a running buffer.

2.1.12.1.2 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 70°C by storing in an oven.

2.1.12.2 Western blots

2.1.12.2.1 10% SDS

10 g of SDS was dissolved in 10 ml of deionised water.

2.1.12.2.2 10% Ammonium persulphate

1 g of ammonium persulphate was dissolved in 10 ml of deionised water.

2.1.12.2.3 5X Tris-glycine electrophoresis buffer

0.125 M Tris, 0.96 M glycine, 0.5% SDS, the pH of the solution was adjusted to 8.3.

2.1.12.2.4 Membrane transfer buffer

25 mM Tris (pH 8.3), 192 mM glycine, 0.1% SDS and 20% methanol.

2.1.12.2.5 2X SDS-PAGE gel-loading buffer

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

2.1.12.2.6 1M Tris buffer

121.12 g of Tris was dissolved in 1 litre of deionised water and the pH of the solution was adjusted to 8.0 using HCl.

2.1.12.2.7 5M NaCl

292 g of NaCl was dissolved in 1 litre of deionised water.

2.1.12.2.8 1X Tris buffered saline (TBS)

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

2.1.12.2.9 Blocking solution

5%, 3% or 1% of non-fat milk powder was dissolved in 1X Phosphate buffered saline to make the blocking solution. For western blot detecting phospho-proteins, 5% of Bovine serum albumin (BSA) was dissolved in 1X TBS.

2.1.12.2.10 Wash solution

To make the wash solution, 5 ml of 10% Triton-X was added to 995ml of 1X Phosphate buffered saline. For western blot detecting phospho-proteins, 1X TBS was used.

2.1.12.3 Gel staining

2.1.12.3.1 SDS PAGE gel staining solution

Staining solution was made by dissolving 0.25 g of Brilliant Blue R-250 in 100 ml of destaining solution.

2.1.12.3.2 SDS PAGE gel destaining solution

Methanol, dd H₂O and glacial acetic acid were mixed in the ratio of 450:450:100 to make the destaining solution.

2.1.13 Yeast reagents

2.1.13.1 10X LiAc

1 M lithium acetate solution was prepared in deionised water.

2.1.13.2 20% Tri-Chloro Acetic Acid (TCA)

20 g of TCA was dissolved in 100 ml of sterile deionized water.

2.1.13.3 50% PEG 3350

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

2.1.13.4 Loading dye (Urea buffer)

Loading dye for yeast cell lysate containing 8M Urea, 50 mM Tris (pH 6.8), 2% SDS, 0.04% bromophenol blue was prepared.

2.1.14 His tag pull-down assay

2.1.14.1 Equilibration buffer for Ni-NTA beads

50 mM Tris (pH 8.0), 100 mM NaCl

2.1.14.2 Lysis Buffer (His-tag pull-down assay)

50 mM Tris (pH 8.0), 100 mM NaCl, 5% NP40, 200 mM PMSF and protease inhibitor.

2.1.14.3 Elution Buffer for Ni-NTA beads

50 mM Tris (pH 8.0), 100 mM NaCl and 250 mM imidazole.

2.1.15 Tissue culture

2.1.15.1 Freezing media

The freezing media was prepared by adding 10% of dimethyl sulfoxide (DMSO) in FBS and filter sterilized through a 0.2µm filter.

2.1.15.2 Lysis buffer for mammalian cells

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 (PMSF), protease inhibitor cocktail containing 10 µg/ml of leupeptin, pepstatin, and aprotinin each.

2.1.15.3 Transfection reagent

A 2 mg/ml solution of Polyethyleneimine PEI (Linear) (Sigma, St. Louis, Mo, USA) was prepared in deionised water. PEI solution was then filter sterilized through a 0.2µm filter. 5µg of PEI was used per microgram of DNA for transfection in mammalian cells.

2.1.15.4 Calcium phosphate transfection reagents for virus production

2.1.15.4.1 2X HBS Buffer

50 mM HEPES (pH 7.05), 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na₂PO₄

2.1.15.4.2 2M CaCl₂

14.72 g of CaCl₂ was dissolved in 50 ml of sterile deionized water.

2.1.15.4.3 25 mM Chloroquine solution

0.193 g of Chloroquine salt was dissolved in 15 ml of sterile deionized water and filters sterilized.

2.2 Methods

2.2.1 *Escherichia coli* cells

2.2.1.1 Growth conditions and maintenance of *E. coli* cells

E. coli was either cultured in LB broth or maintained on LB agar plates at 37°C. *E. coli* transformed with plasmids containing ampicillin or kanamycin selection marker were cultured in LB-broth or grown on LB agar plates supplemented with 75 µg/ml of ampicillin or 25µg/ml of kanamycin.

2.2.1.2 Glycerol stock of *E. coli*

For long term preservation, *E. coli* were maintained in glycerol stock as described. Single colony of the *E. coli* strain was inoculated in 5 ml LB medium and cultured until mid log phase. 200 µl of 80% glycerol solution was mixed with 800 µl of the bacterial culture and then transferred to a cryo-vial and stored at -80°C.

2.2.1.3 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 until to an OD₆₀₀ of 0.5. The culture was then transferred to sterile centrifuge falcons and store in ice until further use. The cells were centriuged at 4470 x *g* at 4°C for 5 minutes. The supernatant was discarded and the pellet was resuspended in 25 ml of ice cold sterile 100 mM MgCl₂. The cells were then centriuged at 4470 x *g* for 10 minutes at 4°C. The supernatant was discarded and the cell pellet was resuspended in 5 ml of sterile ice cold 100 mM CaCl₂ and incubated on ice 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 200µl aliquots in eppendorf tubes. The cells and the reagents were always maintained at 4 °C.

2.2.1.4 Transformation of competent *E. coli* cells

Frozen competent cells in eppendorf tubes were first thawed on ice. For DNA amplification, 1 µl of DNA was added to 50 µl of competent cells. For subcloning of plasmids, 20 µl of ligation reaction was added to 100 µl of competent cells. The tubes were incubated on ice for 20 minutes and heated at 42°C in a water bath for 90 seconds. They were then placed on ice immediately for 30 minutes. Cells were plated on LB agar plates containing the selective antibiotic and incubated at 37°C overnight.

2.2.1.5 Isolation of plasmid DNA from *E. coli*

A single bacterial colony was inoculated in 4 ml of LB containing antibiotic and grown overnight at 37°C. The cells were centrifuged at 4470 x g at 4°C for 5 minutes. The plasmid DNA was isolated from the bacterial cells pellet according to the manual provided in the plasmid purification kit.

2.2.2 *Saccharomyces cerevisiae* culture and manipulations

2.2.2.1 Growth and maintenance of *S. cerevisiae*

S. cerevisiae strains were cultured in YPUAD liquid culture as well as streaked on YPUAD agar plates and maintained at 24°C. Strains harboring plasmids encoding for a nutrient selection marker were grown in Synthetic Defined (SD) liquid media or SD agar plates for proper selection. To perform functional

studies on the proteins expressed in *S. cerevisiae*, the strains were streaked on two YPUAD agar plates, one plate was maintained at 24°C while the other was maintained at 37°C to examine the growth of the strain at their respective temperatures.

2.2.2.2 *S. cerevisiae* glycerol stocks preparation

A single colony of *S. cerevisiae* was streaked on YPUAD agar plate and grown at 30°C for 3 to 4 days. A loopful of cells was inoculated in a cryo-vial containing 500 µl of YPUAD and 20% glycerol. The cells were then stored at -80°C.

2.2.2.3 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. Culture after reaching the OD of 0.8 was then transferred to 50 ml centrifuge tube and centrifuged at 1000 x g for 5 minutes. Supernatant was discarded; pellet was washed with 30 ml of dd H₂O and centrifuged again at 1000 x g for 5 minutes. The supernatant was discarded; pellet was resuspended in 1 ml of 100 mM LiAc and transferred to a 1.5 ml eppendorf tube. This time the cell suspension was centrifuged at 14,100 x g in a micro-centrifuge for 15 seconds. The supernatant was discarded and the pellet was resuspended 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 centrifuged at 14,100 x g 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, 72 µl of dd

H₂O, 10 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. After heat shock, the cells were centrifuged again at 3,300 x g for 15 seconds, the supernatant was discarded and the pellet was resuspended in 400 µl of dd H₂O. 100 µl of this cells suspension was plated on selective media plates and incubated at 24°C until the colonies reached sufficient growth for further analysis.

2.2.2.4 Yeast two hybrid assay

The yeast two hybrid assay was used to identify protein-protein interaction. Proteins, whose interactions were to be verified, cloned into these two plasmids, pAS2-1 or pACT2 which contain Gal4 binding domain and Gal4 activation domain respectively. The interaction of the proteins would bring the binding domain and the activation domain in close proximity and drive the transcription of the reporter genes, HIS3 (Fig. 2.1). *S. cerevisiae* strain PJ69-4A was first transformed with bait plasmid pAS2-1 followed by prey plasmid pACT2 with proteins of interest cloned in and grown on SD plates lacking Trp and Leu. Patching of the transformed *S. cerevisiae* cells were done on two different plates, SD plates lacking Trp and Leu as a control and SD plates lacking Trp, Leu, His and supplemented with 2mM 3AT to check for the interactions between the proteins. 3AT act as an inhibitor for non-specific activation of the transcription of the reporter genes.

Figure 2.1 Yeast two-hybrid principle. Gal4 is the yeast transcription factor that normally control gene responsible for galactose metabolism. Each Gal4 responsive gene contains a target site called UAS. When Gal4 binds with UAS transcription is activated from downstream promoter. In this system Gal4 DNA- binding (DNA-BD) and activation domain (AD) express as fusion proteins.

2.2.2.5 Yeast cell lysis

A loopful of cells were inoculated in 25ml of YPUAD and incubated overnight with shaking at 30°C. The culture was diluted to an OD₆₀₀ of 0.2 in 25 ml of fresh YPUAD media next day and grown (incubated with shaking) to an OD₆₀₀ of 0.8 at 30°C. OD₆₀₀ of 7 units of cells were harvested by centrifugation of the cells at 1000 x g for 5 minutes at RT. The supernatant was decanted and the cell pellet was washed once with deionized water. The cells were centrifuged at 1000 x g at RT for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 1 ml of 1X PBS and transferred to an eppendorf tube. The cells were centrifuged at 14,100 x g for 30s and PBS was removed. The cell pellet was resuspended with 240 µl mixture containing 1.85 M NaOH and 1.06 M β-mercaptoethanol and incubated on ice for 10 min. An equal volume of 20% of TCA was added to the tube and was incubated on ice for an additional 10 min. The resulting pellet was obtained by centrifugation at 14,100 x g for 10 min. The protein pellet was washed in ice-cold acetone. The pellet was then resuspended in 100 µl of loading dye (urea buffer). 15 µl of sample was loaded in 10% SDS-PAGE gel when western blot analysis was required.

2.2.2.6 Fluorescence visualization of yeast cells

Cells were grown to exponential phase were harvested at 0.6 x g for 5 min. They were washed twice with PBS, resuspended in PBS and applied to a microscope slide. Fluorescence imaging was performed using an Olympus microscope with CoolSNAP HQ camera (Roper Scientific). Fluorescence intensities of 100 cells were quantified using the Metamorph software (Molecular devices, CA, USA).

2.2.3 Mammalian cell culture

2.2.3.1 Growth and maintenance of mammalian cells

HEK293T cells and Amphotropic packaging cells were maintained in DMEM (Dulbecco modified Eagle medium) supplemented with 10% FBS and 1% penicillin-streptomycin. Jurkat (clone E6-1) T-cells were maintained in RPMI supplemented with 10% FBS, 1% penicillin-streptomycin.

2.2.3.2 Passaging and trypsinizing of mammalian cells

Mammalian cells were washed once with 1X PBS and detached from the culture flask by incubating them in 1mL of 1X trypsin EDTA solution (Gibco 10X) for 2 minutes at 37°C. Trypsinization of cells were stopped by adding 4 mL of pre-warmed DMEM media supplemented with 10% FBS subsequently cells were transferred at the desired density to a new tissue culture flask. The cells were then cultured at 37°C in a 5% CO₂ incubator. HEK293T cells or Amphotropic packaging cells were trypsinised and split once every 2-3 days at 1:7 dilutions when they become 90% confluent. Jurkat T-cells were split once

every 2-3 days. Jurkat T-cells were maintained at the density of 2×10^5 - 2×10^6 cells per ml.

2.2.3.3 Preparation of mammalian cell stocks

For adherent cells, cells were first trypsinised and then resuspended in the appropriate growth media. For both the adherent and suspension cells, the number of cells and cell viability were determined using the hemocytometer, cell counter and Trypan Blue exclusion dye. 5×10^6 to 1×10^7 cells (per ml of freezing medium) were centrifuged at $0.4 \times g$ for 4 min. The supernatant was discarded and cell pellet was resuspended in freezing medium. The cell suspension was then aliquoted into cryogenic storage vials and stored at -80°C in an isopropanol cryobox, that has a controlled freezing rate, overnight. The frozen cells were then transferred to liquid nitrogen tank.

2.2.3.4 Thawing of cells

Cryo-vials were taken from liquid nitrogen and re-suspended in 4.0 ml of DMEM medium supplemented with 10% FBS until the frozen medium had melted. The thawed cells were then transferred to 20 mL of fresh DMEM medium with 10% FBS and transferred in to culture flask. Growth medium was replaced after 24 hours with fresh DMEM + 10% FBS and 1% Penicillin/Streptomycin.

2.2.3.5 Transfection of adherent mammalian cells

HEK293T cells were grown in 6-well plates to 70% confluency for one day before transfection. $4.0\mu\text{g}$ of DNA was added to $90\mu\text{l}$ of serum-free medium

(SFM). The mixture was then added to 10 μ l of PEI and 90 μ l of SFM. The DNA/PEI mixture was then allowed to stand for 10 min at RT. After 10 min of incubation at RT, this reaction mixture was added drop-wise onto the cells. The cells were incubated for 12 hrs at 37°C in the humidified chamber before changing the media to new growth media. After 36 hrs of incubation at 37 °C in a 5% CO₂-humidified incubator, cells were ready for further analysis.

2.2.3.6 Transfection of amphotropic cells for retrovirus production

Amphotropic cells were transfected with retroviral plasmid (PSIR-EGFP) containing WASP S1 shRNA (Sigma-Aldrich) (Appendix. 2) using calcium phosphate transfection method. The virus supernatant 48 hrs after transfection was used to knockdown the endogenous WASP expression in Jurkat T-cells. Amphotropic cells were grown in complete DMEM media up to 80-90 % confluency in a 10 cm culture dish. Prior to transfection, the cells media was changed with fresh 8 ml DMEM media containing 25 μ g of chloroquine. The cells were then incubated in CO₂ incubator for 5 min. Meanwhile, 80 μ g of DNA was mixed with 124 μ l of 2M CaCl₂ and make up the volume 1 ml of total with water. 1 ml of 2X HBS was then added to DNA/CaCl₂ mixture and bubbled with 5 ml of serological pipette with a pipetman for 15 sec. Then this transfection mixture was quickly added to the cells in a dropwise manner. Cells were then incubated for 12 hours in CO₂ incubator before changing with fresh complete DMEM media.

2.2.3.7 Retrovirus vector mediated RNAi

Amphotropic packaging cells were grown on 10 cm culture dish and were transfected with 80µg of plasmid containing shRNA (Refer to Appendix 2) using calcium phosphate transfection method (Described in section 2.2.3.6). After 48 hrs of transfection, the cells supernatant was collected and filtered using 0.45 micron filter. 10µg/ml of polybrene was then added to the filtered virus supernatant. A 60 mm culture dish was coated with 10µg/ml of fibronectin overnight at 4°C prior to the addition of the virus supernatant. The plate was washed once with sterile 1X PBS. The virus containing supernatant was then added to the fibronectin coated plate and centrifuged at 3148 x g for 90 minutes at 4°C. 2×10^6 Jurkat cells were resuspended in fresh 4ml of complete RPMI media. The virus supernatant was removed from the plate after centrifugation and the Jurkat T-cell suspension was added into the 60 mm plate. The cells were then incubated at 37 °C in a 5% CO₂-humidified incubator for 3-4 days. When the infected cells were confluent, cells with green fluorescent were sorted using FACS Aria cell sorter, the FL1-FITC filter. These cells were further grown at 37 °C in a 5% CO₂-humidified incubator for 3-4 days before analysis of endogenous WASP expression.

2.2.3.8 Microporation of Jurkat cells

Jurkat T-cells were resuspended in new growth media a day before microporation. The number of cells and cell viability were determined using the hemocytometer, cell counter and Trypan Blue exclusion. 5×10^6 cells were centrifuged at 400 x g for 4 min. The supernatant was discarded and cell pellet was washed once with PBS. The cell pellet was then resuspended in 100µl of

Resuspension buffer R (provided in microporation kit from Neon transfection system, Life Technology, CA, USA) and 10µg of DNA. Using microporator-mini (MP-100, NanoEN Tek Inc. Seoul, Korea), the microporation was performed at RT with the parameter of 1500 pulse voltage, 10ms pulse width and 3 pulse number. After which, cells were distributed into 10 cm culture dish containing 10 ml of growth media and incubated at 37 °C in a 5% CO₂-humidified incubator. After 36 hrs of incubation, cells were ready for further analysis. For stable WASP expression, Jurkat cells after 36 hrs of microporation with WASP constructs were selected with neomycin (1.5mg/ml) (G-418, PAA, P02-012) for 7 days. Media with G418 was changed once every 2 to 3 days for a week until WASP is stably expressed in the cells. For exogenous WASP expression, WASP gene resistant to S1 shRNA (primer refer in appendix 2) was cloned in PEPCG vector (Refer in appendix. 1) containing the WASP promoter and RFP at the C terminus of WASP. In order to enhance the protein WASP-RFP fusion protein expression, a SV40 intron was cloned at the C terminus of RFP (Fig. 2.2). The CMV promoter was also used in the same vector for WASP expression in Jurkat T-cells, but a very high expression of WASP under CMV promoter was found to be lethal for Jurkat T-cells.

Figure 2.2 Schematic representation of WASP construct used for expression in Jurkat T-cells. WASP promoter was used in this study to express exogenous WASP in Jurkat T-cells.

2.2.3.9 Mammalian cell lysis

Adherent cells were trypsinised and resuspended in the appropriate growth media. For both the adherent and suspension cells, the number of cells and cell viability were determined using the hemocytometer, cell counter and Trypan Blue exclusion. 2×10^6 cells were centrifuged at $400 \times g$ for 4 min. The supernatant was discarded and the cells pellet was washed with PBS and then resuspended in 50 μ l of lysis buffer (materials 2.1.15.2). Cells were left on ice for 1 hr and unlysed cells were removed by centrifugation at $14,500 \times g$ for 10 min. 50 μ l (equal amounts) of 2X loading dye and 2 μ l of 1M dTT was added to the lysate. Protein concentration was quantified with Bio-Rad DC protein assays. The lysate was boiled at 100°C for 5 min and stored at -80°C until further use.

2.2.4 Cell based assays

2.2.4.1 Binding assay of Jurkat T-cells

Purified ECM components were diluted with 0.1M sodium bicarbonate buffer (pH 9.1) to reach a final concentration of $0.14\mu\text{g}/100\mu\text{l}$ for human fibronectin and $1\mu\text{g}/100\mu\text{l}$ of BSA was used as a negative control. They were then coated on 96-well plate and incubated either overnight at 4°C or 2 hrs incubation at 37°C . After coating, each well was blocked with 100 μ l 0.2% (w/v) BSA at 37°C and washed once with serum-free DMEM. Cells were centrifuged at $400 \times g$, resuspended in serum free medium and incubated for half an hour at 37°C humidified CO_2 incubator. After which 4 μ l Calcein AM was added into 1ml of cells suspension and incubated for another 30 minutes at 37°C . Cells were then pelleted and resuspended in serum-free DMEM. 2×10^4 cells in 50 μ l serum-free DMEM were added into each well and incubated for 20 min at 37°C . The

unbound cells were removed by repeated washing with serum free DMEM. The percentage of bound cells to the ECM were determined using FL600 microplate fluorescence reader with absorption/emission wavelengths as 494/517nm.

2.2.4.2 Analysis of Jurkat T-cell migration using the Dunn chemotaxis chamber

2.2.4.2.1 Washing of Dunn chamber and glass coverslips

Glass coverslips (18 X 18 mm) (Hawksley & Sons Ltd, Lancing, Sussex, UK) were individually washed with detergent in deionised water and rinsed 6 times with deionised water. They were then soaked in fuming HCl for 10 min and washed with deionised water to remove all traces of HCl. The coverslips were stored in 100% ethanol until further use. The Dunn chamber (Weber Scientific International Ltd. West Sussex, UK) was cleaned immediately after use. It was first washed with detergent in deionised water and rinsed 6 times with deionised water. It was then placed in a glass beaker filled with 100% acetone to be washed for 10 min with agitation. Thereafter, the chamber was rinsed 6 times with deionized water and soaked in 30% hydrogen peroxide for further 10 min. The chamber was then rinsed 6 times with deionised water again and was stored in 100% ethanol until further use. The bridge of the chamber was not touched at all times during the washing [253]. The coverslips stored in 100% ethanol were flamed until all ethanol has evaporated. The coverslip was then coated with 2µg/ml of fibronectin in PBS for 1 hour at 37°C CO₂ incubator. Jurkat T-cells suspended in RPMI 1640 supplemented with 10% FBS (2.25×10^6 in 120µl) were loaded on to fibronectin coated coverslip, allowed to attach on coverslip for 10 min and then unbound cells were gently

removed by washing with cell culture media. The Dunn chamber outer and inner well was filled with complete RPMI media. The coverslip with attached cells was then inverted on the Dunn chamber wells with leaving a narrow slit at one edge for draining and refilling the outer well. The outer well media was then drained out using an absorbent tissue applied to filling slit. RPMI media containing chemokine (SDF-1 α 5nM) was gently filled to the outer well using the syringe with fine needle. All the edges of coverslip sealed using a mixture of hot wax (1:1:1: mixture of paraffin wax, bees wax and Vaseline). Cells were incubated for 10 min at 37°C in the 5% CO₂-humidified incubator.

Figure 2.3 The assembled Dunn chamber. The Dunn Chamber contains a circular inner well and a circular outer well, separated by an annular bridge which is 20 μ m lower than the surrounding glass. This leaves a gap between the coverslip and the bridge, allowing the migration of cells across the bridge.

2.2.4.2.2 Time-lapse microscopy

Migrating cells on the annular bridge between the inner and outer wells were observed using an inverted microscope (Olympus IX81) with 10X objective lens. Time-lapsed images were digitally captured every 2 minutes for a period of 3 hours using an Olympus CoolSNAP HQ camera. Images were processed into videos using the Metamorph software (Molecular Devices, CA, USA).

Migration paths of the cells were traced and analysed from the series of images using Metamorph software. The data were plotted with Microsoft Excel. 20 cells were analysed for each set of experiment and experiment was repeated thrice.

2.2.4.3 Transwell migration assay.

Transwell migration assay was performed using 24-well transwell of size 6.5mm (Catalog No-3421) polycarbonate membrane insert with pore size 5.0µm. For transwell migration assay, Jurkat T-cells were first serum starved for two hrs and then added to the upper chamber in 100µl of migration buffer (RPMI-1640). Migration buffer (600µl) containing 100ng/ml of SDF-1α was added to the lower well. Cells were then incubated for another 2 hrs in CO₂ incubator. Cells migrated to the lower well and initial cell number were counted using hemocytometer. Percentage of cells migrated was calculated as ratio of number of cells migrated to lower well to the initial cell number.

2.2.4.4 Jurkat T-cells activation and IL-2 production.

Jurkat T-cells were maintained in complete RPMI media as described in section 2.2.3.2 Endogenous WASP expression was knockdown in Jurkat T-cells by retrovirus transduction method (*Section 2.2.3.8*). These WASP^{KD} Jurkat T-cells were then microporated with WASP_R (shRNA resistant) or WASP_R mutants and selected with neomycin for one week. Selected cells (1 X 10⁶ cells per well of 12 well plate) were then activated with plate bound anti-Human CD3 clone OKT3 (2µg/ml) and anti-Human CD28 antibody (2µg/ml) (eBioscience) for 8 hrs. Coating of 6 well plates with both antibodies was performed by keeping overnight at 4°C in 0.2M bicarbonate buffer (pH 8.0).

Before seeding cells, coated plate was washed with complete RPMI media twice for 10 min each at room temperature. Cells were flush out from plate after 8 hrs of incubation and total RNA was isolated according to manual provided with RNA-Sol from E.Z.N.A DNA/RNA isolation system (Omega Bio-tek). Total RNA was then reverse transcribed and quantitative real time PCR was performed for interleukin-2 (IL-2) and mitochondrial ribosomal protein L27 (MRPL27) as endogenous control.

2.2.4.5 Actin staining in Jurkat T-cells.

Stable WASP or WASP mutants expressing WASP^{KD} Jurkat T-cells were seeded on anti-CD3/anti-CD28 coated coverslip as described in section 2.2.4.4. Cells after seeding on antibody coated coverslips were incubated for 30 min in humidified CO₂ incubator. Cells after incubation were fixed with formaldehyde for 20 min, washed three times and stained with Alexa Flour 594 phalloidin. The mean surface area (in μm^2) and average number of membrane projections per cell of each population were calculated using metamorph software.

2.2.5 DNA manipulation

2.2.5.1 Agarose gel electrophoresis

0.5 $\mu\text{g/ml}$ of Ethidium Bromide was added to 1% agarose in 1X TAE and the gel was left to set. 6X DNA Loading buffer was added to the samples and they were loaded in the wells of the gel, submerged in 1X TAE. DNA was resolved by running the gel at 110V for 50 min. DNA was visualized under the long wavelength UV light.

2.2.5.2 DNA extraction from agarose gel

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 (QIAquick Gel extraction Kit).

2.2.5.3 DNA subcloning

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^{1/2} 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 ddH₂O 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 then 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*.

2.2.5.4 Verification of recombinant plasmid DNA constructs

The recombinant plasmid DNA was verified by checking with restriction enzymes that cut at restriction enzyme sites that are unique to it. The digested

DNA was then resolved by agarose gel electrophoresis. The bands obtained in the gel were checked to verify for the correct sizes.

2.2.5.5 DNA quantification

NanoDrop is the one of the most sensitive method to quantify the DNA concentration with minimal consumption of sample. Nanodrop can measure as little as 1µl of DNA, significantly lower down the sample volume. Concentration of the DNA sample was calculated by using NanoDrop. Software will generate the stander curve using the DNA concentrations along with the negative control and gives the DNA concentration in ng/µl. OD_{260}/OD_{280} ratio was checked to confirm the purity of DNA sample. OD_{260}/OD_{280} less than 1.8 indicate impurity caused by proteins and/or other matter capable of absorbing UV light. OD_{260}/OD_{280} higher than 2.0 indicates possibility of RNA contamination in the DNA sample. OD_{260}/OD_{280} between 1.8 and 2.0 indicate the absorption due to DNA thus all DNA samples which had OD_{260}/OD_{280} between 1.8 to 2.0 were selected for further experiments.

2.2.5.6 Polymerase chain reaction (PCR)

Polymerase chain reaction was performed to specifically amplify DNA from very small amount of DNA. All PCR reactions were performed using the Peltier Thermal Cycle PTC-100. A 50 µl reaction using BIOTOOLS system was set up as follows:

- 5.0µl of 5x BIOTOOLS Reaction Buffer
- 5.0µl of 2 mM dNTP mix
- 1.0µl of 5' primer (10 µM)
- 1.0µl of 3' primer (10 µM)
- 0.5µl of Template DNA
- 0.5µl of BIOTOOLS DNA Polymerase (1 U/µL)
- Deionised water (topped up to 50 µl)

The thermal cycle conditions used were:

Initial Denaturation: 95°C for 10 min

- Denaturation: 95°C for 1 min
- Annealing: 55 °C for 1 min
- Extension: 72°C for 30 sec per kb (30 cycles)
- Final Extension: 72°C for 5 min

The PCR reaction was cycled 30 times for optimal amplification of the target fragment. The primers used in this study are listed in Appendix 2. The PCR products were analysed by Agarose gel electrophoresis (Section 2.2.5.1).

2.2.5.7 Reverse transcription of RNA to cDNA

1-5µg of RNA was added with 1µl of DEPC-treated water and 1µl of Random primers. The mixture was incubated at 70°C for 5 min and incubated on ice immediately for 5 min. A reaction mixture prepared as follows was added to the RNA and incubated at 37°C for 5 min.

Reaction mixture:

- 6 µl of 5X reaction buffer
- 3µl of 0.1M DTT
- 0.5 µl of 25mM dNTPs
- 9 µl of DEPC-treated water

The tube was transferred to the ice and incubated for 5 min. 0.5 µl of Reverse Transcriptase was added to the tube and it was incubated for 60 min at 42°C. The Reverse Transcriptase was then heat inactivated at 70°C for 5 min. The tube containing the cDNA was stored at 4°C.

2.2.5.8 Real-time PCR (RT-PCR)

Total RNA was isolated from the cells using the RNAeasy kit and reversed-transcribed to cDNA according to method 2.2.5.7. 25 µl Real-time PCR reaction was set up as follows:

- 12.5µl of SYBR Green Master Mix
- 2.5µl of cDNA (Diluted 5X)
- 2.5µl of diluted primers (Forward + Reverse, 50nm in 25µl)
- 7.5µl of DNAase/RNAase free water.

Each reaction was performed in triplicates and the primers used are listed in Appendix 2. The RT-PCR was performed on 7500 Real-Time PCR system (Applied Biosystems) using the comparative C_T method and the relative quantification of WASP expression was calculated using the formulae below:

$$C_{t_{\text{target gene}}} - C_{t_{\text{Endogenous control}}} = \Delta C_t$$

$$C_{t_{\text{target gene}}} - C_{t_{\text{Reference sample}}} = \Delta\Delta C_t$$

The standard deviation was calculated using the following formula: $S = \sqrt{(S_1^2 + S_2^2)}$

2.2.5.9 Site directed mutagenesis (SDM)

QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA, USA) or Overlap Extension technique was used to generate the mutation of interest at specific locations in the target DNA.

2.2.5.10 SDM kit

Site-directed mutagenesis was performed according to the instructions provided in the manual of the Site-directed mutagenesis kit. After performing the PCR for mutagenesis, the PCR product was transformed into competent cells and plated onto LB plate containing ampicillin or kanamycin. The plasmid DNA from different clones was extracted and sequenced to identify the clone bearing the mutation of interest.

2.2.5.11 Overlap extension PCR

In the overlap extension PCR, as illustrated in figure 2.4, two halves of the gene of interest are first amplified separately with an outer flanking primer and a mutagenesis primer each (primers a and b, primers c and d). The two mutagenesis primers are complementary to each other. The PCR products from the two PCR reactions (AB and CD) are purified and mixed together to be used as template for the third PCR reaction. In the third PCR reaction, the two outer flanking primers (primers a and d) are used to amplify the final full length PCR product (AD). Finally, the end product obtained is the gene of interest bearing the desired mutation.

Figure 2.4 Schematic diagram of the overlap extension PCR. b and c are the mutagenesis primers while a and d are flanking primers. AD is the final PCR product which contains the desired mutation.

2.2.5.12 DNA precipitation

For 20 μ l of DNA mix, 2.22 μ l of 3M NaOAc (1/10 of DNA mix) and 44.44 μ l of 100% ethanol (2X of DNA and NaOAc mix) was added. The mixture was incubated at -20°C for 2 hours or more and centrifuged at 14,100 x g for 10 min. The resulting pellet was washed with 70% ethanol. The pellet was air-dried and dissolved in appropriate amount of deionised water or 1X TE buffer according to the pellet size.

2.2.6 SDS-PAGE electrophoresis

2.2.6.1 Sample preparation for SDS-PAGE electrophoresis

Bradford assay was performed to measure the total amount of protein in samples. The protein sample was first mixed with 2X loading buffer (*Section 2.1.12.2.5*) and heated at 100 °C for about 5 minutes. 25 µg of the protein sample was used to run the SDS-PAGE.

2.2.6.2 SDS-PAGE gel preparation

A 10% SDS-PAGE gel was prepared as follows:

Resolving gel:

Components	ml
1.5 M Tris-HCl, pH 8.8	1.3
10% SDS	0.05
30% Acrylamide/Bis-acrylamide	1.7
10% ammonium persulfate (APS)	0.05
TEMED	0.002
H ₂ O	1.9

Stacking gel:

Components	ml
1.0 M Tris-HCl, pH 6.8	0.38
10% SDS	0.03
30% Acrylamide/Bis-acrylamide	0.5
10% ammonium persulfate (APS)	0.03
TEMED	0.003
H ₂ O	2.1

Proteins in samples were resolved by loading the appropriate amounts in the SDS-PAGE gel and the gel was run at 90V for 120 min, until the dye front has migrated past the end of the gel. The gel was then further processed either for the staining of proteins or Western blot analysis.

2.2.6.3 Western blot analysis

Western blot is the technique which is used for specific detection of proteins on the basis of size of proteins through SDS-PAGE technique. Electroblothing was

performed on the proteins which had been resolved through SDS-PAGE. The nitrocellulose membrane, gel and filter papers were soaked in the transfer buffer for about 5 minutes. The nitrocellulose membrane and the protein gel were overlaid on each other and sandwiched between 4 filter papers on each side and placed in the transfer cassette. The transfer cassette was then placed inside the transfer cell in the correct orientation such that the transfer of the proteins would take place in the direction from the protein gel and on to the nitrocellulose membrane. The transfer was carried out at a constant voltage of 85 V for 2 hrs at 4°C.

2.2.6.4 Immunoblotting and chemoluminescence

After blotting, the membrane was removed from the transfer cassette and blocked in the 3-5% skim milk solution in PBS (5% BSA in Tris buffer (pH 8.0) for phospho-protein) for 30 min at room temperature. Primary antibody was incubated with the membrane overnight with appropriate dilution (for dilutions see Table 2.4) 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 2.5) conjugated with Horseradish peroxidase (HRP) for 1 hour at room temperature. The membrane was then washed and protein bands were detected with PICO (Pierce) or ECLTM Western blotting detection reagent (Amersham Biosciences) as per the instructions of manufactures.

2.2.6.5 Hexa histidine-tag pull down assay

His tag pull down assay was performed to examine the interaction between the two proteins expressed in HEK293T cells. The DNA encoding for the polyhistidine-tag (His-tag) was fused to the C-terminal of the DNA encoding for one of the two proteins and the DNA encoding for GFP was fused to the C-terminal of the DNA encoding for the other protein. Both the GFP fusion protein and the HisTag fusion proteins were expressed in the HEK293T cells. HEK293T cells were grown in 10 cm² culture dish. 36 hours after post-transfection, the cells were lysed by adding 500µl of lysis buffer (*Section 2.1.14.2*) and incubating on ice for 1 hour. 200µl of beads were aliquoted into eppendorf tubes for each lysis sample. The Ni-NTA beads (Invitrogen, Carlsbad, CA, USA) were washed 3X with 1ml of deionised water at 100G (lowest speed) for 5 min. 500µl of equilibration buffer (*Section 2.1.14.1*) was then added to the beads and the tubes were rocked for 1 hour at 4°C. The beads were further washed with 400µl of equilibration buffer. Tubes containing the lysed cells were centrifuged at 14,100 x g for 5 min to remove cells that were not lysed and the supernatant was transferred to a new tube. 30ul of the supernatant was aliquoted out as whole cell lysate. 50ul of beads or equivalent amounts were added to the supernatant. The proteins were allowed to bind to the beads by rocking the tubes for 3 hours at 4°C. The beads were centrifuged for 5 min at 0.1 x g and the flow-through was stored in new tubes. Beads were washed 6 times with lysis buffer and then bound protein on beads was eluted with elution buffer (*Section 2.1.14.3*) containing 250 mM imidazole. Eluted proteins in the supernatant, flow-through and whole cell lysate were precipitated by adding an equal volume of 20% TCA (250ul) and incubated on

ice for 10 min. The precipitated proteins were centrifuged at 14,100 x g for 10 min to obtain a pellet. The pellets were washed with ice-cold acetone. The acetone was removed and the pellets were air-dried for 2 min. The pellets were resuspended in 60ul of 2X SDS-PAGE loading dye and 1M dTT (4ul in 100ul) and subsequently boiled at 100°C for 5 min. The samples were stored at -20°C. 15ul of the samples were loaded in each lane of the SDS-PAGE gel and Western blot was performed.

3. Results

3.1 Identification and characterization of the I30 region of WASP

3.1.1 Introduction

The actin cytoskeleton together with the actin associated proteins plays a critical role in many cellular processes, including cell signalling, cell activation and cell division. WASP family of proteins: WASP, N-WASP, WAVE1-3 are involved in regulation and reorganization of actin cytoskeleton [118]. WASP is expressed in hematopoietic cells while its homologue N-WASP is expressed ubiquitously [141]. Although WASP and N-WASP are homologue and share similar domain structure, N-WASP can only partially compensate the functional defects of WASP-deficient cells of hematopoietic origin. T-cell development is found to be normal in individual conditional WASP^{KO} or N-WASP^{KO} mice. However, the development of T-cells was markedly altered in WASP/N-WASP conditional double knockout mice [60], suggesting that WASP and N-WASP have some unique as well as redundant functions. To date, the unique and redundant roles of WASP and N-WASP in T-cells have not been well characterized. Therefore, in this study, the molecular and functional differences between N-WASP and WASP in T-cells were characterized.

3.1.2 Expression of N-WASP is lower than WASP in hematopoietic cells

Recently, WASP expression was compared with N-WASP expression in mouse macrophage cell line (RAW264.7) and found to be 15-fold higher relative to N-

WASP [97]. However, this quantification was at the protein level based on antibody detection which recognized both WASP and N-WASP. It is possible that the reduced amount of N-WASP expression as compared to WASP expression in T-cells may not be sufficient to compensate WASP functions in WASP-deficient T-cells. Therefore, the expression levels of WASP and N-WASP were first compared at transcription level in the cells of hematopoietic origin. In order to compare the endogenous level of N-WASP and WASP, total RNA was isolated from Jurkat T-cells, human monocyte cell line THP-1 cells, mouse macrophage cell line RAW264.7 cells and human B-cell line Raji B-cells.

WASP and N-WASP levels using specific primers were detected by quantitative real-time PCR. Real time specific primers for mitochondrial ribosomal protein L27 (MRPL27) were used as endogenous control for cell lines of human origin (Jurkat T-cells, Raji B-cells, HEK293T cells and THP-1 cells) whereas, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) specific primers were used as endogenous control for cell line of mouse origin (RAW264.7 cells). The real time quantification showed that the expression of WASP mRNA was significantly higher than N-WASP mRNA in all the four cell lines of hematopoietic origin. Expression of WASP was approximately 2.5 fold higher in Jurkat T-cells, 3.9 fold higher in THP-1 monocyte cells, 2.8 fold higher in RAW264.7 macrophage cells and 1.6 fold higher in Raji B-cells relative to N-WASP expression (Fig. 3.1). In order to check the specificity of real time PCR primers, expressions of WASP and N-WASP were compared in HEK293T cells. Expression of WASP was negligible compared to N-WASP in HEK293T cells indicating that both the primers used in this study were

specific. The expression levels of WASP and N-WASP at transcriptional level were also compared in activated (with anti-CD3 alone or both anti-CD3/CD28 antibodies) and non-activated primary T-cells. Similar to expression levels of WASP and N-WASP observed in transformed cell lines (in above experiment), the expression of WASP was 18.7 fold higher in non-activated and 40 fold (activated with anti-CD3 alone) or 32 fold higher (activated with both anti-CD3/CD28) relative to N-WASP expression (Fig. 3.2).

Figure 3.1 Expression of N-WASP is lower compared to WASP expression in the cells of hematopoietic origin. Quantitative real time PCR analysis of WASP and N-WASP in Jurkat T-cells, THP-1 cells, Raji B-cells, RAW264.7 cells and HEK293T cells. *** represent $P < 0.001$ (unpaired student's t-test).

Figure 3.2 Expression of N-WASP is lower compared to WASP expression in primary T-cells. Quantitative real time PCR analysis of WASP and N-WASP in non-activated or activated primary T-cells. *** represent $P < 0.001$ (unpaired student's t-test).

These results imply that while N-WASP is expressed in cells of hematopoietic origin, it is at a much lower level than WASP expression at transcription level. However, this is a quantification of mRNA and higher level of mRNA could be due to the instability of WASP compared to N-WASP.

3.1.3 Knocking down of WASP expression in Jurkat T-cells.

Loss of WASP expression has a pronounced effect on T-cells functions as T-cells from WAS patients were found to have intrinsic abnormalities such as impaired CD3 mediated T-cell activation, defects in cell proliferation and IL-2 gene transcription [156]. N-WASP expression was found to be relatively lower than WASP expression in the cells of hematopoietic origin (Fig. 3.1 & 3.2). Therefore, in order to identify whether exogenous expression of N-WASP can rescue for the loss of WASP in T-cells, WASP deficient Jurkat T-cell line was first generated. For generation of WASP^{KD} Jurkat T-cells, an RNAi method was used in which stable integration of lentiviral or retroviral vectors encoding shRNA or siRNA into the genome of the cells can down regulate the expression of WASP for several generations. Two lentiviral vectors encoding siRNA targeting the WASP coding region and 3' UTR, W7si and W8si (Appendix 2) were designed based on the studies done by Olivier et al [164]. The efficacies of these two siRNA plasmid vectors were determined in both HEK293T cells and Jurkat T-cells. Both the siRNA plasmid vectors were able to knockdown the expression of exogenous WASP in HEK293T cells but in Jurkat T-cells, were unable to knockdown endogenous WASP expression (Data not shown). Subsequently, lentiviral vectors encoding W7, W8 and S1 (shRNA targeting sequence from Sigma) (Appendix 2) were designed and tested in both the cell lines. Similar results were obtained and no knockdown of WASP

expression in Jurkat T-cells was observed (Data not shown). Epstein-Barr (EBV) based vectors (shown to be maintained episomally) [86] encoding shRNA targeting WASP coding region were tested however, there was no knockdown of WASP expression observed in Jurkat T-cells (Data not shown).

(A)

(B)

Figure 3.3 Stable transduction of Jurkat T-cells for knocking down of WASP expression. (A) Sequence representing the S1 WASP shRNA and target site in the WASP gene. (B) Amphotrophic cells were transfected with S1 WASP shRNA for retrovirus production. 48 hrs after transfection, virus supernatant used to transduce Jurkat T-cells. 3 days after transduction, the GFP positive cells were sorted by FACS.

Finally, a retroviral plasmid vector (PSIR; Clontech) encoding S1 WASP shRNA (Sigma-Aldrich) (Fig. 3.3A) targeting WASP coding region was used. This retroviral vector also encodes GFP which allow the identification and sorting of transduced cells. Amphotropic packaging cells [113] were used to generate viral particles by transfecting these packaging cells with plasmid containing S1 WASP shRNA using calcium phosphate method. The

transfection efficiency of the amphotropic cells was high (nearly 80%) (Fig. 3.3B) Forty eight hours post-transfection, virus supernatant was collected and centrifuged on fibronectin coated plate. These viral particles coated plates were used to transduce Jurkat T-cells. The transduction efficiency of Jurkat T-cell using spinoculation method was 43% (Fig. 3.3B). The transduced Jurkat T-cells stably expressing GFP were sorted by flow cytometry (Fig. 3.3B).

Figure 3.4 Knocking down of endogenous WASP expression in Jurkat T-cells. (A) Western blot analysis of the expression level of WASP in Jurkat T-cells transduced with S1 WASP shRNA encoding vector using anti WASP (α -WASP) and anti GAPDH (α -GAPDH) was used as loading control. Lane 1: wild type (WT) Jurkat T-cells (control), lane 2: Jurkat T-cells transduced with S1 WASP shRNA (WASP^{KD} Jurkat T-cells). (B) WASP mRNA level was quantified by real-time PCR in WT and WASP^{KD} Jurkat T-cells. *** represent $P<0.001$ compared to WT Jurkat T-cells (unpaired student's t-test).

Western blot analysis of sorted Jurkat T-cells showed effective knockdown of endogenous WASP expression (Fig. 3.4A). This was further validated by real-time PCR which showed that mRNA level of WASP in WASP^{KD} Jurkat T-cells was 68% less compared to wild type (WT) Jurkat T-cells (Fig. 3.4B). N-WASP and WASP have more than 50% sequence homology [67,141], thus there was a possibility that the S1 WASP shRNA could target N-WASP as well. In order to check for the specificity of the S1 WASP shRNA, both WASP and N-WASP tagged with GFP were expressed in HEK293T cells with or without the

plasmid expressing S1 WASP shRNA. Western blot analysis showed that the S1 WASP shRNA was specific for WASP as N-WASP expression was not affected by S1 WASP shRNA (Fig. 3.5A). Similarly, S1 WASP shRNA did not affect the expression of N-WASP in Jurkat T-cells (Fig. 3.5B & C).

Figure 3.5 S1 WASP shRNA was specific to WASP and did not affect N-WASP expression. (A) Expression of WASP-GFP, N-WASP-GFP and WASP_R-GFP in HEK293T cells transfected with or without S1 WASP shRNA using anti-GFP (α-GFP) and anti-GAPDH (α-GAPDH) was used as loading control. (B) Western blot analysis of expression of N-WASP in WT and WASP^{KD} Jurkat T-cells using anti N WASP (α-N-WASP) and anti GAPDH (α-GAPDH) was used as loading control. (C) mRNA level of N-WASP quantified by real-time PCR in WT and WASP^{KD} Jurkat T-cells. (D) Sequence representing WASP_R contains four silent mutations that rendered it resistant to the WASP specific S1 WASP shRNA.

Human WASP specific S1 WASP shRNA (used for knocking down of WASP expression in Jurkat T-cells) can also affect the expression of exogenous

WASP, therefore there was a need to construct S1 WASP shRNA resistant version of human WASP. Through site directed mutagenesis, S1 WASP shRNA resistant WASP (WASP_R) was constructed by introducing four silent mutations at WASP C-terminal region targeted by the S1 WASP shRNA (Fig. 3.5D). WASP_R was then tested in HEK293T cells to verify that it was resistant to S1 WASP shRNA mediated knockdown. Western blot analysis showed that WASP_R is expressed well compared to WASP when transfected together with S1 WASP shRNA (Fig. 3.5A).

3.1.4 WASP^{KD} Jurkat T-cells are defective in chemotaxis to SDF-1 α .

Defects in T-cells migration is one of the primary cause of immune-deficiencies in WAS patients. Impaired T-cell homing and defect in T-cell migration towards chemokine SDF-1 α has been reported [76,200,234] in WASP-deficient T-cells. SDF-1 α is a small chemokine that serves as a potent chemo-attractant for lymphocytes [2,231]. During embryogenesis, SDF-1 α directs the migration of hematopoietic cells from fetal liver to bone marrow [84]. The only known receptor for SDF-1 α is a seven-transmembrane G-protein-coupled receptor, CXCR4 which is constitutively expressed by T lymphocytes, regulates directional migration [55] and also accumulates at the immunological synapse [144]. More than 150,000 binding sites in a single T-cell for SDF-1 α have been reported [87]. Besides CXCR4, Jurkat T-cells also express low level of other chemokine receptors such as CCR1-10 [134]. In contrast to CXCR4, other chemokine receptors such as CCR5 only express in activated T-cells and direct migration along CCL3, CCL4 and CCL5 gradient

[56]. Interaction of SDF-1 α to its receptor CXCR4 has been shown to mediate activation of downstream signalling molecules such as ZAP-70, SLP-76 [166], Lck, Crk-L, Nck and Pyk2 [163] which are regulate lymphocytes migration. Moreover, tyrosine phosphorylation of WASP and its association with Nck has been reported in Jurkat T-cells after stimulation with SDF-1 α , resulting in Jurkat T-cell chemotaxis [162]. All these studies suggest the importance of SDF-1 α in T lymphocytes activation and chemotaxis. Therefore, in this study, SDF-1 α was used as chemokine source to analysis the chemotaxis of WASP or WASP mutants expressing Jurkat T-cells.

In order to identify if knocking down of WASP expression impaired the chemotaxis of WASP^{KD} Jurkat T-cells, the chemotactic response of WT and WASP^{KD} Jurkat T cells towards SDF-1 α gradient was analysed using Dunn chamber. Using time-lapse video microscopy, the migration tracks of WT and WASP^{KD} Jurkat T-cells on the annular bridge of the Dunn chamber were traced (Method. 2.2.4.2.2). As shown in vector plot (Fig. 3.6A), WT Jurkat T-cells migrated preferentially towards the source of SDF-1 α , whereas migration of WASP^{KD} Jurkat T-cells towards SDF-1 α was impaired. In addition, migration velocities of WASP^{KD} Jurkat T-cells (0.98 μ m/min) were significantly reduced compared to WT Jurkat T-cells (1.84 μ m/min) (Fig. 3.6B). In order to analyse the overall directionality of migrated cells, circular histograms were plotted using the final X, Y coordinate values of migrated cells. Based on circular histograms, 85% of WT Jurkat T-cells lies in the 40° arc facing the chemokine source while only 15% of the WASP^{KD} Jurkat T-cells migrated within the 40° arc facing the chemokine source (Fig. 3.6C) suggesting that the migration of

WASP^{KD} Jurkat T-cells was random. The defective chemotaxis of WASP^{KD} Jurkat T-cells toward SDF-1 α was confirmed using transwell migration assay. WT or WASP^{KD} Jurkat T-cells (2×10^5 cells) were loaded in upper well of transwell and were allowed to migrate towards SDF-1 α (100 ng/ml) containing media in the lower well. The cells migrated to the lower well were counted and plotted as percentage of cells migrated (Number of cells migrated to the lower well/Initial cell number loaded to the upper well) X 100. The migration of WASP^{KD} Jurkat T-cells in transwell migration assay was significantly impaired which is consistent with the Dunn chamber chemotaxis results (29.06% of total WASP^{KD} cells migrated to the lower well containing SDF-1 α compared to 51.99% of total WT Jurkat T-cells) (Fig. 3.7). All together, it can be conclude that WASP is essential for chemotactic migration of Jurkat T-cells and partial knockdown of WASP expression was sufficient to cause the impairment.

Figure 3.6 Knocking down of WASP expression impaired the motility of Jurkat T-cells towards SDF-1 α . (A) Vector plots of migration paths of 20 randomly chosen cells from WT and WASP^{KD} Jurkat T-cells exposed to a gradient of SDF-1 α in the Dunn chamber. Time-lapsed images were captured over 3.0 hrs at intervals of 2.0 min. Intersection of the X and Y axes are the starting point of each cell. The source of SDF-1 α was at the top. (B) Migration velocity of 60 randomly chosen cells (20 cells each from 3 sets of experiments). * represent $P < 0.05$ compared to WT Jurkat T-cells (unpaired student's t-test). (C) Circular histograms which showing percentage of cells where the final positions of cells lie in each of the sectors (20°).

Figure 3.7 Impaired motility of WASP^{KD} Jurkat T-cells towards SDF-1 α in transwell migration assay. WT and WASP^{KD} Jurkat T-cells (2×10^5 cells in 100 μ l media) were loaded in upper well and allowed to migrate towards SDF-1 α (100 ng/ml) containing media in the lower well for 3.0 hrs. Cells migrated to the bottom well were counted and plotted as percentage of cells migrated. Experiment was repeated thrice. *** represent $P < 0.001$ compared to WT Jurkat T-cells (unpaired student's t-test).

3.1.5 WASP^{KD} Jurkat T-cells has enhanced binding to fibronectin

Integrins (transmembrane receptors) mediate interaction of cells or tissues with the extracellular matrix (ECM) [63]. Integrins VLA-4 ($\alpha_4\beta_1$) and VLA-5 ($\alpha_5\beta_1$) on T-cells were found to mediate adhesion to fibronectin [104,148]. Although, the expression of VLA-4 and VLA-5 are normal in WASP^{-/-}, WIP^{-/-} and DKO T-cells [76] the dynamic assembly and disassembly of focal adhesions are require for cell migration. WASP^{KD} Jurkat T-cells were impaired in chemokine induced directional migration (Fig. 3.6). Therefore, it is possible that knocking down of WASP expression may affect binding of Jurkat T-cells to fibronectin. To investigate the role of WASP in cell adhesion, cell adhesion assays were performed using WT and WASP^{KD} Jurkat T-cells.

Cell adhesion assay was performed in 96 well microtiter plate coated with fibronectin and BSA coated plate was used as a negative control. Jurkat T-cells (WT and WASP^{KD}) plated on fibronectin or BSA coated tissue culture wells and cells were incubated for 20 min. The binding efficiency of both WT and WASP^{KD} Jurkat T-cells to fibronectin was quantified (Refer to section 2.2.4.1). Initially 1µg/100µL of fibronectin and 1µg/100µL of BSA was used but significant difference between WT and WASP^{KD} Jurkat T-cells was not observed. This concentration might be too high and have saturated the receptor on the cell surface of both the cell types. Therefore, a titration to find the optimal fibronectin concentration was performed and finally a concentration of 0.15 µg/100µL of fibronectin was used.

Figure 3.8 WASP^{KD} Jurkat T-cells adhere more efficiently to fibronectin. (A) Binding assay was performed to compare the cell adhesion capability of WT and WASP^{KD} Jurkat T-cells to fibronectin and BSA (negative control) coated surface. 2×10^4 cells were incubated on fibronectin coated plate (0.15µg/100µl) for 20 min. The bound cells were quantified and plotted. WASP^{KD} Jurkat T-cells showed significantly higher binding than WT Jurkat T-cells to fibronectin. ** represent $P < 0.01$ compared to WT Jurkat T-cells (unpaired student's t-test). (B) Western blot analysis of expression of β1 integrin in WT and WASP^{KD} Jurkat T-cells. Anti GAPDH (α-GAPDH) was used as loading control.

As shown in figure 3.8A, the adhesion of WASP^{KD} Jurkat T-cells (87.7%) to fibronectin (0.15µg/100µl) was significantly higher than WT Jurkat T-cells

(62.3%), whereas adhesion of both WT and WASP^{KD} Jurkat T-cells to BSA were negligible. This increase in WASP^{KD} Jurkat T-cell adhesion to fibronectin was not due to the change in $\beta 1$ integrin expression compared to WT Jurkat T-cells (Fig. 3.8B). Thus, the binding assay showed that loss of WASP leads to increased adhesion of Jurkat T-cells to fibronectin and this might be one of the reasons for migration defect of WASP^{KD} Jurkat T-cells.

3.1.6 WASP^{KD} Jurkat T-cells are defective in anti-CD3/anti-CD28 stimulated actin cytoskeleton organization

WASP regulates actin network during various immunological responses by activating the Arp2/3 complex [215]. Defective actin reorganization and polymerization in response to anti-CD3 stimulation has been reported in WASP-deficient T-cells [77] suggesting the role of WASP in membrane projections formation. Thus, to investigate the role of WASP in TCR/CD28 stimulated rearrangement of actin cytoskeleton, WT and WASP^{KD} Jurkat T-cells were plated on anti-CD3/anti-CD28 coated coverslip (2 μ g/ml each) and incubated for 30 min. As a control, both WT and WASP^{KD} Jurkat T-cells were plated on poly-l-lysine coated coverslip and incubated for same period of time. The cells were fixed with formaldehyde, stained with Alexa 594 phalloidin (Red) and analysed by fluorescence microscopy. As shown in figure 3.9A, in both WT and WASP^{KD} Jurkat T-cells, actin accumulated at the periphery of the cells and the cells had spread well upon stimulation with immobilized anti-CD3/anti-CD28 antibodies (Fig. 3.9B). A number of small actin rich membrane projections were observed in WT Jurkat T-cells after stimulation, which were significantly more compared to WASP^{KD} Jurkat T-cells (Fig.

3.9C). The actin rich membrane projections on T lymphocytes have been shown to be the grouping sites for surface molecules including CD4, CCR5, CXCR5 [198]. In contrast to the cells on anti-CD3/anti-CD28, both the cell types on poly-l-lysine coated coverslip were found to be round (not spread) and formed actin ring on the membrane (Fig. 3.9A & B). This result suggests that WASP is important for actin cytoskeleton reorganization in the presence of both anti-CD3 (TCR) and anti-CD28 (costimulatory antibody).

Figure 3.9 WASP is critical for CD3/CD28 activated actin cytoskeleton rearrangement. (A) WT or WASP^{KD} Jurkat T-cells were plated on anti-CD3/anti-CD28 antibodies coated coverslip, incubated for 30 min, fixed and stained with Alexa 594 phalloidin. (B) The mean surface area of 30 randomly chosen cells in each set of three independent experiments was quantified using Metamorph software and plotted. (C) The average number of projections per cell was quantified for 30 cells in each set of experiment and experiment was repeated thrice. ** $P < 0.01$ compared to WT Jurkat T-cells, *** $P < 0.001$ compared to poly-l-lysine coated surface (unpaired student's t-test).

3.1.7 Expression of N-WASP does not rescue chemotactic defects of WASP^{KD} Jurkat T-cells

WASP^{KD} Jurkat T-cells are defective in chemotaxis towards SDF-1 α (Fig. 3.6). In order to confirm that the chemotactic defect is due to WASP knockdown, reconstitution of WASP expression in WASP^{KD} Jurkat T-cells was performed. To express exogenous WASP in WASP^{KD} Jurkat T-cells, S1 WASP shRNA resistant version of WASP (WASP_R) was made (Described in section 3.1.3).

In order to differentiate between exogenous and endogenous WASP, WASP_R or its mutants were tagged with RFP (WASP_R-RFP or mutant-RFP) and cloned in neomycin resistant plasmid (Appendix 1). These constructs were expressed under the WASP promoter (since high expression of WASP under the CMV promoter was found to be lethal to Jurkat T-cells). The neomycin resistant plasmid used in this study has ability to replicate episomally for several generations. In order to generate stable cell lines expressing exogeneous WASP_R or its mutants, WASP^{KD} Jurkat T-cells were microporated with respective WASP constructs followed by neomycin selection for one week (described in method 2.2.3.8). The percentage of cells stably expressing exogeneous gene was determined by expressing RFP_{CMV} (RFP gene cloned in the same plasmid under the CMV promoter, since WASP promoter is a weak promoter compared to CMV promoter and RFP expression under WASP promoter was difficult to observe using fluorescence microscope). Using this method to generate stable cell lines, more than 95% of cells were found to express the transfected gene (Fig 3.10). WASP^{KD} Jurkat T-cells stably expressing WASP_R-RFP or RFP alone (control) were analysed for their

chemotactic response towards SDF-1 α using Dunn Chamber and time-lapse microscopy.

Figure 3.10 Generation of stable cell line. RFP gene cloned in neomycin resistant plasmid under the CMV promoter was microporated in WASP^{KD} Jurkat T-cells followed by neomycin selection for one week. The percentage of cells stably expressing RFP was observed using fluorescence microscope.

Figure 3.11 Expression of N-WASP does not rescue the chemotactic defect of WASP^{KD} Jurkat T-cell. (A) Vector plots of migration paths of 20 randomly chosen cells from WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) N-WASP-RFP exposed to a gradient of SDF-1 α in the Dunn chamber. Time-lapse images were captured over 3.0 hrs at 2.0 min intervals. Intersection of the X and Y axes are the starting point of the each cells. (B) Percentage of cells where the final positions of cells lie in each of the sectors (20°) was determined by circular histogram. The source of SDF-1 α was at the top.

Reconstitution of WASP expression (WASP_R-RFP) in WASP^{KD} Jurkat T-cells rescued the impaired chemotactic response towards SDF-1 α (Fig. 3.11A) while expression of RFP alone was unable to rescue the chemotactic response towards SDF-1 α . In order to test whether N-WASP can rescue the migration defect of WASP^{KD} Jurkat T-cells, N-WASP-RFP was microporated in WASP^{KD} Jurkat T-cells and selected with neomycin for one week. Similar to control (RFP) cells, N-WASP-RFP expressing WASP^{KD} Jurkat T-cells were defective in chemotaxis towards SDF-1 α in Dunn chamber assay. Based on circular histogram, the directionality of migration of N-WASP-RFP expressing cells was random. Compared to 80% of WASP_R-RFP expressing cells, only 20% of N-WASP-RFP expressing cells lie in the 40° arc facing the chemokine source, which was similar to RFP (control) (10%) expressing cells (Fig. 3.11B).

Figure 3.12 Expression of N-WASP rescued the migration velocity defect of WASP^{KD} Jurkat T-cells. (A) Migration velocity of each cell shown as a mean of velocities of 60 randomly chosen cells (20 cells each from 3 sets of experiments) * $P < 0.05$, ** $P < 0.001$ (unpaired student's t-test). (B) Western blot analysis of the expression levels of WASP and N-WASP in transfected cells after one week of selection with neomycin using appropriate antibodies. α -GAPDH antibody was used as loading control.

Quantitative analysis revealed that N-WASP-RFP expression rescued the migration velocity of WASP^{KD} Jurkat T-cells (2.25µm/min for N-WASP-RFP expressing cells compared to 0.95 µm/min for RFP alone expressing cells) and was similar to WASP_R-RFP expressing cells (2.30µm/min) (Fig. 3.12A). The chemotactic defect of N-WASP-RFP expressing cells was not due to its poor expression (Fig. 3.12B).

Figure 3.13 Expression of N-WASP does not rescue the chemotactic defect of WASP^{KD} Jurkat T-cell in transwell migration assay. Transwell migration assay was performed with WT, WASP^{KD} Jurkat T-cells or WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) N-WASP-RFP. 2 X 10⁵ cells in 100µl media were loaded in upper well and allowed to migrate towards SDF-1α (100 ng/ml) containing media in the lower well for 3.0 hrs. Cells migrated to the bottom well were counted and plotted as percentage of cells migrated. Experiment was repeated thrice. ** *P* < 0.01, *** represent *P* < 0.001 (unpaired student's t-test).

In order to confirm the inability of N-WASP-RFP in rescuing the chemotactic defect of WASP^{KD} Jurkat T-cells, a transwell migration assay was performed. Similar to chemotactic defect observed in Dunn chamber assay, the migration of N-WASP-RFP expressing WASP^{KD} Jurkat T-cells in transwell migration assay was significantly impaired towards SDF-1α (34.45% of N-WASP-RFP expressing cells migrated to lower well of transwell containing SDF-1α which was similar to RFP expressing cells or WASP^{KD} Jurkat T-cells, 32.74% or

29.06% respectively). However, expression of WASP_R-RFP in WASP^{KD} Jurkat T-cells rescued the chemotaxis in transwell migration assay (51.24% of total WASP_R-RFP expressing WASP^{KD} Jurkat T-cells migrated towards SDF-1 α containing media in lower well which was similar to 51.99% WT Jurkat T-cells) (Fig. 3.13). Taken together, it can be concluded that N-WASP can rescue the migration velocity but it does not rescue the directional migration defect of WASP^{KD} Jurkat T-cells.

3.1.8 N-WASP expression does not reduce the adhesion of WASP^{KD} Jurkat T-cells to fibronectin

Result from section 3.1.5 showed that knocking down of WASP expression caused a significant increase in Jurkat T-cells adhesion to fibronectin. In order to characterize whether reconstitution of WASP expression affect cell-ECM adhesion, WASP^{KD} Jurkat T-cells stably expressing RFP or WASP_R-RFP or N-WASP-RFP were incubated on fibronectin coated plate as described in methods 2.2.4.1. Expression of exogenous WASP_R-RFP significantly reduced the adhesion of WASP^{KD} Jurkat T-cells to fibronectin (58.1% of total WASP_R-RFP compared to 82.6% of total RFP expressing cell adhere to fibronectin) (Fig.3.14). In contrast to WASP_R-RFP, expression of N-WASP-RFP (78.0% of total) did not reduce adhesion to fibronectin and behaved similar to WASP^{KD} Jurkat T-cells. Thus, this result suggests that N-WASP is not able to compensate for WASP function in adhesion of Jurkat T-cells to fibronectin.

Figure 3.14 Expression of N-WASP does not reduce adhesion of WASP^{KD} Jurkat T-cells to fibronectin. Binding assay was performed to compare the cell adhesion capability of WT, WASP^{KD} Jurkat T-cells or WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) N-WASP-RFP to fibronectin coated surface. 2×10^4 cells were incubated on fibronectin coated plate (0.15μg/100μl) for 20 min and the bound cells were quantified and plotted. ** represent $P < 0.01$ (unpaired student's t-test).

3.1.9 Expression of N-WASP does not rescue the membrane projection defect of WASP^{KD} Jurkat T-cells.

Result from section 3.1.6 showed that WASP^{KD} Jurkat T-cells form fewer membrane projections in response to anti-CD3/anti-CD28 stimulus. In order to investigate whether reconstitution of WASP expression or expression of N-WASP in WASP^{KD} Jurkat T-cells rescue the formation of membrane projection, WASP^{KD} Jurkat T-cells stably expressing WASP_R-RFP or N-WASP-RFP or RFP (control) were loaded on anti-CD3/anti-CD28 coated plates and incubated for 30 min. Cells were fixed and stained with Alexa 594 phalloidin (Red). The RFP signal from WASP_R-RFP or N-WASP-RFP does not interfere in visualising actin by Alexa 594 phalloidin. Reconstitution of WASP expression in WASP^{KD} Jurkat T-cells rescued the formation of membrane projections after stimulation with anti-CD3/anti-CD28 coated plates. However, expression of N-WASP does not rescue the membrane

projection defect of WASP^{KD} Jurkat T-cells (Fig. 3.15 A & C). Expression of N-WASP does not affect spreading of Jurkat T-cell on stimulatory surface (Fig. 3.15B). N-WASP has been shown to be essential factor in microspike formation in COS-7 cells under EGF stimulation [240], which could be due to the different cell type and different kind of stimulus. These results suggest that N-WASP does not regulate actin cytoskeleton organization in T-cells upon TCR ligation. This could be due to the inability of N-WASP to be activated upon TCR ligation.

Figure 3.15 Expression of N-WASP does not regulate actin remodelling after TCR stimulation. (A) WASP^{KD} Jurkat T-cells expressing (1) RFP alone, (2) WASP_R-RFP, (3) N-WASP-RFP were plated on anti-CD3/anti-CD28 antibodies coated plate, incubated for 30 min, fixed and stained with Alexa 594 phalloidin. (B) The mean surface area of 30 randomly chosen cells in set of three independent experiments was quantified using Metamorph software and plotted. (C) The average number of projections per cell was quantified for 30 cells in each set of experiment and experiment was repeated thrice. ** $P < 0.01$, * $P < 0.05$ compared to WASP_R-RFP expressing WASP^{KD} Jurkat T-cells (unpaired student's t-test).

3.1.10 Expression of N-WASP rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells

Interleukin-2 (IL-2) production is necessary for growth and function of T-cells during an immune response [199]. It has been well documented that T-cells from WAS patients and WASP^{KO} mouse are defective in proliferation and IL-2 secretion in response to T-cell receptor (TCR) triggered by immobilized anti-CD3 antibody [201,248]. In order to characterize whether WASP^{KD} Jurkat T-cells are defective in IL-2 gene transcription, WT and WASP^{KD} Jurkat T-cells (1 X 10⁶ cell per well of 12-well plate) were stimulated with plate bound anti-CD3/anti-CD28 (2µg/ml each) antibody as described (methods 2.2.4.4). Consistent with previous studies in WASP^{KO} mouse and WAS patients, WASP^{KD} Jurkat T-cells were defective in IL-2 gene transcription which was rescued by exogenous WASP_R-RFP expression. Similar to WASP_R-RFP, expression of N-WASP-RFP also rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells (Fig. 3.16).

Figure 3.16 N-WASP expression rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells. Total RNA was isolated from WT, WASP^{KD} Jurkat T-cells or WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) N-WASP-RFP after incubating 8.0 hrs with anti-CD3/anti-CD28 antibodies (2µg/ml each). Quantitative real time PCR was performed and quantified. *** represent $P < 0.001$ (unpaired student's t-test).

Although N-WASP did not to rescue the chemotaxis of WASP^{KD} Jurkat T-cells, it rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells suggesting that N-WASP can rescue some of the defects of WASP^{KD} Jurkat T-cells. Together these results suggest that WASP and N-WASP have some overlapping roles in T-cell functions.

3.1.11 Differential recruitment of N-WASP and WASP to the TCR activation site

WASP has been shown to localize at TCR activation site when T-cells were stimulated on anti-CD3 coated plates [24]. The localization of WASP and its mutants in this study has been performed using WT Jurkat T-cells as WASP^{KD} Jurkat T-cells were stably expressing GFP (retroviral vector used to knockdown endogenous WASP expression also encodes GFP) therefore, cannot be use further for colocalization study. In order to study the localization of WASP and N-WASP relative to actin during TCR stimulation, WT Jurkat T-cells expressing WASP-RFP or N-WASP-RFP or RFP alone were stimulated on anti-CD3 antibody coated plate for 5 min (at this time point, cells spreading was maximal and cells form the strongest contact with the stimulatory plane). Cells were fixed and stained with Alexa 488 phalloidin. This localization study of WASP during T-cell activation is based on a published method [24]. As shown in figure 3.17, WASP-RFP is mainly co-localized with actin at the circumferential ring at the perimeter of the spread cell. Cells did not spread when dropped on to poly-l-lysine coated coverslip. N-WASP-RFP also localized at the actin rich circumferential ring however, in addition, a strong localization of N-WASP-RFP at the center (nucleus) of the spread cell was also observed. Localization of RFP alone was diffused and showed no accumulation

at the circumferential ring. Thus, suggesting that the cellular distribution of N-WASP is different from WASP during TCR stimulation.

Figure 3.17 Localization of WASP and N-WASP to the TCR activation site. WT Jurkat T-cells expressing WASP-RFP or N-WASP-RFP or RFP alone were plated on (A) Poly-L-Lysine coated coverslip or (B) stimulatory coverslip (anti-CD3 antibody coated) and incubated for 5.0 min. Cells were fixed and stained with Alexa 488 phalloidin.

3.1.12 WASP has a 30 amino acid insertion which is absent in

N-WASP

Expression of N-WASP can rescue some of the defects of WASP^{KD} Jurkat T-cells, suggesting that WASP and N-WASP may possess some molecular differences in functions-structure/sequence. Both WASP and N-WASP are similar in terms of protein length (502 a.a. for WASP and 505 a.a for N-WASP). However, protein sequence analysis of WASP and N-WASP revealed

that WASP contains a continuous stretch of 30 amino acids region (a.a.158-187) (Fig. 3.18), which is present between BR and WH1 domain of WASP. This 30 amino acid region of WASP was named as I30 region of WASP.

Figure 3.18 Schematic diagram illustrating the common domains shared by the WASP and N-WASP. WASP possess proline rich 30 amino acid region (a.a.158-187) which is present between WASP homology domain 1 (WH1) and basic region (BR).

Previously, it has been reported that a peptide of WASP comprising I30 region can block the interaction between WASP and Btk (Bruton's tyrosine kinase) [59]. Btk has been shown to phosphorylates WASP at Tyr²⁹¹ [16] which is important for WASP activation [58,170]. A point mutation (G187C) in I30 region of WASP has been reported to cause X-linked thrombocytopenia [96]. Thus, these studies suggest that the I30 region may plays an important role in WASP mediated functions and may account for the functional differences between N-WASP and WASP. In order to elucidate the functions of I30 region, a deletion mutant of WASP lacking this 158-187 amino acids region (referred as WASP^{ΔI30}) was constructed.

3.1.13 I30 region of WASP mediates interaction with SH3 domain containing proteins

Many SH3 domain-containing tyrosine kinases and adaptor proteins such as Lck, Fyn, Btk, Itk, Nck, Grb2, and PST-PIP reported to interact with the proline rich region of WASP (WASP^{PRR}) [25,196]. This interaction of adaptor proteins and tyrosine kinases with WASP^{PRR} is crucial for TCR induced localization of WASP at immunological synapse [44]. I30 region of WASP contains nine proline residues out of 30 amino acids (30% proline) (Fig. 3.18). Therefore, it is possible that SH3 domain containing proteins beside PRR of WASP may also binds to the I30 region of WASP. Thus, a smaller peptide of WASP without PRR (WASP₁₃₈₋₂₆₅) was constructed to characterize the interaction between I30 region and SH3 domain containing proteins. In order to elucidate the interaction between SH3 domain containing proteins and I30 region of WASP, a yeast two hybrid assay was performed using WASP₁₃₈₋₂₆₅ and I30 deleted mutant WASP₁₃₈₋₂₆₅Δ^{I30} as a bait (Fig. 3.19).

Figure 3.19 Schematic representation of peptides of WASP used for yeast two hybrid assay. Gal4BD-WASP₁₃₈₋₂₆₅ or Gal4BD-WASP₁₃₈₋₂₆₅^{ΔI30} fusion peptides were used to study the interaction between I30 region of WASP and SH3 domain containing proteins.

Table 3.1: Yeast Two Hybrid assay: I30 region of WASP interacts with SH3 domain containing proteins.

	BD-Vector	BD-WASP ₁₃₈₋₂₆₅	BD-WASP ₁₃₈₋₂₆₅ ^{ΔI30}
AD-Fusion protein	-T-L-H	-T-L-H	-T-L-H
AD-Vector	-	-	-
AD-Fyn	-	+	-
AD-Fgr	-	+	-
AD-Lyn	-	+	-
AD-Hck	-	+	-
AD-Nck1	-	+	-
AD-Toca1	-	+	-
AD-Grb2	-	+	-
AD-Src	-	+	-
AD-PIK	-	-	-
AD-Tec	-	-	-
AD-VASP	-	-	-
AD-Lck	-	-	-
AD-PSTPIP	-	-	-
AD-Btk	-	-	-
AD-Abl	-	-	-
AD-IRSp53	-	-	-
AD-Cortactin	-	-	-

The yeast strain PJ69-4A was co-transformed with plasmids expressing the BD fusion proteins and the AD fusion proteins in combinations as indicated in the table. The transformants were streaked on histidine lacking SD minimal media plates and incubated at 30°C for 5 days. The growth of the strains on the plates (+) was observed after 5 days of incubation at 30°C. (-) sign indicates lack of interaction. BD-Binding domain, AD-Activation domain.

S. cerevisiae strain PJ69-4A was co-transformed with plasmids expressing Gal4 DNA binding domain fusion proteins and activation domain fusion proteins as indicated in table 3.1. Transformed cells were selected on Trp⁻ Leu⁻

S. cerevisiae strain PJ69-4A was co-transformed with plasmids expressing Gal4 DNA binding domain fusion proteins and activation domain fusion proteins as indicated in table 3.2. Transformed cells were selected on Trp⁻ Leu⁻ (control plates) and Trp⁻ Leu⁻ His⁻ plates were used for checking interaction between BD-WASP₁₃₈₋₂₆₅ or BD-WASP₁₃₈₋₂₆₅^{G187C} fusion proteins and AD-SH3 fusion proteins. As shown in table 3.2, the missense mutation G187C of WASP abolished the interaction of WASP₁₃₈₋₂₆₅^{G187C} with Toca1 suggesting that Gly¹⁸⁷ is critical for WASP^{I30}-Toca1 interaction.

Table 3.2: Yeast Two Hybrid assay. Missense mutation G187C of WASP causes the loss of interaction with Toca1

	BD-Vector	BD-WASP ₁₃₈₋₂₆₅	BD-WASP ₁₃₈₋₂₆₅ ^{G187C}
AD-Fusion protein	-T-L-H	-T-L-H	-T-L-H
AD-Vector	-	-	-
AD-Fyn	-	+	+
AD-Fgr	-	+	+
AD-Lyn	-	+	+
AD-Hck	-	+	+
AD-Nck1	-	+	+
AD-Toca1	-	+	-
AD-Grb2	-	+	+
AD-Src	-	+	+

S. cerevisiae strain PJ69-4A was co-transformed with plasmids expressing the BD fusion proteins and the AD fusion proteins in combinations as indicated in the table. The transformants were streaked on histidine lacking SD minimal media plates and incubated at 30°C for 5 days. The growth of the strains on the plates (+) was observed after 5 days of incubation at 30°C. (-) sign indicates lack of interaction. BD-Binding domain, AD-Activation domain.

3.1.15 Toca1 binds to WASP at both the PRR and I30 region

Results from yeast two hybrid assay (Table. 3.1) indicate that I30 region of WASP interacts with SH3 domain of Toca1 and the point mutation G187C which causes XLT abolishes WASP^{I30}-Toca-1 interaction (Table 3.2). Toca1 has been shown to be essential for N-WASP activation mediated by Cdc42 [89]. In order to confirm the interaction between WASP^{I30} and Toca1, a His-tag pull down assay was performed. Full length WASP-6XHis or deletion mutants of WASP (Fig. 3.21A) were ectopically expressed together with Toca1 in HEK293T cells. The WASP-6XHis or deletion mutants were pulled down from the HEK293T cell lysate using Ni-Agarose beads and the proteins bound to the beads were analyzed by immunoblotting with anti-His and anti Toca1 antibodies.

Toca1 binds to the full length WASP, WASP^{ΔI30} and WASP₁₃₈₋₃₂₀. However, WASP₁₃₈₋₃₂₀^{ΔI30} (which is devoid of both WASP^{PRR} and I30 region) failed to interact with Toca1. The interaction of Toca1 with WASP^{ΔI30} could be due to the presence of PRR of WASP which had interacted with SH3 domain of Toca1. Degradation products of WASP were observed in full length WASP, WASP^{ΔI30} and WASP₁₃₈₋₃₂₀ but not with WASP₁₃₈₋₃₂₀^{ΔI30} suggesting that I30 region might be a possible target site for proteases. Taken together, this result confirmed the interaction of Toca1 with I30 region of WASP (Fig. 3.21B).

Figure 3.21 WASP has two binding sites for interaction with Toca1. (A) Schematic diagram showing the WASP and WASP deletion mutants generated for His-tag pull down assay. (B) HEK293T cells were transfected with Toca1 together with (1) Vector (2) WASP-His, (3) WASP^{ΔI30}-His, (4) WASP₁₃₈₋₃₂₀-His (5) WASP₁₃₈₋₃₂₀^{ΔI30}-His. The WASP or WASP deletion mutants were isolated by His-tag pull-down assay. Western blot analysis of WASP or WASP deletion mutants was detected using anti-His and Toca1 was detected using anti-Toca1 antibody. * represent the degradation product of WASP or its deletion mutants.

3.1.16 Deletion of I30 region of WASP partially opens the closed conformation of WASP.

WASP exists in two different conformations [213]. In the resting state WASP exists in closed conformation and WIP stabilizes the closed conformation of WASP. After T-cell receptor ligation, Rho family of GTPase such as Cdc42 and tyrosine kinases bind and activates WASP [26,44,81] relieving it from its

closed conformation. In order to study the effect of deletion of I30 region (WASP^{ΔI30}) and missense mutation (WASP^{G187C}) present in I30 region on the conformational changes of WASP, a Bi-molecular Fluorescence Complementation (BiFC) assay was performed. This method is based on the complementation of two halves of a single YFP molecule. The YFP molecule was split into two fragments; YFP₁₋₁₅₄ was ligated to the N-terminus of WASP while YFP₁₅₅₋₂₃₈ was ligated to the C-terminus of WASP. Similarly, YFP molecule was fused to WASP^{ΔI30} and WASP^{G187C}. When WASP is in its closed conformation, YFP fluorescence is expected due to close proximity of the two YFP fragments. As the conformation changes to an open one due to activation, fluorescence intensity will be reduced. In this way, conformational changes in WASP can be tracked effectively (Fig. 3.22A). WASP in the absence of WIP has been shown to be unstable in the cytoplasm of the cell [180]. Thus, a nuclear localization signal (NLS) was ligated to the N-terminus of the sensor molecule to target it to the nucleus away from cytoplasmic proteases forming NLS-YFP₁₋₁₅₄-WASP-YFP₁₅₅₋₂₃₈ (WASP sensor), NLS-YFP₁₋₁₅₄- WASP^{ΔI30}-YFP₁₅₅₋₂₃₈ (WASP^{ΔI30} sensor) and NLS-YFP₁₋₁₅₄- WASP^{G187C}-YFP₁₅₅₋₂₃₈ (WASP^{G187C} sensor) under the control of Vrp1 promoter in YEplac112 (Trp) plasmid (Figure 3.22B). Previously, it has shown that WASP sensor adopts an auto-inhibited closed conformation and this closed conformation was due to intramolecular complementation and not due to intermolecular complementation [125]. In addition, WIP has been shown to stabilize the closed conformation of WASP as fluorescence signal of WASP sensor was increased in the presence of WIP [125].

Figure 3.22 (A). Schematic representation of a protein tagged with two halves of a YFP molecule to the two termini. When the WASP molecule adopts a close conformation fluorescence emission occurs and in the open conformation there is no fluorescence emission observed. **(B) Schematic diagram showing fusion constructs used for BiFC assay.** Constructs were cloned with NLS ligated to the N-terminus, under the control of Vrp1 promoter. The fusion constructs were expressed from YEplac112 (Trp) or YEplac181(Leu) or YEplac195 (Ura) plasmids.

In order to investigate whether deletion of I30 region of WASP or missense mutation G187C of WASP affects the conformation of WASP^{ΔI30} or WASP^{G187C} sensor, yeast strain PJ69-4A was transformed with WASP sensors (WT or WASP^{ΔI30} or WASP^{G187C}) (Trp) together with either NLS-WIP or empty vector (Ura). The transformants were then selected on plates deficient in Tryptophan and Uracil and subsequently analyzed using fluorescence

microscopy (Fig. 3.23A). The fluorescence signals from 100 cells were quantified and plotted (Fig. 3.23B).

Figure 3.23 The I30 region of WASP is critical for mounting closed conformation.

(A) *S. cerevisiae* cells were transformed with empty vector (Ura) or NLS-WIP (Ura) together with (1) WASP sensor (Trp) or (2) WASP^{ΔI30} sensor (Trp) or (3) WASP^{G187C} sensor (Trp) and selected on SD (-Trp-Ura) plates. The cells were grown at 24°C in selective media and YFP signals in these cells were subsequently analyzed using fluorescence microscopy. Bar = 5μm.

(B) Fluorescence signal quantification from 100 *S. cerevisiae* cells expressing empty vector (Ura) or WIP (Ura) together with (1) WASP sensor (Trp) or (2) WASP^{ΔI30} sensor (Trp) or (3) WASP^{G187C} sensor (Trp) using metamorph software. *** $P < 0.001$ (Unpaired students t-test).

(C) Analysis of expression of the WASP sensors in *S. cerevisiae* cells using anti-GFP (α-GFP) antibody (anti-GFP antibody also recognizes YFP; therefore it is used to control the amount of sensors). Anti-Hexokinase (α-Hex) antibody was used as loading control.

As shown in figure 3.23 A & B the fluorescence intensity of WASP^{ΔI30} and WASP^{G187C} sensors were similar to WASP sensor in the absence of WIP. However the YFP fluorescence of all the three WASP sensors was significantly increased in the presence of WIP. Compared to WASP sensor + WIP, the YFP fluorescence intensity of WASP^{ΔI30} sensor + WIP was significantly reduced. However, the YFP fluorescence intensity of WASP^{G187C} sensor + WIP was similar to WASP sensor + WIP (Fig. 3.23 A & B). Western blot analysis showed equal expression of all three WASP sensors either in the presence or absence of WIP indicating that increase in fluorescence intensity of WASP sensors was due to increase in stability of closed conformation of WASP and was not due to increase in the expression of WASP sensor molecules in the presence of WIP (Fig. 3.23C). These results suggest that deletion of I30 region of WASP partially opens the WASP closed conformation in the presence of WIP and this reduction in YFP fluorescence intensity was not due to a weakened interaction between WASP^{ΔI30} and WIP (Data not shown). However, mutation in the I30 region of WASP (WASP^{G187C}) which causes XLT does not affect WASP closed conformation.

3.1.17 Toca1 or Nck1 are unable to reduce YFP fluorescence intensity of WASP^{ΔI30} in the presence of WIP.

Previously, it has been shown that Toca1 and Nck1 can relieve WASP from its closed conformation even in the presence of WIP [125]. Result from yeast two hybrid assay showed that both Nck1 and Toca1 interact with I30 region of WASP (Table 3.1).

Figure 3.24 Conformation of WASP^{Δ130} is not altered by Nck1 or Toca1. (A) *S. cerevisiae* cells were transformed with WASP or WASP^{Δ130} sensor (Trp) + NLS-WIP (Ura) together with (1) empty Leu plasmid, or Leu plasmids expressing (2) NLS-Toca1 or (3) NLS-Nck1 and selected on SD (-Trp-Leu-Ura) plates. The transformants were grown phase at 24°C in selective media and YFP signals in these cells were subsequently analyzed using fluorescence microscopy. Bar= 5μm. (B) Fluorescence signal quantification from 100 *S. cerevisiae* cells expressing WASP or WASP^{Δ130} sensor (Trp) + NLS-WIP (Ura) together with (1) empty Leu plasmid, (2) NLS-Toca1 or (3) NLS-Nck1 using metamorph software. ****P*<0.001 compared to WASP sensor + NLS-WIP + Vector expressing cells. ***P*<0.01 (Unpaired students t-test). (C) Analysis of expression of the WASP or WASP^{Δ130} sensor (Trp) + NLS-WIP (Ura) in *S. cerevisiae* cells using anti-GFP (α-GFP) (anti-GFP antibody also recognizes YFP, therefore it is used to control the amount of sensors) and anti-WIP antibody. Anti-Hexokinase (α-Hex) antibody was used as loading control.

To investigate whether Toca1 and Nck can cause changes in the conformation of WASP^{ΔI30}, BiFC assay was performed. Yeast strain PJ69-4A was first transformed with WASP sensor or WASP^{ΔI30} sensor (Trp) together with NLS-WIP (Ura). The transformants were then co-transformed with Leu plasmids expressing either NLS-Toca1 or NLS-Nck1 or empty Leu plasmid. The cells were then selected on plates deficient in Tryptophan, Leucine and Uracil and were subsequently analyzed using fluorescence microscopy. As shown in figure 3.24 A & B, the fluorescence intensity of WASP sensor + WIP was reduced significantly when co-expressed with either Toca1 or Nck1. Deletion of I30 region of WASP partially disrupts the WASP auto-inhibitory conformation in the presence of WIP (Fig. 3.23 A & B). However, there was no further reduction in the fluorescence intensity of WASP^{ΔI30} + WIP observed either in the presence of Toca1 or Nck1 (Fig. 3.24 A & B). In addition, WASP sensor + WIP + Toca1 or WASP sensor + WIP + Nck1 had significantly lower fluorescence intensity than WASP^{ΔI30} sensor + WIP + Toca1 or WASP^{ΔI30} sensor + WIP + Nck1 respectively (Fig. 3.24 A & B). The expression of WASP and WASP^{ΔI30} sensors was also comparable in all respective co-transformants (Fig. 3.24C) suggesting that the differences in fluorescence intensities observed were not due to the difference in protein expression. All together, these results suggesting that Nck1 or Toca1 can relieve the auto-inhibitory state of WASP sensor but have no effect on the changes in partial open conformation of WASP^{ΔI30} sensor. The significantly reduced fluorescence intensity of WASP sensor + WIP + Toca1 or Nck1 compared to WASP^{ΔI30} sensor + WIP + Toca1 or Nck1 suggest the involvement of I30 region of WASP in opening of WASP auto-inhibitory conformation mediated by Toca1 or Nck1.

3.1.18 Glycine¹⁸⁷ of WASP is critical for Toca1 mediated conformation changes of WASP.

As mentioned in section 3.1.16, a point mutation in the I30 region of WASP (WASP^{G187C}) did not affect WASP conformation in the presence of WIP. Therefore, the effect of Toca1 or Nck1 on the conformational change of WASP^{G187C} sensor in the presence WIP was further analysed using BiFC assay. Nck1 but not Toca1 was able to reduce the YFP fluorescence intensity of WASP^{G187C} sensors even in the presence of WIP (Fig. 3.25 A & B). This reduction of fluorescence intensity in the presence of Nck1 was not due to reduced expression of WASP^{G187C} sensor (Fig. 3.26). As shown in yeast two hybrid assay, a small peptide of WASP with a point mutation G187C (WASP₁₃₈₋₂₆₅^{G187C}) abolished WASP^{I30}-Toca1 interaction (Table 3.2) but not with Nck1 suggesting that Gly¹⁸⁷ is critical for WASP^{I30}-Toca1 interaction. Deletion of I30 region (comprising Gly¹⁸⁷) in full length WASP (WASP^{ΔI30}) was found to interact with Toca1 due to presence of proline rich region of WASP (Fig. 3.21B). All these results when taken together, suggest that Toca1 can still interact with WASP^{G187C} (through PRR of WASP) but this interaction does not alter the auto-inhibitory conformation of WASP suggesting that Gly¹⁸⁷ is critical for Toca1 mediated opening of WASP conformation.

Figure 3.25 Conformation of WASP^{G187C} is not altered by Toca1 (A). (A) *S. cerevisiae* cells were transformed with WASP sensor or WASP^{G187C} sensor (Trp) + NLS-WIP (Ura) together with (1) empty Leu plasmid, or Leu plasmids expressing (2) NLS-Toca1 or (3) NLS-Nck1 and selected on SD (-Trp-Leu-Ura) plates. The cells were grown at 24°C in selective media and YFP signals in these cells were subsequently analyzed using fluorescence microscopy. Bar= 5μm. (B) Fluorescence signal quantification from 100 *S. cerevisiae* cells expressing WASP sensor or WASP^{G187C} sensor (Trp) + NLS-WIP (Ura) together with (1) empty Leu plasmid, (2) NLS-Toca1 or (3) NLS-Nck1 using metamorph software. *** $P < 0.001$ compared to WASP sensor + NLS-WIP + Vector (Unpaired student's t-test)

Figure 3.26 Conformation of WASP^{G187C} is not altered by Toca1 (B). Western blot analysis of expression of the WASP sensor or WASP^{G187C} sensor (Trp) + WIP (Ura) transformed together with (1) empty Leu plasmid, or Leu plasmids expressing (2) NLS-Toca1, (3) NLS-Nck1 using anti-GFP (α -GFP) (anti-GFP antibody also recognize YFP, therefore it is used to control the amount of sensors) and anti-WIP antibody. Anti-Hexokinase (α -Hex) antibody was used as loading control.

3.1.19 Hck binds to WASP at both the PRR and I30 region of WASP

Beside Cdc42-Toca1 mediated WASP activation, tyrosine kinases have also been reported to regulate WASP activity in a Cdc42 independent manner [58]. Src family of tyrosine kinase such as Hck is found to be exclusively expressed in hematopoietic cells, which binds to the PRR of WASP via its SH3 domain and phosphorylates WASP at conserved tyrosine residue (Tyr²⁹¹) [58,193]. WASP phosphorylation Tyr²⁹¹ is thought to alter the charges on amino acid thus affect the WASP auto-inhibited conformation. Results from yeast two hybrid data showed that deletion of I30 region abolish WASP₁₃₈₋₂₆₅ ^{Δ I30}-Hck interaction (Table 3.1). In order to confirm the interaction between I30 region of WASP and Hck, a His-tag pull down assay was performed.

Figure 3.27 WASP has two binding sites for Hck. HEK293T cells were transfected with Hck together with (1) Vector (2) WASP-His, (3) WASP^{ΔI30}-His, (4) WASP₁₃₈₋₃₂₀-His (5) WASP₁₃₈₋₃₂₀^{ΔI30}-His. The WASP or WASP deletion mutants were isolated by His-tag pull-down assay. Western blot analysis of WASP or WASP deletion mutants was detected using anti-His and Hck was detected using anti-GFP antibody. * represent the degradation product of WASP or its deletion mutants.

Full length WASP-6XHis or deletion mutants of WASP tagged with histidine (Fig. 3.21A) were ectopically expressed together with Hck tagged with GFP in HEK293T cells. The WASP-6XHis or deletion mutants were pulled down from the HEK293T cell lysate using Ni-Agarose beads and the protein bound to the beads were analyzed by immunoblotting with anti-His and anti-GFP antibodies. Similar to result obtained with Toca1 (Fig. 3.21B), Hck interacted with full length WASP, WASP^{ΔI30} and WASP₁₃₈₋₃₂₀ but not with WASP₁₃₈₋₃₂₀^{ΔI30} (which is devoid of both the PRR domain and I30 region). The interaction of Hck with WASP^{ΔI30} is due to the presence of PRR of WASP which has interacted with SH3 domain of Hck. Taken together, these results suggest that WASP has two binding sites for Hck, one is PRR and the other is I30 region (Fig. 3.27).

3.1.20 Deletion of I30 region increase WASP^{ΔI30} tyrosine phosphorylation by Hck

The significance of WASP-Tyr²⁹¹ phosphorylation in T-cell development was described in a study using knock-in mouse model of phospho-mutants (Y293E/F, equivalent to Y291 in murine WASP) [30]. It is proposed that in order for kinases to gain access to WASP^{Y291}, binding of an activator such as Cdc42 that can partially destabilize the conformation, should first take place. BiFC data suggest that deletion of I30 region partially opens the WASP closed conformation (Fig. 3.23 A & B). Therefore, it is possible that the partially opened conformation of WASP^{ΔI30} may bypass the requirement of an activator for kinases to gain access to WASP^{Y291}. The results from previous section suggest that Hck interacts with both PRR and I30 region of WASP (Fig. 3.27). Therefore, the ability of Hck to phosphorylate WASP or WASP^{ΔI30} was examined. HEK293T cells were transfected with WASP or WASP^{ΔI30} tagged with Histidine tag, together with vector or Hck tagged with GFP. The transfected cells were lysed and His-tag pull-down assay was performed. The tyrosine-phosphorylation levels of WASP or WASP^{ΔI30} was detected using phosphotyrosine specific antibody, 4G10. Western blot analysis revealed that Hck increase WASP tyrosine phosphorylation when I30 region was deleted from WASP (Fig. 3.28). The increased level of phosphorylated WASP^{ΔI30} detected from cells co-expressing Hck in comparison to the WT counterpart was not due to unequal levels of WASP or WASP^{ΔI30} isolated by the pull-down assay (detected using anti-His antibody). No phosphorylation of WASP was detected when the cells were transfected with WASP or WASP^{ΔI30} constructs without Hck indicating that the phosphorylation levels detected in the western

blots were due to exogenous Hck expression. Thus, these results suggest that deletion of I30 region increases WASP^{ΔI30} tyrosine phosphorylation by Hck.

Figure 3.28 Deletion of I30 region enhanced Hck mediated phosphorylation of WASP. (A) HEK293T cells were transfected with WASP-His or WASP^{ΔI30}-His together with (1) Vector, (2) Hck-GFP. WASP or WASP^{ΔI30} was isolated by His-tag pull-down assay. Western blot analysis of levels of Hck-GFP isolated using anti-GFP (α -GFP) antibody. (B) Tyrosine-phosphorylation levels of WASP or WASP^{ΔI30} was detected using 4G10 antibody. (C) Western blot analysis of levels of WASP or WASP^{ΔI30} isolated using anti-His (α -His) antibody.

3.1.21 WASP^{ΔI30} or WASP^{G187C} can rescue the growth defect of *S. cerevisiae las17Δ* strain

Results from the section 3.1.16 suggest that deletion of I30 region partially opens the auto-inhibitory WASP conformation, resulted in increase in WASP^{ΔI30} phosphorylation by Hck (Fig. 3.28). Therefore, there is a possibility of global change in the conformation of WASP^{ΔI30} leading to defects in the functions of WASP-WIP complex. Las17p is the yeast homologue of WASP and *las17Δ* strain (IDY166) can grow at 24°C but does not grow at elevated temperature 37°C [126]. Our laboratory has shown that expression of human WASP does not rescue the growth defect of *las17Δ* strain. However, expression of Human WASP together with Human WIP can rescue the growth

defects of *S. cerevisiae las17Δ* strain [180]. In order to determine whether WASP^{ΔI30} or WASP^{G187C} can rescue the growth defects of *las17Δ* strain, WASP or WASP^{ΔI30} or WASP^{G187C} transformed together with or without WIP in *las17Δ* yeast strain. Transformed cells were selected on Trp⁻ Leu⁻ plates. Colonies from the selection plate were streaked onto YPUAD agar plates and grown either at 24°C or 37°C for 3 days. As shown in figure 3.29A, WASP or WASP^{ΔI30} or WASP^{G187C} together with WIP can rescue the growth defect of *las17Δ* strain. Western blot analysis showed comparable expression of WT WASP or WASP mutants (WASP^{ΔI30} or WASP^{G187C}) (Fig. 3.29B).

Figure 3.29 WASP^{ΔI30} or WASP^{G187C} together with WIP can rescue the growth defect of *las17Δ* strain. (A) Viability at 24 and 37°C of *las17Δ* yeast strain IDY166 transformed with full length WT WASP or WASP^{ΔI30} or WASP^{G187C} expressing plasmids with WIP or empty vector (Vect). Each strain was streaked on YPUAD agar, incubated at 24 as well as at 37°C. (B) Western blot analysis of WASP and WASP^{ΔI30} or WASP^{G187C} expressed with WIP or empty vector (Vect) in *las17Δ* yeast strain IDY166 at 24°C using anti-WASP antibody. Anti-Hexokinase antibody was used as endogeneous control.

This result suggests that deletion of I30 region of WASP (WASP^{ΔI30}) or a point mutation in the I30 region (WASP^{G187C}) does not affect the basic activity of WASP. Both the mutants can rescue the growth defect of *las17Δ* strain suggesting that formation of functional WASP-WIP complex is not abolished.

3.1.22 WASP^{G187C} but not WASP^{ΔI30} rescue the migration defect of WASP^{KD} Jurkat T-cells

As deletion of I30 region of WASP altered the WASP conformation, resulting in increased Tyr²⁹¹ phosphorylation of WASP^{ΔI30} by Hck (Fig. 3.28), it is possible that it may affect WASP function in receptor mediated cytoskeleton response of T-cells. Hck is expressed exclusively in the hematopoietic lineages and it plays an important role in coupling chemoattractant stimuli to actin cytoskeleton rearrangement which is critical for chemotaxis of neutrophils and monocytes [61,206,254]. Therefore, an in vitro chemotaxis assay was performed using Dunn chamber to address whether WASP^{ΔI30}-RFP expression would influence the chemotaxis of WASP^{KD} Jurkat T-cells towards chemokine SDF-1 α . As shown in figure 3.30A, reconstitution of WASP expression in WASP^{KD} Jurkat T-cells by stably expressing (selected with neomycin for one week after microporation) WASP_R-RFP was able to rescue the chemotaxis of WASP^{KD} Jurkat T-cells towards SDF-1 α . However, chemotactic response of WASP^{KD} Jurkat T-cells expressing WASP_R^{ΔI30}-RFP or RFP alone (control) were significantly compromised (Fig. 3.30A). Quantitative analysis revealed that the migration speed of WASP^{KD} Jurkat T-cells expressing WASP_R^{ΔI30}-RFP (2.1 μ m/min) was comparable to cells expressing

WASP_R-RFP (2.3μm/min) and was lower for RFP (control) expressing cells (0.95μm/min) (Fig. 3.30B). Indeed, it was observed that WASP_R^{Δ130}-RFP expressing WASP^{KD} Jurkat T-cells frequently changed their direction compared to WASP_R-RFP expressing cells. The circular histogram showing the overall directionality of cell migration revealed that 80% of total WASP_R-RFP expressing cells compared to only 22.32% of WASP_R^{Δ130}-RFP expressing cells moved within 40° arc facing the chemokine source, indicating that the migration of WASP_R^{Δ130}-RFP expressing cells were random (Fig. 3.31A).

Figure 3.30 Expression of WASP^{Δ130} does not rescue the chemotaxis of WASP^{KD} Jurkat T-cell (A). (A) Vector plots of migration paths of 20 randomly chosen cells from WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{Δ130}-RFP, (4) WASP_R^{G187C}-RFP exposed to a gradient of SDF-1α in the Dunn chamber. Time-lapse images were captured over 3.0 hrs at intervals of 2.0 min. Intersection of the X and Y axes are the starting point of each cell. The source of SDF-1α was at the top. (B) Migration velocity of each cell shown as a mean of velocities of 60 randomly chosen cells (20 cells each from 3 sets of experiments) ** represent $P < 0.01$ compared to RFP (control) expressing cells (unpaired student's t-test).

The chemotactic defect of WASP_R^{ΔI30}-RFP expressing WASP^{KD} Jurkat T-cells was not due to the poor expression of WASP_R^{ΔI30}-RFP as western blot analysis showed its expression was comparable to WASP_R-RFP expression (Fig. 3.31B). A point mutation in the I30 region of WASP (WASP_R^{G187C}-RFP) when expressed in WASP^{KD} Jurkat T-cells behaved similar to WASP_R-RFP expressing cells (preferentially migrated towards the SDF-1α gradient) (Fig. 3.30A). Infact the migration speed of WASP_R^{G187C}-RFP (2.02μm/min) expressing cells was comparable to WASP_R-RFP expressing cells (2.3μm/min) (Fig. 3.30B).

Figure 3.31 Expression of WASP^{ΔI30} does not rescue the chemotaxis of WASP^{KD} Jurkat T-cell (B). (A) WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{ΔI30}-RFP, (4) WASP_R^{G187C}-RFP exposed to a gradient of SDF-1α in the Dunn chamber. Percentage of cells where the final positions of cells lie in each of the sectors (20°) was determined by circular histogram. The source of SDF-1α was at the top. (B) Analysis of the expression levels of (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{ΔI30}-RFP, (4) WASP_R^{G187C}-RFP in transfected cells after one week of selection with neomycin using appropriate antibodies.

The overall directionality of WASP_R^{G187C}-RFP expressing cells (60% of total), according to circular histogram plotted was comparable to WASP_R-RFP expressing cells (80% of total) (Fig. 3.31A), indicating that the point mutation (G187C in WASP I30 region) has a minimal effect on directional migration of Jurkat T-cells. The migration defect of WASP^{KD} Jurkat T-cells expressing WASP_R^{ΔI30}-RFP was further confirmed by transwell migration assay. The migration of WASP_R^{ΔI30}-RFP expressing WASP^{KD} Jurkat T-cells in transwell migration assay towards SDF-1α was significantly impaired, which is consistent with the Dunn chamber results (38.88% of total WASP^{KD} + WASP_R^{ΔI30}-RFP cells migrated to lower well compared to 51.24% of total WASP^{KD} + WASP_R-RFP Jurkat T-cells) (Fig. 3.32). The migration of WASP^{KD} + WASP_R^{G187C}-RFP Jurkat T-cells (55.48%) in transwell migration assay was similar to WASP^{KD} + WASP_R-RFP Jurkat T-cells (Fig. 3.32).

Figure 3.32 Expression of WASP^{ΔI30} does not rescue the chemotaxis of WASP^{KD} Jurkat T-cell in transwell migration assay. WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{ΔI30}-RFP, (4) WASP_R^{G187C}-RFP cells were loaded in upper well and allowed to migrate towards SDF-1α (100 ng/ml) containing media in the lower well for 3.0 hrs. Cells migrated to the bottom well were counted and plotted as percentage of cells migrated. Experiment was repeated thrice. * $P < 0.05$, ** $P < 0.01$, *** represent $P < 0.001$ compared to RFP (control) transfected WASP^{KD} Jurkat T-cells (unpaired student's t-test).

Taken together, these results suggest that the I30 region of WASP plays an essential role in establishing or maintaining the directionality of Jurkat T-cells movement towards chemokine whereas a point mutation in I30 region (G187C) does not affect chemotactic migration.

3.1.23 Expression of WASP^{ΔI30} or WASP^{G187C} reduced the adhesion of WASP^{KD} Jurkat T-cells to fibronectin

In contrast to WASP, N-WASP expression in WASP^{KD} Jurkat T-cells did not reduce the adhesion of cells to fibronectin (Fig. 3.14). WASP consists of a continuous stretch of 30 amino acid (I30 region) which is absent in N-WASP (Fig 3.18). Therefore, the involvement of I30 region in Jurkat T-cell adhesion was determined. WASP^{KD} Jurkat T-cells expressing exogenous WASP (WASP_R-RFP) or WASP mutants (WASP_R^{ΔI30}-RFP or WASP_R^{G187C}-RFP) or RFP alone (control) added to 96-well plates coated with fibronectin (1.5μg/ml) and the bound cells were quantified after washing off unbound cells. As shown in figure 3.33, expression of WASP_R-RFP (62.6%) or WASP_R^{ΔI30}-RFP (38.13%) or WASP_R^{G187C}-RFP (35.2%) significantly reduced the adhesion of WASP^{KD} Jurkat T-cells compared to RFP (82.6%) expressing cells. In addition, the adhesion of WASP_R^{ΔI30}-RFP (38.13%) or WASP_R^{G187C}-RFP (35.2%) expressing cells to fibronectin was substantially reduced compared to WASP_R-RFP (62.6%) expressing WASP^{KD} Jurkat T-cells. The significant reduction of adhesion of WASP_R^{ΔI30}-RFP or WASP_R^{G187C}-RFP expressing cells to fibronectin compared to WASP_R-RFP expressing cells, suggesting the possibility of increase in WASP turnover leading to random motility. Thus, the

result of binding assay indicates that I30 region of WASP may regulate T-cell-ECM adhesion.

Figure 3.33 Expression of WASP^{ΔI30} or WASP^{G187C} reduced the adhesion of WASP^{KD} Jurkat T-cells to fibronectin. Binding assay was performed to compare the cell adhesion capability of WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{ΔI30}-RFP, (4) WASP_R^{G187C}-RFP to fibronectin coated surface. 2×10^4 cells were added and allowed to adhere to fibronectin coated plate (0.15μg/100μl) for 20 min. The bound cells were quantified and plotted. ** represent $P < 0.01$ (unpaired student's t-test).

3.1.24 Expression of WASP^{ΔI30} or WASP^{G187C} does not rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells

I30 region of WASP interacts with SH3 domain containing proteins (Section 3.1.13). It might be possible that besides interaction with SH3 domain containing proteins, I30 region of WASP is responsible for interaction with other unknown WASP regulatory proteins and loss of these interactions might be responsible for defective chemotaxis of WASP_R^{ΔI30}-RFP expressing cells. Besides defective chemotaxis, deletion of I30 region of WASP might affect the other WASP mediated functions. WASP has been shown to be important for T-cell activation [43]. Therefore, the role of I30 region of WASP in T-cell activation was determined. WT Jurkat T-cells or WASP^{KD} Jurkat T-cells expressing WASP_R-RFP or WASP_R^{ΔI30}-RFP or WASP_R^{G187C}-RFP or RFP

alone (control) cells were stimulated on anti-CD3/anti-CD28 coated plates as described in methods 2.2.4.4. Quantitative real time analysis revealed that the expression of exogenous WASP (WASP_R-RFP) rescued while the expression of WASP_R^{ΔI30}-RFP or WASP_R^{G187C}-RFP did not rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells (Fig. 3.34). This result suggests that I30 region of WASP is important for its function in IL-2 gene transcription. Moreover, a point mutation in I30 region of WASP (G187C) is sufficient to abolish WASP mediated IL-2 gene transcription.

Figure 3.34 Expression of WASP^{ΔI30} or WASP^{G187C} does not rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells. Total RNA was isolated from WT or WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{ΔI30}-RFP, (4) WASP_R^{G187C}-RFP 8.0 hrs after incubation with anti-CD3/anti-CD28 antibodies (2μg/ml each). Quantitative real time PCR was performed and quantified. *** represent $P < 0.001$ compared to WT Jurkat T-cells (unpaired student's t-test).

3.1.25 Expression of WASP^{ΔI30} does not rescue the membrane projection defect of WASP^{KD} Jurkat T-cells.

Deletion of I30 region of WASP abolished WASP function in chemotaxis and IL-2 gene transcription (Section. 3.1.22 & 3.1.24). In order to identify the role of I30 region of WASP in TCR induced actin cytoskeleton organization,

WASP^{KD} Jurkat T-cells expressing WASP_R^{ΔI30}-RFP or WASP_R^{G187C}-RFP or RFP alone were induced with plate bound anti-CD3/anti-CD28 coated antibodies (described in methods 2.2.4.5). Compared to WASP_R^{G187C}-RFP or WASP_R-RFP, expression of WASP_R^{ΔI30}-RFP does not rescue the defect of WASP^{KD} Jurkat T-cells in TCR stimulated membrane projections formation suggesting the importance of I30 region in actin cytoskeleton reorganization (Fig. 3.35 A & C). IL-2 production and actin polymerization are the independent activities of WASP [93]. Actin polymerization and reorganization (formation of lamellipodia and filopodia) plays a major role in cell spreading and cell migration [121]. Deletion of I30 region of WASP did not affect spreading of Jurkat T-cells on anti-CD3/anti-CD28 coated plates (Fig. 3.35B), but abolished chemotaxis, membrane projections formation and IL-2 gene transcription. On the other hand a point mutation WASP^{G187C} only affected IL-2 gene transcription suggesting the possibility of regulation of WASP activities by its I30 region by through its interactions with different regulators. These results indicate that I30 region of WASP independently regulates its dual functions; actin cytoskeleton organization through VCA domain of WASP (which is important for chemotaxis) and IL-2 gene transcription (which is dependent on WASP region comprising glycine at 187 position of WASP).

Figure 3.35 Expression of WASP^{ΔI30} does not regulate actin remodelling after TCR stimulation.

(A) WASP^{KD} Jurkat T-cells expressing (1) RFP alone, (2) WASP_R-RFP, (3) WASP_R^{ΔI30}-RFP, (4) WASP_R^{G187C}-RFP were plated on anti-CD3/anti-CD28 antibodies coated plate, incubated for 30 min, fixed and stained with Alexa 594 phalloidin. (B) The mean surface area of 30 randomly chosen cells in each three independent experiments was quantified using Metamorph software and plotted. (C) The average number of projections per cell was quantified for 30 cells in each set of experiment and experiment was repeated thrice. ** $P < 0.01$, * $P < 0.05$ compared to WASP_R-RFP expressing WASP^{KD} Jurkat T-cells (unpaired student's t-test).

3.1.26 The I30 region does not regulates WASP localization at TCR activation site

The I30 region of WASP is critical for interaction with SH3 domain containing proteins (Table 3.1) Therefore, the involvement of I30 region of WASP in recruiting WASP to the TCR activation site was determined. WT Jurkat T-cells expressing WASP^{ΔI30}-RFP or WASP^{G187C}-RFP were stimulated on anti-CD3 coated plate as described in section 3.1.11.

Figure 3.36 Deletion of I30 region of WASP does not affect its localization at TCR activation site. WT Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP^{ΔI30}-RFP, (4) WASP^{G187C}-RFP were plated on stimulatory coverslip (anti-CD3 antibody coated) and incubated for 5.0 min. Cells were fixed and stained with Alexa 488 phalloidin.

As shown in figure 3.36, deletion of I30 region of WASP does not affect WASP^{ΔI30} recruitment to the periphery of the cells where it colocalized with the actin ring. Similarly, WASP^{G187C}-RFP is also recruited to the circumferential actin ring. WASP has been shown to bind to many cytoplasmic proteins and therefore, there are several mechanisms have been proposed for WASP recruitment at TCR activation sites. This result suggests that I30 region of WASP may not be crucial for WASP recruitment to the TCR activation site.

3.1.27 Insertion of I30 region of WASP in N-WASP rescued the chemotaxis of WASP^{KD} Jurkat T-cells

Expression of N-WASP did not rescue the chemotaxis of WASP^{KD} Jurkat T-cells (Section 3.1.7) Moreover, deletion of I30 region of WASP abolished

WASP function in rescuing the chemotaxis of WASP^{KD} Jurkat T-cells suggesting the importance of WASP^{I30} region in chemotaxis towards SDF-1 α (Section. 3.1.22). Therefore, it was hypothesised that insertion of WASP-I30 region in N-WASP may rescue the chemotactic defects of WASP^{KD} Jurkat T-cells. The I30 region of WASP was inserted in N-WASP at a region between WH1 domain and basic region using overlapping PCR cloning method. Three reverse primers (contains a smaller part of I30 region) were used to insert I30 region of WASP in N-WASP (Appendix 2). This modified N-WASP construct was referred to as N-WASP-I30 (Fig. 3.37).

Figure 3.37 Schematic diagrams representing the insertion of I30 region of WASP in N-WASP. WASP-I30 region was inserted in N-WASP between WH1 domain and basic region of N-WASP.

In order to compare the expression of WASP, N-WASP and N-WASP-I30 in Jurkat T-cells, these construct were tagged with His-tag and cloned in neomycin resistant plasmid. WASP_R-His, N-WASP-His, N-WASP-I30-His and vector alone were microporated in WASP^{KD} Jurkat T-cells and subsequently selected with neomycin for one week. Subsequently, the chemotactic responses of neomycin selected cells (stable cells) towards SDF-1 α were analysed using Dunn chamber.

Figure 3.38 Expression of N-WASP-I30 rescued the chemotactic defect of WASP^{KD} Jurkat T-cell (A). (A) Vector plots of migration paths of 20 randomly chosen cells from WASP^{KD} Jurkat T-cells expressing (1) Vector, (2) WASP_R-His, (3) N-WASP-His, (4) N-WASP-I30-His exposed to a gradient of SDF-1α in the Dunn chamber. Time-lapse images were captured over 3.0 hrs at intervals of 2.0 min. Intersection of the X and Y axes are the starting point of each cell. The source of SDF-1α was at the top. (B) Migration velocity of each cell shown as a mean of velocities of 60 randomly chosen cells (20 cells each from 3 sets of experiments) * $P < 0.05$, ** represent $P < 0.01$ compared to vector expressing cells (unpaired student's t-test).

As shown in figure 3.38, expression of WASP_R-His rescued the chemotactic defect of WASP^{KD} Jurkat T-cells whereas N-WASP-His expressing cells had impaired migration towards the chemokine gradient and behaved similar to empty vector expressing WASP^{KD} Jurkat T-cells (Fig. 3.38A). However, expression of N-WASP-I30-His rescued the impairment in chemotaxis of WASP^{KD} Jurkat T-cells. The migration velocities (μm/min) of WASP^{KD} Jurkat T-cells expressing N-WASP-His (2.26 μm/min), or N-WASP-I30-His (1.82

$\mu\text{m}/\text{min}$) was comparable to WASP_R-His expressing cells (1.89 $\mu\text{m}/\text{min}$) (Fig.3.38B).

Figure 3.39 Expression of N-WASP-I30 rescued the chemotactic defect of WASP^{KD} Jurkat T-cell (B). WASP^{KD} Jurkat T-cells expressing (1) Vector, (2) WASP_R-His, (3) N-WASP-His, (4) N-WASP-I30-His exposed to a gradient of SDF-1 α in the Dunn chamber. Percentage of cells where the final positions of cells lie in each of the sectors (20°) was determined by circular histogram. The source of SDF-1 α was at the top.

Circular histogram analysing the overall directionality of the cell motility showed that 66.6% of total N-WASP-I30-His expressing cells lie within the 40° arc facing the chemokine source which was comparable to WASP_R-His expressing cells (73.3%), whereas only 26.6% of N-WASP-His expressing cells migrated within the 40° arc facing the chemokine source (Fig. 3.39). The ability of N-WASP-I30-His to rescue the migration defects of WASP^{KD} Jurkat T-cells was confirmed by transwell migration assay. Similar to chemotactic

defect observed in Dunn chamber, the migration of N-WASP-His expressing cells was significantly impaired in transwell migration assay (39.3% of total WASP^{KD} + N-WASP-His cells, 37.7% of total WASP^{KD} + Vector cells migrated to the lower well containing SDF-1 α compared to 57.04% total of WASP^{KD} + WASP_R-His Jurkat T-cells). 54.0% of N-WASP-I30-His expressing WASP^{KD} Jurkat T-cells migrated towards the chemokine source which was comparable to WASP_R-His expressing cells (Fig. 3.40A).

Figure 3.40 Expression of N-WASP-I30 rescued the chemotactic defect of WASP^{KD} Jurkat T-cell in transwell migration assay. (A) WASP^{KD} Jurkat T-cells expressing (1) Vector, (2) WASP_R-His, (3) N-WASP-His, (4) N-WASP-I30-His were loaded in upper well and allowed to migrate towards SDF-1 α (100 ng/ml) containing media in the lower well for 3.0 hrs. Cells migrated to the bottom well were counted and plotted as percentage of cells migrated. Experiment was repeated thrice. ** $P < 0.01$ compared to vector transfected WASP^{KD} Jurkat T-cells (unpaired student's t-test). (B) Analysis of the expression levels of (1) Vector, (2) WASP_R-His, (3) N-WASP-His, (4) N-WASP-I30-His in transfected cells after one week of selection with neomycin using anti-His antibody for respective proteins expression and GAPDH was used as loading control.

Western blot analysis showed that expression of N-WASP-His was slightly higher compared to WASP_R-His or N-WASP-I30-His (Fig. 3.40B). This could be because of I30 mediated increase in turnover of N-WASP-I30 which is critical for Jurkat T-cell chemotaxis. Taken together, these results suggest that

I30 region of WASP is important for directional migration of Jurkat T-cells towards the chemokine source SDF-1 α . Insertion of I30 region of WASP in N-WASP enables N-WASP to mediate Jurkat T-cells chemotaxis.

3.1.28 N-WASP-I30 behaved similar to WASP in terms of Jurkat T-cells adhesion to fibronectin.

Expression of N-WASP-I30 rescued the chemotactic defects of WASP^{KD} Jurkat T-cells (Section 3.1.27) suggesting that insertion of I30 region of WASP enabled N-WASP to behave similar to WASP. In order to study the adhesion property of cells expressing N-WASP-I30 to fibronectin, a binding assay was performed. Binding assay for WASP^{KD} Jurkat T-cells expressing vector or WASP_R-His or N-WASP-His or N-WASP-I30-His was performed (methods 2.2.4.1).

Figure 3.41 Expression of N-WASP-I30 reduces adhesion of WASP^{KD} Jurkat T-cells to fibronectin. Binding assay was performed to compare the cell adhesion capability of WASP^{KD} Jurkat T-cells expressing (1) Vector, (2) WASP_R-His, (3) N-WASP-His, (4) N-WASP-I30-His to fibronectin coated surface. 2×10^4 cells were allowed to adhere to fibronectin coated plate (0.15 μ g/100 μ l) for 20 min. Bound cells were quantified and plotted as percentage of cells adheres to fibronectin. * $P < 0.05$, ** represent $P < 0.01$ compared to vector expressing cells (unpaired student's t-test).

As shown in figure 3.41, expression of exogenous N-WASP-I30-His (58.5% of total cells bound to fibronectin) significantly reduced the adhesion of WASP^{KD}

Jurkat T-cells to fibronectin, which was comparable to WASP_R-His (63.0% of total cells bound to fibronectin) expressing cells. However, the cells expressing N-WASP-His (75.2%) did not reduce the adhesion of WASP^{KD} Jurkat T-cells, which were significantly higher than WASP_R-His expressing cells. Thus, this result suggests that insertion of I30 region of WASP in N-WASP reduces cell-ECM adhesion and enables N-WASP to behave like WASP.

3.1.29 Expression of N-WASP-I30 rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells.

Result from section 3.1.10 showed that N-WASP can rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells. In order to determine whether insertion of I30 region of WASP in N-WASP affects the ability of N-WASP in IL-2 gene transcription, WT Jurkat T-cells or WASP^{KD} Jurkat T-cells expressing vector alone, WASP_R-His, N-WASP-His or N-WASP-I30-His were induced on anti-CD3/anti-CD28 coated plate as described in methods 2.2.4.4.

Figure 3.42 Expression of N-WASP-I30 rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells. Total RNA was isolated from WT or WASP^{KD} Jurkat T-cells expressing (1) Vector, (2) WASP_R-His, (3) N-WASP-His, (4) N-WASP-I30-His 8.0 hrs after incubation with anti-CD3/anti-CD28 antibodies (2µg/ml each). Quantitative real time PCR was performed and quantified. *** represent $P < 0.001$ compared to WT Jurkat T-cells (unpaired student's t-test).

As shown in figure 3.42, the expression of N-WASP-I30-His in WASP^{KD} Jurkat T-cells rescued the IL-2 gene transcription defect (comparable to WT or WASP^{KD} Jurkat T-cells expressing N-WASP-His or WASP_R-His). This suggests that insertion of I30 region of WASP in N-WASP does not affect N-WASP ability to rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells.

3.1.30 Expression of N-WASP-I30 rescued the membrane projection formation after TCR stimulation

Expression of N-WASP was unable to rescue the TCR/CD28 induced fewer membrane projections formation defect of WASP^{KD} Jurkat T-cells (Fig. 3.15). Whether insertion of I30 region in N-WASP corrected the anti-CD3/anti-CD28 stimulated actin cytoskeleton organization was analysed. WASP^{KD} Jurkat T-cells expressing N-WASP-I30-His were subjected to anti-CD3/anti-CD28 stimulation and stained with Alexa 594 phalloidin (described in methods 2.2.4.5). Insertion of I30 region of WASP in N-WASP does not affect spreading of Jurkat T-cells on anti-CD3/anti-CD28 coated plates (Fig. 3.43B). As shown in figure 3.43 A & C, expression of N-WASP-I30-His rescued the membrane projections formation defect of WASP^{KD} Jurkat T-cells (determined by average number of projections per cell) upon anti-CD3/anti-CD28 stimulation which was comparable to WASP_R-His expressing cells. Thus, suggesting that I30 region is sufficient to mediate interactions require for TCR/CD28 costimulation induced actin cytoskeleton reorganization.

Figure 3.43 Expression of N-WASP-I30 is able to remodel actin cytoskeleton upon stimulation with anti-CD3/anti-CD28 antibodies. (A) WASP^{KD} Jurkat T-cells expressing (1) RFP alone, (2) WASP_R-His, (3) N-WASP-His, (4) N-WASP-I30-His were plated on anti-CD3/anti-CD28 antibodies coated plate, incubated for 30 min, fixed and stained with Alexa 594 phalloidin. (B) The mean surface area of 30 random cells in each three independent experiments was quantified using Metamorph software and plotted. (C) The average number of projections per cell was quantified for 30 cells in each set of experiment and experiment was repeated thrice. ** $P < 0.01$, * $P < 0.05$ compared to WASP_R-His expressing WASP^{KD} Jurkat T-cells (unpaired student's t-test).

3.1.31 Insertion of I30 region in N-WASP recruits N-WASP at TCR activation site

N-WASP is mainly localized at the center (nuclear localization) of the activated Jurkat T-cell (Section 3.1.11). Therefore, the localization pattern of N-WASP-I30 relative to the polymerized actin after TCR stimulation was analysed. N-WASP-I30-RFP was mainly located to the periphery of the fully spread cell and it colocalised with actin ring (Fig. 3.44). Beside peripheral

localization; central localization (nuclear) of N-WASP-RFP was also observed, which was absent in case of N-WASP-I30-RFP localization pattern. This suggests that interactions through the I30 region of WASP might be targeting N-WASP-I30 to the TCR activation site.

Figure 3.44 N-WASP-I30 is able to localize at TCR activation site. WT Jurkat T-cells expressing (1) RFP, (2) WASP-RFP, (3) N-WASP-RFP, (4) N-WASP-I30-RFP were plated on stimulatory coverslip (anti-CD3 antibody coated) and incubated for 5.0 min. Cells were fixed and stained with Alexa 488 phalloidin.

In this study, the I30 region of WASP has been identified as one of the molecular and functional differences between WASP and N-WASP in terms of Jurkat T-cell functions as summarised in table 3.3. Deletion of I30 region of WASP impaired WASP functions in Jurkat T-cells chemotaxis, IL-2 production and membrane projections formation after stimulation with anti-CD/anti-CD28 antibodies. Insertion of WASP-I30 region in N-WASP corrected all the functional defects observed in WASP^{KD} Jurkat T-cells (Table 3.3).

Table 3.3 List of constructs used and functional assays performed in this study

Constructs Assays	WASP	WASP^{ΔI30}	WASP^{G187C}	N-WASP	N-WASP-I30
Chemotaxis towards SDF-1α	Rescued	<u>Impaired</u>	Rescued	<u>Impaired</u>	Rescued
Migration velocity	Rescued	Rescued	Rescued	Rescued	Rescued
Adhesion to Fibronectin	Rescued	<u>Reduced</u>	<u>Reduced</u>	<u>More</u>	Rescued
IL-2 gene activation	Rescued	<u>Impaired</u>	<u>Impaired</u>	Rescued	Rescued
Membrane projections	Rescued	<u>Impaired</u>	Rescued	<u>Impaired</u>	Rescued
Cell spreading on anti-CD3/anti-CD28 antibody	Normal	Normal	Normal	Normal	Normal
Colocalization with actin ring at TCR	Colocalized with actin at TCR	Colocalized with actin at TCR	Colocalized with actin at TCR	Mainly located at center of cell	Colocalized with actin at TCR

A summary of WASP, N-WASP and their mutants or chimera used in this study and their functional assays performed. All the assays except colocalization study were performed in WASP^{KD} Jurkat T-cells. The results obtained in respective assays are mentioned in this table. The WASP mutants or N-WASP or N-WASP-I30 in respective assays are compared with WT WASP.

The I30 region of WASP is important for interaction with SH3 domain containing proteins or may be with unknown proteins which are not identified in this study and this could be the possible reason for why N-WASP is unable to rescue the functional defects of WASP^{KD} T-cells. Therefore, from this study it can be concluded that I30 region of WASP is crucial for WASP mediated Jurkat T-cell functions.

3.2 Molecular characterization of disease causing mutations in WASP

3.2.1 Introduction

A large number of point mutations in WASP have been identified from patients with different degree of severity such as WAS, XLT and XLN [103]. However the links between mutations and disease have not been characterized. Out of 52 WASP missense mutations reported [96], 40 missense mutations are found in the WH1 domain of WASP, whereas the remaining 12 missense mutations are present outside the WH1 domain. These 12 missense WASP mutants were able to rescue the growth defect of *las17Δ* yeast strain suggesting that these mutations do not affect the activity of mutants in basic actin dynamics [181]. Amongst the 12 mutations outside the WH1 domain, 5 mutations causing classic WAS are located in the PRR (S339Y/ P373S) (WASPbase) and the VCA domain (P459S, K476E and D485N) of WASP [96]. Even after a number of studies it is still not clear why the mutation in proline rich region of WASP and WH1 domain of WASP causes the disease. Therefore, characterizing the molecular defect of mutations (WASP^{P373S}, WASP^{S339Y}) at proline rich region of WASP will lead to a better understanding of the regulation of WASP by SH3 domain containing proteins.

3.2.2 WASP^{PRR} missense mutant (S339Y or P373S) can interact with SH3 domain containing proteins.

Beside Cdc42 mediated WASP activation, SH3 domain containing proteins (tyrosine kinases and adaptor proteins) are known to interact with PRR of WASP and regulates WASP activation [69,215]. Two point mutations (339/373, Ser-Tyr/Pro-Ser) at exon 10 of WASP gene (PRR domain) has been

reported to occur together in patients and leads to WAS (WASPbase). Therefore, it is possible that WASP^{PRR} missense mutation may affect the interaction of WASP with SH3 domain containing proteins. However, it is not clear whether both the mutations are required to cause the disease.

In order to identify the effect of WASP^{PRR} missense mutation individually (WASP^{S339Y} or WASP^{P373S}) or both together (WASP^{S339Y/P373S}) on interaction with SH3 domain containing proteins, a yeast two hybrid assay was performed using Gal4BD expressing WASP^{S339Y} or WASP^{P373S} or WASP^{S339Y/P373S} (WASP^{SP/YS}) as bait. *S. cerevisiae* strain PJ69-4A was co-transformed with plasmids expressing Gal4 DNA binding domain fusion proteins and activation domain fusion proteins as indicated in table 3.4. Transformed cells were grown on Trp⁻Leu⁻ (control plates) or Trp⁻Leu⁻His⁻ plates for checking interaction between BD fusion protein and AD fusion protein. As shown in table 3.4, WASP^{PRR} missense mutants did not affect the interaction of WASP with SH3 domain containing proteins. This result suggests that Serine 339 and Proline 373 may not be crucial for interaction of WASP with SH3 domain containing proteins.

Table 3.4 WASP^{PRR} mutants can interact with SH3 domain containing proteins

	BD-Vector	BD-WASP	BD-WASP ^{S339Y}	BD-WASP ^{P373S}	BD-WASP ^{SP/YS}
AD-Fusion protein	-T-L-H	-T-L-H	-T-L-H	-T-L-H	-T-L-H
AD-Vector	-	-	-	-	-
AD-Fyn	-	+	+	+	+
AD-Fgr	-	+	+	+	+
AD-Lyn	-	+	+	+	+
AD-Hck	-	+	+	+	+
AD-Nck	-	+	+	+	+
AD-Toca1	-	+	+	+	+
AD-Grb2	-	+	+	+	+
AD-Src	-	+	+	+	+
AD-PIK	-	+	+	+	+
AD-Tec	-	+	+	+	+
AD-VASP	-	+	+	+	+
AD-Lck	-	+	+	+	+
AD-PSTPIP	-	+	+	+	+
AD-Btk	-	+	+	+	+
AD-Abl	-	+	+	+	+
AD-IRSp53	-	+	+	+	+
AD-Cortactin	-	+	+	+	+

The yeast strain PJ69-4A was co-transformed with plasmids expressing the BD fusion proteins and the AD fusion proteins in combinations as indicated in the table. The transformants were streaked on histidine lacking SD minimal media plates. The growth of the strains on the plates (+) were observed after 5 days of incubation at 30°C. (-) sign indicates lack of interaction. BD-Binding domain, AD-Activation domain.

Results from section 3.1.13 showed that I30 region of WASP interacts with 8 out of 17 tyrosine kinases or adaptor proteins. Therefore, it is possible that the interaction of WASP^{PRR} mutants observed with SH3 domain containing proteins may be because of the presence of I30 region of WASP which is

masking the effect of WASP^{PRR} mutants. To rule out this possibility, a smaller peptide of WASP or WASP^{SP/YS} mutant (a.a. 306-407) which is devoid of I30 region was cloned in bait plasmid (Fig. 3.45). These two small peptides of WASP (Fig. 3.45) were used to study for interaction with eight tyrosine kinases and adaptor proteins that interacted with I30 region of WASP.

Figure 3.45 Schematic representation of peptides of WASP used for yeast two hybrid assay. Gal4BD-WASP₃₀₆₋₄₀₇ or Gal4BD-WASP₃₀₆₋₄₀₇^{SP/YS} fusion peptides were used to study the interaction with SH3 domain containing proteins interacted with I30 region of WASP.

Table 3.5 WASP₃₀₆₋₄₀₇^{SP/YS} can interacts with SH3 domain containing proteins

	BD-Vector	BD-WASP ₃₀₆₋₄₀₇	BD-WASP ₃₀₆₋₄₀₇ ^{SP/YS}
AD-Fusion protein	-T-L-H	-T-L-H	-T-L-H
AD-Vector	-	-	-
AD-Fyn	-	+	+
AD-Fgr	-	+	+
AD-Lyn	-	+	+
AD-Hck	-	+	+
AD-Nck1	-	+	+
AD-Toca1	-	+	+
AD-Grb2	-	+	+
AD-Src	-	+	+

The yeast strain PJ69-4A was co-transformed with plasmids expressing the BD fusion proteins and the AD fusion proteins in combinations as indicated in the table. The transformants were streaked on histidine lacking SD minimal media plates. The growth of the strains on the plates (+) were observed after 5 days of incubation at at 30°C. (-) sign indicates lack of interaction. BD-Binding domain, AD-Activation domain.

As shown in table 3.5, WASP₃₀₆₋₄₀₇^{SP/YS} peptide interacted with all the eight tyrosine kinases and adaptor proteins which have been shown to interact with I30 region of WASP (Table 3.1). Taken together, these results suggest that WASP^{PRR} mutations causing WAS do not affect interaction of WASP with SH3 domain containing tyrosine kinases and adaptor proteins.

3.2.3 WASP^{PRR} mutants are able to form functional complex with WIP

Results from section 3.1.21 suggest that WASP and WIP have to form a functional complex for actin polymerization in order to rescue the growth defects of *las17Δ* yeast cells. In order to analyse the effect of WASP^{PRR} mutants (WASP^{S339Y}, WASP^{P373S}, WASP^{SP/YS}) on their ability to form functional WIP-WASP complex, WASP or WASP^{PRR} mutants (Trp⁻) were transformed into *las17Δ* cells (IDY166) together with either WIP (Leu⁺) or empty Leu plasmid. Transformed cells were selected on Trp⁻ Leu⁺ plates. Colonies from the selection plate were streaked onto YPUAD agar plates and grown at either 24°C or 37°C for 3 days.

The WASP^{PRR} mutants did not affect the ability of WASP to rescue the growth defect of *las17Δ* cells (Fig. 3.46A). Western blot analysis showed equal expression of WASP or WASP^{PRR} mutants in *las17Δ* cells (Fig. 3.46B). The results indicate that the WASP^{PRR} mutants are able to form functional WASP-WIP complex. Most importantly this result shows that the activity of both the N-terminus (interaction with WIP) and C-terminus (actin polymerization) of WASP^{PRR} mutants are still maintained.

Figure 3.46 Co-expression of WASP or WASP^{PRR} mutants with WIP suppressed the growth defects of *las17Δ* cells. (A) Viability at 24 and 37°C of *las17Δ* yeast strain IDY166 transformed with full length WT WASP or WASP^{S339Y} or WASP^{P373S} or WASP^{SP/YS} expressing plasmid with WIP or empty vector (Vect). Each strain was streaked for single colonies on YPUAD agar and incubated at 24 as well as at 37°C. (B) Western blot analysis of WASP or WASP^{PRR} mutants expressed with WIP or empty vector (Vect) in *las17Δ* yeast strain at 24°C using anti-WASP antibody. Anti-Hexokinase antibody was used as loading control.

3.2.4 WASP^{S339Y} and WASP^{SP/YS} missense mutants adopt partial open conformation in the presence of WIP

Although WASP^{PRR} missense mutants did not affect interaction of WASP with SH3 domain containing proteins (Table 3.4); they might affect changes in the conformation of WASP. To analyse whether WASP^{PRR} missense mutations leads to changes in the conformation, yeast strain PJ69-4A was transformed with WASP sensor (WT or WASP^{S339Y} or WASP^{P373S} or WASP^{SP/YS}) (Trp) together with either NLS-WIP or empty vector (Ura). The transformants were selected on plates deficient in Tryptophan and Uracil and cells were subsequently analyzed using fluorescence microscopy (Fig. 3.47). The

fluorescence signals from 100 cells were quantified and plotted (Fig. 3.48A). As shown in figure 3.47 & 3.48A the fluorescence intensity of WASP^{S339Y}, WASP^{P373S} and WASP^{SP/YS} sensors were similar to WT WASP sensor in the absence of WIP. However the YFP fluorescence intensity of all the WASP sensors was significantly enhanced when expressed together with WIP suggesting the WIP stabilizes the closed conformation of all the WASP sensors.

Figure 3.47 WASP^{S339Y} and WASP^{SP/YS} missense mutants adopt partial open conformation (A). *S. cerevisiae* cells were transformed with empty vector (Ura) or NLS-WIP (Ura) together with (1) WASP sensor (Trp) or (2) WASP^{S339Y} sensor (Trp) or (3) WASP^{P373S} sensor (Trp) or WASP^{SP/YS} sensor (Trp) and selected on SD (-Trp-Ura) plates. The transformants were grown at 24°C in selective media and YFP signals in these cells were subsequently analyzed using fluorescence microscopy. Bar = 5μm.

The YFP fluorescence intensity of WASP^{S339Y} sensor + WIP or WASP^{SP/YS} sensor + WIP but not WASP^{P373S} sensor + WIP was significantly reduced

compared to WASP sensor + WIP. However, the fluorescence intensity of WASP^{SP/YS} sensor or WASP^{S339Y} sensor was significantly increased in the presence of WIP compared to absence of WIP (Fig. 3.48A) indicating that WASP^{SP/YS} or WASP^{S339Y} sensor exists in partially open conformation. The reduced fluorescence intensity of WASP^{SP/YS} sensor could be the effect of WASP^{S339Y} mutation alone as fluorescence intensity of WASP^{P373S} sensor was similar to WASP sensor in the presence of WIP.

Figure 3.48 WASP^{S339Y} and WASP^{SP/YS} missense mutants adopt partial open conformation (B). (A) Fluorescence signal quantification from 100 *S. cerevisiae* cells expressing empty vector (Ura) or WIP (Ura) together with (1) WASP sensor (Trp) or (2) WASP^{S339Y} sensor (Trp) or (3) WASP^{P373S} sensor (Trp) or WASP^{SP/YS} sensor (Trp) using metamorph software. *** $P < 0.001$ compared to WASP sensor + NLS-WIP (Unpaired student's t-test). (B) Analysis of expression of the WASP sensors in *S. cerevisiae* cells using anti-GFP (α -GFP) antibody (anti-GFP antibody also recognizes YFP; therefore it is used to control the amount of sensors). Anti-Hexokinase (α -Hex) was used as a loading control.

Western blot analysis showed the similar expression of all WASP sensors either in the presence or absence of WIP indicating that the increase in fluorescence intensities of all the WASP sensors in the presence of WIP was not due to increased expression of sensor molecules (Fig. 3.48B). These results suggest that the WASP^{PRR} point mutation S339Y, alter the WASP's closed conformation even in the presence of WIP.

3.2.5 Toca1 or Nck1 does not relieve the auto-inhibition of WASP^{P373S} sensor

WASP and N-WASP are multi-domain proteins which have been shown to be regulated by number of other interacting proteins. Yeast two hybrid data (Table 3.4) suggest that WASP^{P373S} interacts with both Toca1 and Nck1. Furthermore, the interaction of either Toca1 or Nck1 with WASP₃₀₆₋₄₀₇^{SP/YS} (a smaller peptide of WASP devoid of I30 region and bear both WASP^{PRR} mutations together) was not inhibited (Table 3.5). Although WASP^{P373S} mutant does not affect interaction with both Toca1 and Nck1, it might affect the formation of functional WASP-Toca1 or WASP-Nck1 complex. Result from previous section revealed that WASP^{P373S} similar to WASP in the presence of WIP exists in closed conformation.

Figure 3.49 Toca1 or Nck1 does not reduce the YFP fluorescence intensity of WASP^{P373S} sensor (A). (A) *S. cerevisiae* cells were transformed with WASP or WASP^{P373S} sensor (Trp) + NLS-WIP (Ura) together with (1) empty Leu plasmid, or Leu plasmids expressing (2) NLS-Toca1 or (3) NLS-Nck1 and selected on SD (-Trp-Leu-Ura) plates. The transformants were grown at 24°C in selective media and YFP signals in these cells were subsequently analyzed using fluorescence microscopy. Bar= 5μm. (B) Fluorescence signal quantification from 100 *S. cerevisiae* cells expressing WASP or WASP^{P373S} sensor (Trp) + NLS-WIP (Ura) together with (1) empty Leu plasmid, (2) NLS-Toca1 or (3) NLS-Nck1 using metamorph software. ****P*<0.001 (Unpaired student's t-test).

The effect of Toca1 or Nck1 on conformation changes of WASP^{P373S} sensor was analysed. The yeast cells expressing WASP^{P373S} sensor (Trp) together with WIP (Ura) were transformed with plasmid expressing Toca1 or Nck1 or empty plasmid (Leu) and cells were selected on plates deficient in Tryptophan, Uracil

and Leucine. Analysis of fluorescence intensity (Fig. 3.49 A & B) revealed that both Toca1 and Nck1 did not cause a decrease in the fluorescence intensity of WASP^{P373S} sensor. However, both Toca1 and Nck1 reduced the fluorescence intensity of WT WASP sensor (Fig. 3.49B). Western blot analysis showed the similar expression of WASP^{P373S} sensor in all respective co-transformants (Fig. 3.50). Taken together, these results indicate that both Toca-1 and Nck1 can disrupt the auto-inhibitory loop of WASP even in the presence of WIP which is consistent with previous report [125], but fail to relieve the auto-inhibition of WASP^{P373S} mutant.

Figure 3.50 Toca1 or Nck1 does not reduce the YFP fluorescence intensity of WASP^{P373S} sensor (B). Analysis of expression of the WASP or WASP^{P373S} sensor (Trp) + NLS-WIP (Ura) in *S. cerevisiae* cells transformed together with (1) empty Leu plasmid, or Leu plasmids expressing (2) NLS-Toca1, (3) NLS-Nck1 using anti-GFP (α -GFP) or anti-WIP antibody. Anti-Hexokinase antibody (α -Hex) was used as loading control.

3.2.6 Toca1 or Nck1 further reduced the YFP fluorescence of WASP^{S339Y} sensor in the presence of WIP

Results from section 3.2.4 showed that WASP^{SP/YS} and WASP^{S339Y} exists in a partial open conformation even in the presence of WIP. In order to analyse the effect of Toca1 and Nck1 on conformation changes of WASP^{S339Y} or WASP^{SP/YS}, yeast cells expressing WASP sensor or WASP^{S339Y} sensor or

WASP^{SP/YS} sensor (Trp) together with WIP (Ura) were transformed with plasmids expressing Toca1 or Nck1 or empty plasmid (Leu). The transformants were selected on SD plates lacking Tryptophan, Uracil and Leucine. The colonies grown on selection plates were analyzed using fluorescence microscopy (Fig. 3.51).

Figure 3.51 Both Toca1 and Nck1 caused further reduction in the fluorescence intensity of WASP^{S339Y} sensor (A). *S. cerevisiae* cells were transformed with WASP or WASP^{S339Y} or WASP^{SP/YS} sensor (Trp) + NLS-WIP (Ura) together with (1) empty Leu plasmid, or Leu plasmids expressing (2) NLS-Toca1 or (3) NLS-Nck1 and selected on SD (-Trp-Leu-Ura) plates. The cells were grown at 24°C in selective media and YFP signals in these cells were observed using fluorescence microscopy. Bar= 5μm.

Analysis of fluorescence intensity (Fig. 3.52A) revealed that both Toca1 and Nck1 further reduced the fluorescence from WASP^{S339Y} sensor and had no effect on WASP^{SP/YS} sensor. The western blot analysis of WASP^{S339Y} or

WASP^{SP/YS} sensors showed their similar expression in all the respective transformants (Fig. 3.52B).

Figure 3.52 Both Toca1 and Nck1 caused further reduction in the fluorescence intensity of WASP^{S339Y} sensor (B). (A) Fluorescence signal quantification from 100 *S. cerevisiae* cells expressing WASP sensor or WASP^{S339Y} sensor or WASP^{SP/YS} sensor (Trp) + NLS-WIP (Ura) together with (1) empty Leu plasmid, (2) NLS-Toca1 or (3) NLS-Nck1 using metamorph software. ** $P < 0.01$ compared to WASP^{S339Y} sensor + NLS-WIP expressing cells, *** $P < 0.001$ (Unpaired student's t-test). (B) Analysis of expression of the WASP sensor or WASP^{S339Y} sensor or WASP^{SP/YS} sensor (Trp) + NLS-WIP (Ura) in *S. cerevisiae* cells using anti-GFP (α -GFP) (anti-GFP antibody also recognizes YFP, therefore it is used to control the amount of sensors) and anti-WIP antibody. Anti-Hexokinase (α -Hex) antibody was used as loading control.

These results indicate that Toca1 or Nck1 induces conformational changes of WASP^{S339Y} sensor suggesting formation of a functional WASP^{S339Y}-Toca1 or

WASP^{S339Y}-Nck1 complex. Toca1 or Nck1 had no effect on WASP^{SP/YS} sensor indicating that possible effect of mutation (P373S) which may be inhibiting the further changes in conformation of WASP^{SP/YS} sensor by Toca1 or Nck1.

3.2.7 WASP^{PRR} mutations decrease the Hck-mediated WASP tyrosine phosphorylation.

Although the WASP^{PRR} mutations do not abolish the interaction of WASP with Hck (Table 3.4 & 3.5), it alters the closed conformation of WASP (Section 3.2.4). Hck has been shown to interact with WASP at both proline rich region and I30 region of WASP (Section 3.1.19). Deletion of I30 region partially open up the closed conformation of WASP, resulted in increase in Hck mediated WASP^{ΔI30} phosphorylation suggesting that changes in conformation affect WASP phosphorylation by Hck. Therefore, the effect of WASP^{PRR} mutants on Hck mediated tyrosine phosphorylation of WASP was analysed.

Figure 3.53 Hck does not phosphorylate WASP^{PRR} mutants efficiently. (A) HEK293T cells were transfected with WASP-His or WASP^{S339Y}-His or WASP^{P373S}-His or WASP^{SP/YS}-His together with (1) Vector, (2) Hck-GFP. WASP or WASP^{PRR} mutants were isolated by His-tag pull-down assay. Western blot analysis of levels of Hck-GFP isolated using anti-GFP (α -GFP) antibody. (B) Tyrosine-phosphorylation levels of WASP or WASP^{PRR} mutants were detected using 4G10 antibody. (C) Western blot analysis of levels of WASP or WASP^{PRR} mutants isolated using anti-His (α -His) antibody. (D) Quantification of western blots using densitometry. Image J software was used to perform densitometry. Band densities were measured as area under the curve. * represent $P < 0.05$ compared to WASP (Unpaired student's t-test).

HEK293T cells were transfected with WASP or WASP^{PRR} mutants tagged with Histidine tag (WASPS^{339Y}-His, WASPS^{P373S}-His, WASPS^{SP/YS}-His) together with vector or Hck, tagged with GFP. The cells after 36 hrs of transfection were lysed and His-tag pull-down assay was performed. The tyrosine-phosphorylation levels of WASP or WASP^{PRR} mutants were detected using phosphotyrosine specific antibody, 4G10. Western blot analysis revealed that WASP^{PRR} mutants do not abolish interaction of WASP with Hck (Fig. 3.53A) which is consistent with yeast two hybrid data (Section. 3.2.2). Quantification of western blots using densitometry analysis revealed that WASP^{PRR} mutants reduce the Hck mediated tyrosine phosphorylation of WASP (Fig. 3.53 B & D). No WASP tyrosine phosphorylation was detected when the cells were transfected with WASP or WASP^{PRR} mutants without Hck indicating that the phosphorylation levels detected in the western blots were due to exogenous Hck expression. Thus, these results indicate that WASP^{PRR} mutations decrease the Hck mediated WASP tyrosine phosphorylation.

3.2.8 WASP^{PRR} mutants do not rescue migration defect of WASP^{KD} Jurkat T-cells

WASP^{PRR} mutations do not alter the function of WASP-WIP complex (Fig. 3.46A) (actin polymerization) in order to suppress the growth defects of *las17Δ* cells. However, they alter the changes in conformation resulted in decrease in Hck mediated Tyr²⁹¹ phosphorylation (Fig. 3.53). Therefore, it is possible that these mutations may affect WASP mediated T-cell functions. WASP^{KD} Jurkat T-cells stably expressing (microporated and selected with neomycin) WASP^{PRR}

mutants tagged with RFP (WASP^{S339Y}-RFP or WASP^{P373S}-RFP or WASP^{SP/YS}-RFP) were used to study the T-cell functions.

In order to analyze the effect of WASP^{PRR} mutations in their ability to rescue the chemotaxis of WASP^{KD} Jurkat T-cells, Dunn chamber migration assay was performed. As shown in figure 3.54, exogenous WASP expression (WASP_R-RFP) rescued the chemotaxis of WASP^{KD} Jurkat T-cells; however RFP (control) expressing cells were unable to migrate towards the increasing concentration of SDF-1 α . Similar to RFP expressing cells, all the WASP^{PRR} mutants expressing cells did not rescue the chemotaxis of WASP^{KD} Jurkat T-cells (Fig. 3.54).

Figure 3.54 Expression of WASP^{PRR} mutants do not rescue the chemotactic defect of WASP^{KD} Jurkat T-cell. Vector plots of migration paths of 20 randomly chosen cells from WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{S339Y}-RFP, (4) WASP_R^{P373S}-RFP, (5) WASP_R^{SP/YS}-RFP exposed to a gradient of SDF-1 α in Dunn chamber. Time-lapse images were captured over 3.0 hrs at 2.0 min intervals. Intersection of the X and Y axes are the starting point of each cell. The source of SDF-1 α was at the top.

Circular histograms illustrating the overall directionality of the cell motility showed that only 20% of total WASP_R^{S339Y}-RFP expressing cells moved to the position within 40° arc facing the chemokine source compared to 80% of total WASP_R-RFP expressing cells. About 30% of total WASP_R^{P373S}-RFP expressing cells and only 20% of total WASP_R^{SP/YS}-RFP expressing cells migrated within 40° arc facing the chemokine source, indicating that WASP_R^{PRR} mutants expressing cells were migrated in random directions (Fig. 3.55)

Figure 3.55 Expression of WASP^{PRR} mutants do not rescue the directionality of WASP^{KD} Jurkat T-cell. WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{S339Y}-RFP, (4) WASP_R^{P373S}-RFP, (5) WASP_R^{SP/YS}-RFP exposed to a gradient of SDF-1 α in Dunn chamber. Percentage of cells where the final positions of cells lie in each of the sectors (20°) was determined by circular histogram. The source of SDF-1 α was at the top.

The chemotactic defect observed in WASP^{KD} Jurkat T-cells expressing WASP_R^{PRR} mutants was not due to their poor expression as western blot analysis showed their expression were comparable to WASP_R-RFP expression (Fig. 3.56A). The quantitative analysis revealed that the migration speed of WASP^{KD} Jurkat T-cells expressing WASP_R^{S339Y}-RFP (1.73μm/min), WASP_R^{P373S}-RFP (1.96μm/min) and WASP_R^{SP/YS}-RFP (1.75μm/min) were similar to WASP_R-RFP (2.3μm/min) expressing cells and was lower for RFP alone (control) expressing cells (0.95μm/min) (Fig. 3.56B) suggesting that WASP^{PRR} mutants are able to rescue the migration speed of WASP^{KD} Jurkat T-cells.

Figure 3.56 Expression of WASP^{PRR} mutants rescued the migration velocity of WASP^{KD} Jurkat T-cell. (A) Analysis of the expression levels of (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{S339Y}-RFP, (4) WASP_R^{P373S}-RFP, (5) WASP_R^{SP/YS}-RFP in transfected cells after one week of selection with neomycin using appropriate antibodies. (B) Migration velocity of each cell shown as a mean of velocities of 60 randomly chosen cells (20 cells each set from 3 sets of experiments) * *P* < 0.05, ** *P* < 0.01 compared to RFP expressing WASP^{KD} Jurkat T-cells (unpaired student's t-test).

In order to confirm the chemotactic defect of WASP^{KD} Jurkat T-cells expressing WASP_R^{PRR} mutants toward SDF-1α, a transwell migration assay was performed. Similar to chemotactic defects observed in a Dunn chamber, the migration of WASP^{KD} Jurkat T-cells expressing WASP_R^{PRR} mutants were

significantly impaired in transwell migration assay (40.0% of total WASP^{KD} + WASP_R^{S339Y}-RFP cells, 40.3% of total WASP^{KD} + WASP_R^{P373S}-RFP cells, 39.10% of total WASP^{KD} + WASP_R^{SP/YS}-RFP cells migrated towards SDF-1 α containing media in lower well compared to 51.24% of WASP^{KD} + WASP_R-RFP Jurkat T-cells migrated) (Fig. 3.57). Taken together, all these data suggest that the expressions of WASP^{PRR} mutants do not rescue the chemotaxis of WASP^{KD} Jurkat T-cells towards chemokine SDF-1 α . These results suggest the importance of PRR domain of WASP in chemotactic migration of Jurkat T-cells.

Figure 3.57 Expression of WASP^{PRR} mutants do not rescue the chemotaxis of WASP^{KD} Jurkat T-cells in transwell migration assay. WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{S339Y}-RFP, (4) WASP_R^{P373S}-RFP, (5) WASP_R^{SP/YS}-RFP were loaded in upper well and allowed to migrate towards SDF-1 α (100 ng/ml) containing media in the lower well for 3.0 hrs. Cells migrated to the bottom well were counted and plotted as percentage of cells migrated. Experiment was repeated thrice. * $P < 0.05$, ** $P < 0.01$ compared to WASP_R-RFP expressing WASP^{KD} Jurkat T-cells (unpaired student's t-test).

3.2.9 Expression of WASP^{P373S} or WASP^{SP/YS} in WASP^{KD} Jurkat T-cells reduces cell to fibronectin adhesion.

Previous results suggest that expression of WASP^{PRR} mutants did not rescue the chemotaxis defect of WASP^{KD} Jurkat T-cells (Section 3.2.8). In order to assess whether these mutations affect cell-ECM adhesion, binding assay was

performed as described in methods 2.2.4.1. As shown in figure 3.58, all the WASP^{PRR} mutants expressing WASP^{KD} Jurkat T-cells had significantly reduced adhesion to fibronectin compared to RFP alone (control) expressing cells (82.6% of total). When compared with WASP_R-RFP (62.6% of total) expressing cells, the adhesion of WASP_R^{S339Y}-RFP expressing cells (60.6% of total) was similar. However, the adhesion of WASP_R^{P373S}-RFP (46.11% of total) or WASP_R^{SP/YS}-RFP (43.63% of total) expressing cells to fibronectin was substantially decreased compared to WASP_R-RFP expressing cells (62.6% of total). A significant reduction in adhesion of WASP^{KD} Jurkat T-cells expressing WASP_R^{P373S}-RFP or WASP_R^{SP/YS}-RFP to fibronectin was observed compared to WASP_R-RFP expressing cells, suggests that proline at 373 position (Pro³⁷³) of WASP may be crucial for regulation of WASP function in Jurkat T-cell-ECM adhesion.

Figure 3.58 Expression of WASP^{PRR} mutation (P373S) reduces adhesion of WASP^{KD} Jurkat T-cells to fibronectin. Binding assay was performed to compare the cell adhesion capability of WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{S339Y}-RFP, (4) WASP_R^{P373S}-RFP, (5) WASP_R^{SP/YS}-RFP to fibronectin coated surface. 2×10^4 cells were allowed to adhere to fibronectin coated plate (0.15μg/100μl) for 20 min. Bound cells were quantified and plotted as percentage of cells adheres to fibronectin ** represent $P < 0.01$ (unpaired student's t-test).

3.2.10 Expression of WASP^{P373S} and WASP^{SP/YS} mutants do not rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells

WASP-deficient T-cells exhibit defect in IL-2 production [54]. In order to analyze whether WASP^{PRR} mutants affect WASP mediated IL-2 gene transcription, WT Jurkat T-cells or WASP^{KD} Jurkat T-cells expressing WASP_R-RFP or WASP^{PRR} mutants were stimulated on anti-CD3/anti-CD28 coated plates as described in methods 2.2.4.4.

As shown in figure 3.59, the expression of WASP_R-RFP or WASP^{PRR} mutant (WASP_R^{S339Y}-RFP) in WASP^{KD} Jurkat T-cells rescued the IL-2 gene transcription defect (behaved similar to WT Jurkat T-cells) while the expression of WASP_R^{P373S}-RFP or WASP_R^{SP/YS}-RFP were unable to rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells (behaved similar to WASP^{KD} Jurkat T-cells). The real time PCR data shows that the point mutation (WASP^{P373S}) either alone or together with WASP^{S339Y} (WASP^{SP/YS}) did not rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells, suggesting that mutation WASP^{P373S} alone is sufficient for inhibiting WASP function in IL-2 gene transcription. This result also suggests that the WASP^{PRR} mutation (S339Y) does not abolish WASP function in TCR dependent IL-2 gene transcription in Jurkat T-cells.

Figure 3.59 WASP^{PRR} mutation (P373S) impaired WASP mediated IL-2 gene transcription.

Total RNA was isolated from WT or WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{S339Y}-RFP, (4) WASP_R^{P373S}-RFP, (5) WASP_R^{SP/YS}-RFP 8.0 hrs after incubation with anti-CD3/anti-CD28 antibodies (2μg/ml each). Quantitative real time PCR was performed and quantified. **P* < 0.05, ***P* < 0.01, *** *P* < 0.001 compared to WT Jurkat T-cells (unpaired student's t-test).

3.2.11 Expression of WASP^{PRR} mutants do not rescue the membrane projection defect of WASP^{KD} Jurkat T-cells.

WASP^{SP/YS} mutant expressing WASP^{KD} Jurkat T-cells was defective in TCR stimulated IL-2 gene transcription (Section. 3.2.10) Therefore, the effect of WASP^{PRR} mutants on TCR induced actin cytoskeleton changes was further analysed. WASP^{PRR} mutants expressing cells WASP^{KD} Jurkat T-cells were stimulated on anti-CD3/anti-CD28 coated plates as described in methods 2.2.4.5. All the WASP^{PRR} mutants expressing cells spread well on anti-CD3/anti-CD28 coated plates (Fig. 3.60B) and formed circular actin ring, but unable to rescue the fewer membrane projections defect of WASP^{KD} Jurkat T-cells (Fig. 3.60 A & C) suggesting that TCR/CD28 costimulation induced actin cytoskeleton reorganization has been affected.

(A)

(B)

(C)

Figure 3.60 WASP^{PRR} mutants were defective towards anti-CD3/anti-CD28 stimulated actin cytoskeleton organization. (A) WASP^{KD} Jurkat T-cells expressing (1) RFP alone, (2) WASP_R-RFP, (3) WASP_R^{S339Y}-RFP, (4) WASP_R^{P373S}-RFP, (5) WASP_R^{SP/YS}-RFP were plated on anti-CD3/anti-CD28 antibodies coated plate, incubated for 30 min, fixed and stained with Alexa 594 phalloidin. (B) The mean surface area of 30 randomly chosen cells in each three independent experiments was quantified using Metamorph software and plotted. (C) The average number of projections per cell was quantified for 30 cells in each set of experiment and experiment was repeated thrice. ** $P < 0.01$, * $P < 0.05$ compared to WASP_R-RFP expressing WASP^{KD} Jurkat T-cells (unpaired student's t-test).

3.2.12 WASP^{PRR} mutants had diffuse pattern of localization at TCR activation site

The PRR of WASP has been shown to be important for WASP recruitment to the TCR contact sites [44]. Thus, the localization pattern of WASP^{PRR} mutants relative to polymerized actin after TCR induction was analyzed. WT Jurkat T-cells expressing WASP^{PRR} mutants were stimulated on anti-CD3 coated plate as described in section 3.1.11.

Figure 3.61 WASP^{PRR} mutants have diffuse pattern of localization at TCR activation site. WT Jurkat T-cells expressing (1) RFP, (2) WASP-RFP, (3) WASP^{S339Y}-RFP, (4) WASP^{P373S}-RFP, (5) WASP^{SP/YS}-RFP were plated on stimulatory coverslip (anti-CD3 antibody coated) and incubated for 5.0 min. Cells were fixed and stained with Alexa 488 phalloidin.

As shown in figure 3.61, there was no colocalization of WAS^{S339Y}-RFP observed with polymerized actin ring at the periphery of the fully spread cell. Infact, small patches of WAS^{S339Y}-RFP were observed inside the cell. Studies of cells expressing WASP^{P373S}-RFP or WAS^{SP/YS}-RFP showed a minor colocalization of these mutants with actin ring while most of these molecules were located inside the cell in a diffused pattern. Thus suggesting that missense mutation in the PRR of WASP causes WAS affected the localization of WASP at the TCR activation site. This study further implies the importance of WASP^{PRR} domain in localization of WASP at TCR activation site.

3.2.13 Yeast two-Hybrid screen to identify WASP interacting novel interactors

Beside two WASP^{PRR} point mutations reported in WAS patients, a large numbers of missense mutations have also been reported to occur in the WH1 domain of WASP which led to WAS or XLT. Some of these WASP^{WH1} point mutations such as R86H, Y107C and A134T have been shown to affect WASP-WIP interaction [205]. Thus, it is still not clear how the majority of remaining point mutations in the WH1 domain of WASP causes the disease. It is possible that beside interaction with WIP, the WH1 domain of WASP also interacts with other unknown proteins, which may participate in many signalling pathways and these missense mutations may be responsible for loss of interaction with unidentified proteins.

In results section 3.1, the I30 region of WASP has been identified, which interacts with some of the SH3 domain containing proteins and account for one of the molecular/functional difference between WASP and N-WASP. Besides GBD, the I30 region of WASP also regulates the changes in the WASP conformation (Fig. 3.23). Missense mutations in GBD of WASP (L270P and I294T) have been identified in the WASP of XLN patients, which disrupted the autoinhibitory WASP conformation, resulted in increased WASP activity [5]. Till now, Rho family of GTPase such as Cdc42 has been known to interact with GBD of WASP, which regulates WASP activation. However, there are some evidences which showed that WASP can be activated without the involvement of Cdc42. Therefore, it is possible that the changes in WASP conformation through its I30 region or GBD may be regulated through I30 or GBD mediated interactions with unknown proteins, which either functions co-

operatively or may bypass the involvement known regulator of WASP. In order to identify novel WASP interacting partners; monocyte cDNA library screening was performed using a smaller peptide of WASP (WASP₁₋₃₂₀; comprising WH1 domain, I30 region, BR and GBD) as bait (Fig. 3.62A).

Figure 3.62 Monocyte cDNA library screening. (A) Schematic representation of Gal4BD-WASP₁₋₃₂₀ fusion construct used in Monocyte cDNA library screening as bait. (B) Expression of BD-WASP₁₋₃₂₀ fusion protein in PJ69-4A yeast strain. (C) WASP^{WH1} Interactors found in Monocyte cDNA library screening

WASP₁₋₃₂₀ was cloned in Gal4BD vector and transformed into PJ69-4A yeast strain. Transformed colonies were selected on Trp⁻ selection plate and the expression of BD-WASP₁₋₃₂₀ fusion protein was analysed (Fig. 3.62B). Subsequently, monocyte cDNA library (Gal4AD) was transformed into PJ69-4A yeast cells expressing BD-WASP₁₋₃₂₀ fusion protein. To check transformation efficiency, transformed colonies were plated on Trp⁻ Leu⁻ selection plates whereas positive interactors were screened on Trp⁻Leu⁻His⁻ plates. In this screen, 24 colonies were identified which were able to grow on agar plates lacking histidine (positive for interaction). Sequence analysis revealed colonies had cDNA encoding WASP Interacting Protein (WIP) and Calcium and integrin binding protein (CIB1) as in-frame fused with Gal4AD,

while the remainder had out of frame (Fig. 3.62C). WIP and CIB1 are known interactors of WH1 domain of WASP. WIP binds to the WH1 domain of WASP and stabilizes the closed conformation of WASP [64,180]. CIB1 was identified as a protein that binds to the cytoplasmic tail of platelet integrin $\alpha\text{IIb}\beta 3$ [150]. WASP–CIB1 complex has been shown to be important in $\alpha\text{IIb}\beta 3$ -mediated cell adhesion, which has a primary role in platelet aggregation [225]. In this library screening, the other WASP^{WH1} interacting novel interactors were not identified.

3.2.14 Identification of WASP^{WH1} missense mutations affecting WASP interaction with CIB1

Apart from WIP, calcium and integrin binding protein (CIB1), a 22 kDa EF-hand integrin and kinase binding protein has also been identified as another WASP^{WH1} domain binding partner. Using yeast two hybrid system, it has been discovered that the N-terminal of CIB1 binds directly to the N-terminal of WASP at residues 1-105 [225]. CIB1 was identified as WASP interacting protein in the monocyte cDNA library screening using WASP₁₋₃₂₀ as bait (Fig. 3.62). In order to analyse the effect of WASP^{WH1} missense mutations that cause WAS or XLT on WASP-CIB1 interaction, a yeast two hybrid assay was performed using Gal4BD expressing WASP₁₋₂₆₅ (WT or WH1 mutants) as bait. *S. cerevisiae* strain PJ69-4A was co-transformed with plasmids expressing Gal4 DNA binding domain fusion proteins and activation domain fused with CIB1 (pAD-CIB1). Transformed cells were selected on Trp⁻ Leu⁻ (control plates) and Trp⁻ Leu⁻ His⁻ plates were used for checking interaction between BD-WASP₁₋₂₆₅ fusion proteins and AD-CIB1 fusion protein.

As shown in figure 3.63 & table 3.6, out of 25 WASP^{WH1} missense mutations tested, six of them (WASP₁₋₂₆₅^{G40V}, WASP₁₋₂₆₅^{T45M}, WASP₁₋₂₆₅^{L46P}, WASP₁₋₂₆₅^{A47D}, WASP₁₋₂₆₅^{P58L} and WASP₁₋₂₆₅^{G70W}) did not interact with CIB1. Out of these six WASP^{WH1} missense mutations identified in yeast two hybrid assay, which abolished interaction of WASP with CIB1, three of them have been reported to cause WAS (WASP^{G40V}, WASP^{P58L} and WASP^{G70W}) whereas other three mutations (WASP^{T45M}, WASP^{L46P}, WASP^{A47D}) have been reported to cause XLT [96]. Thus, it is possible that WASP-CIB1 interaction plays an important role and regulates WASP functions.

Figure 3.63 WASP^{WH1} missense mutations cause loss of interaction of WASP with CIB1. Representative plates showing the interaction between WASP or WASP^{WH1} missense mutants with CIB1. Growth is observed as light streaks against the dark background

Table 3.6 WASP^{WH1} mutants affecting interaction with CIB1

Domain	Amino acid residue	Missense mutation	-T-L	-T-L-H
WH1	-	Wild-Type	+	+
WH1	6	Met > Ile	+	+
WH1	27	Leu > Phe	+	+
WH1	31	Glu > Lys	+	+
WH1	35	Leu > His	+	+
WH1	39	Leu > Pro	+	+
WH1	40	Gly > Val	+	-
WH1	41	Arg > Gly	+	+
WH1	43	Cys > Trp	+	+
WH1	45	Thr > Met	+	-
WH1	46	Leu > Pro	+	-
WH1	47	Ala > Asp	+	-
WH1	52	Gln > His	+	+
WH1	56	Ala > Val	+	+
WH1	58	Pro > Leu	+	-
WH1	64	Trp > Arg	+	+
WH1	70	Gly > Trp	+	-
WH1	73	Cys > Tyr	+	+
WH1	75	Val > Met	+	+
WH1	77	Gln > Gly	+	+
WH1	82	Ser > Pro	+	+
WH1	84	Phe > Leu	+	+
WH1	85	Ile > Thr	+	+
WH1	86	Arg > His	+	+
WH1	99	Gln > Arg	+	+
WH1	105	Leu > Pro	+	+

WT or missense mutants of WASP were examined for interaction with CIB1 using yeast two-hybrid system. Trp⁻ Leu⁻ SD plates were used for transformants selection. Colonies grown on Trp⁻ Leu⁻ His⁻ SD plates were used to determine the interaction with CIB1. (+) sign indicates the presence of growth while (-) sign indicates the absence of growth (loss of interaction). Six WASP missense mutants (BD-WASP₁₋₂₆₅^{G40V}, BD-WASP₁₋₂₆₅^{T45M}, BD-WASP₁₋₂₆₅^{L46P}, BD-WASP₁₋₂₆₅^{A47D}, BD-WASP₁₋₂₆₅^{P58L} and BD-WASP₁₋₂₆₅^{G70W}) were found to affect the interaction with AD-CIB1. BD-Binding domain, AD-Activation domain.

3.2.15 Six WASP^{WH1} missense mutants abolished WASP-CIB1 interaction did not affect WASP interaction with WIP

WIP is a chaperone for WASP and its interaction prevents WASP degradation from cytoplasmic proteases [64,180]. In order to examine if the WASP missense mutants that affect WASP-CIB1 interaction also have any influence on WIP interaction, *S. cerevisiae* strain PJ69-4A was co-transformed with plasmids expressing Gal4 DNA binding domain fusion proteins (Gal4BD-WASP₁₋₂₆₅) (WT or six WASP^{WH1} mutants) and activation domain fused with pAD-WIP. Transformed cells were selected on Trp⁻ Leu⁻ (control plates) and Trp⁻ Leu⁻ His⁻ plates were used for checking interaction between BD-WASP₁₋₂₆₅ fusion proteins and AD-WIP fusion protein.

Figure 3.64 Mutations affecting WASP-CIB1 interaction do not affect WASP-WIP complex formation. *S. cerevisiae* strain PJ69-4A was co-transformed with plasmids expressing the BD-WASP or WASP^{WH1} mutant's fusion proteins and the AD-WIP or empty vector. The transformed colonies were streaked on SD minimal media plates lacking histidine and incubated at 30°C for 5 days.

The results demonstrated that WIP interaction is still present with all six WASP^{WH1} mutants (WASP₁₋₂₆₅^{G40V}, WASP₁₋₂₆₅^{T45M}, WASP₁₋₂₆₅^{L46P}, WASP₁₋₂₆₅^{A47D}, WASP₁₋₂₆₅^{P58L} and WASP₁₋₂₆₅^{G70W}) abolished interaction of WASP with CIB1 (Figure 3.64), thus suggesting that these six WASP^{WH1} mutants only affect WASP-CIB1 interaction.

3.2.16 Varied expression level of WASP^{WH1} missense mutants in WASP^{KD} Jurkat T-cells

Out of six WASP^{WH1} mutations which affecting WASP-CIB1 interaction, two mutations (G40V, G70W) have been reported to express poorly whereas other four mutations (T45M, L46P, A47D, P58L) have reduced expression compared to WT WASP expression in *S. cerevisiae* cells [181]. Therefore, the protein stability of these six WASP^{WH1} mutants (WASP^{G40V}, WASP^{T45M}, WASP^{L46P}, WASP^{A47D}, WASP^{P58L} and WASP^{G70W}) were analysed in WASP^{KD} Jurkat T-cells. WASP^{KD} Jurkat T-cells stably expressing (microporated and selected with neomycin) WT WASP or WASP^{WH1} mutants tagged with RFP (in neomycin resistant construct) were lysed and immunoblotted using α -WASP antibody. As shown in figure 3.65, the expression of WASP^{L46P} and WASP^{A47D} can be readily detected whereas expression levels of other four mutants (WASP^{G40V}, WASP^{T45M}, WASP^{P58L} and WASP^{G70W}) were lower than that of the WT WASP expression. In yeast two hybrid data, all six WASP^{WH1} mutants did not affect their interaction with WIP. Thus, four individual WASP^{WH1} mutations affecting protein stability (WASP^{G40V}, WASP^{T45M}, WASP^{P58L} and WASP^{G70W}) was not due to loss of interaction with WIP.

Figure 3.65 Expression levels of WASP^{WH1} missense mutants in WASP^{KD} Jurkat T-cells. Western blot analysis of the expression levels of (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{G40V}-RFP, (4) WASP_R^{T45M}-RFP, (5) WASP_R^{L46P}-RFP, (6) WASP_R^{A47D}-RFP, (7) WASP_R^{P58L}-RFP, (8) WASP_R^{G70W}-RFP in transfected WASP^{KD} Jurkat T-cells after one week of selection with neomycin using appropriate antibodies.

3.2.17 Expression of WASP^{L46P} and WASP^{A47D} do not rescue the chemotaxis of WASP^{KD} Jurkat T-cells towards SDF-1 α .

Results from section 3.2.16 showed that four WASP^{WH1} missense mutations (G40V, T45M, P58L, G70W) affecting WASP-CIB1 interaction have negligible expression in WASP^{KD} Jurkat T-cells, whereas the other two mutations (L46P, A47D) are readily detected. Therefore, WASP^{L46P} or WASP^{A47D} mutants were analysed for their ability to rescue the chemotactic defects of WASP^{KD} Jurkat T-cells. WASP^{KD} Jurkat T-cells stably expressing WASP_R^{L46P}-RFP or WASP_R^{A47D}-RFP (used to determine expression level in previous result) mutants were analysed for their chemotactic response towards SDF-1 α using Dunn chamber. As shown in vector plot, expression of WASP_R^{L46P}-RFP or WASP_R^{A47D}-RFP did not rescue the chemotactic defect of WASP^{KD} Jurkat T-cells, whereas WASP_R-RFP rescued the chemotactic defect (Fig. 3.66A). Quantitative analysis revealed that the migration speed of WASP_R^{L46P}-RFP (1.46 μ m/min) or WASP_R^{A47D}-RFP (1.36 μ m/min) expressing

cells were significantly reduced compare to WASP_R-RFP expressing cells (2.3μm/min) (Fig. 3.66B).

Figure 3.66 Expression of WASP^{L46P} or WASP^{A47D} did not rescue the chemotaxis of WASP^{KD} Jurkat T-cell (A). (A) Vector plots of migration paths of 20 randomly chosen cells from WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{L46P}-RFP, (4) WASP_R^{A47D}-RFP exposed to a gradient of SDF-1α in the Dunn chamber. Time-lapse images were captured over 3.0 hrs at 2.0 min of intervals. Intersection of the X and Y axes are the starting point of each cell. The source of SDF-1α was at the top. (B) Migration velocity of each cell shown as a mean of velocities of 60 randomly chosen cells (20 cells each from 3 sets of experiments) * represent $P < 0.05$ (unpaired student's t-test).

Circular histogram showing the overall directionality of cell migration revealed that 86.6% of total WASP_R-RFP expressing cells moved to the position within 40° arc facing the chemokine source. Only 21.32% of total WASP_R^{L46P}-RFP or 20.6% of total WASP_R^{A47D}-RFP expressing cells moved within 40° arc facing the chemokine source which was similar to RFP alone (control) expressing

cells (10% of total) indicating that WASP_R^{L46P} or WASP_R^{A47D} mutations affecting the directionality of migration (Fig. 3.67).

Figure 3.67 Expression of WASP^{L46P} or WASP^{A47D} did not rescue the chemotaxis of WASP^{KD} Jurkat T-cell (B). WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{L46P}-RFP, (4) WASP_R^{A47D}-RFP exposed to a gradient of SDF-1α in the Dunn chamber. Percentage of cells where the final positions of cells lie in each of the sectors (20°) was determined by circular histogram. The source of SDF-1α was at the top.

To confirm the chemotactic defect of WASP^{KD} Jurkat T-cells expressing WASP_R^{L46P}-RFP or WASP_R^{A47D}-RFP, a transwell migration assay was performed. 38.8% of total WASP^{KD} + WASP_R^{L46P}-RFP or 35.2% of total WASP^{KD} + WASP_R^{A47D}-RFP Jurkat T-cells migrated towards the SDF-1α containing media in lower well compared to 51.24% of WASP^{KD} + WASP_R-RFP Jurkat T-cells suggesting that WASP_R^{L46P} or WASP_R^{A47D} mutants are unable to rescue the impaired chemotaxis of WASP^{KD} Jurkat T-cells (Fig.

3.68). Taken together, these results suggest that the WASP^{WH1} missense mutation L46P or A47D abolishes WASP function in chemotactic migration of Jurkat T-cells.

Figure 3.68 Expression of WASP^{L46P} or WASP^{A47D} did not rescue the chemotaxis of WASP^{KD} Jurkat T-cells in transwell migration assay. Transwell migration assay was performed with WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{L46P}-RFP, (4) WASP_R^{A47D}-RFP. 2 X 10⁵ cells were loaded in upper well and allowed to migrate towards SDF-1 α (100 ng/ml) containing media in the lower well for 3.0 hrs. Cells migrated to the bottom well were counted and plotted as percentage of cells migrated. Experiment was repeated thrice. * $P < 0.05$, ** $P < 0.01$ compared to WASP^{KD} Jurkat T-cells expressing WASP_R-RFP (unpaired student's t-test).

3.2.18 WASP^{L46P} and WASP^{A47D} mutants behaved similar to WASP in terms of binding of Jurkat T-cells to fibronectin.

WASP^{L46P} and WASP^{A47D} mutations have been found in XLT patients suggesting the extensive effect of these mutations on platelets functions. In previous results, it was identified that these two WASP^{WH1} mutations (WASP^{L46P} or WASP^{A47D}) were unable to rescue the chemotactic defect of WASP^{KD} Jurkat T-cells indicating apart from affecting platelets functions, these mutations also perturb T-cell functions. In order to analyse whether WASP mutations (L46P or A47D) affecting binding of Jurkat T-cells to

fibronectin, adhesion assay was performed as described in methods 2.2.4.1. The adhesion of WASP^{KD} Jurkat T-cells expressing WAS_R-RFP (50.1%) to fibronectin was comparable to WASP_R^{L46P}-RFP (44.1%) or WASP_R^{A47D}-RFP (47.9%) expressing cells and was significantly reduced compared to RFP (control) expressing cells (81.4%) (Fig. 3.69). This result suggests that WASP^{WH1} mutations (L46P or A47D) do not affect WASP functions in Jurkat T-cell-ECM adhesion.

Figure 3.69 Expression of WASP^{L46P} or WASP^{A47D} behaved similar to WASP in terms of Jurkat T-cells adhesion to fibronectin. Binding assay was performed to compare the cell adhesion capability of WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{L46P}-RFP, (4) WASP_R^{A47D}-RFP to fibronectin coated surface. 2 X 10⁴ cells were allowed to adhere to fibronectin coated plate (0.15µg/100µl) for 20 min. Bound cells were quantified and plotted. ** *P* < 0.01 compared to RFP expressing WASP^{KD} Jurkat T-cells (unpaired student's t-test).

3.2.19 Expression of WASP^{L46P} mutant does not rescue IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells

In order to analyze the effect of WASP^{WH1} mutants (WASP^{L46P} or WASP^{A47D}) in IL-2 gene transcription, WT Jurkat T-cells or WASP^{KD} Jurkat T-cells expressing WASP_R-RFP or WASP_R^{L46P}-RFP or WASP_R^{A47D}-RFP or RFP alone (control) cells were stimulated on anti-CD3/anti-CD28 coated plates as

described in methods 2.2.4.4. Quantitative real time analysis for IL-2 gene transcription revealed that expression of WASP_R-RFP or WASP_R^{A47D}-RFP rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells. However, expression of WASP_R^{L46P}-RFP mutant did not rescue the IL-2 gene transcription defect compared to WASP_R-RFP expressing cells (Fig. 3.70) suggesting the WASP mutation (L46P) abolished T-cell receptor mediated cell signalling.

Figure 3.70 Expression of WASP^{L46P} mutant does not rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells. Total RNA was isolated from WT or WASP^{KD} Jurkat T-cells expressing (1) RFP, (2) WASP_R-RFP, (3) WASP_R^{L46P}-RFP, (4) WASP_R^{A47D}-RFP 8.0 hrs after incubation with anti-CD3/anti-CD28 antibodies (2µg/ml each). Quantitative real time PCR was performed and quantified. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to WT Jurkat T-cells (unpaired student's t-test).

3.2.20 Abnormal actin cytoskeleton of WASP^{KD} Jurkat T-cells expressing WASP^{L46P} and WASP^{A47D} mutants

The actin cytoskeleton organization of WASP^{KD} Jurkat T-cells expressing WASP_R^{L46P}-RFP or WASP_R^{A47D}-RFP after anti-CD3/anti-CD28 stimulation was analysed. WASP_R^{L46P}-RFP or WASP_R^{A47D}-RFP expressing cells were activated for 30 min on anti-CD3/anti-CD28 coated plates, fixed and stained with Alexa 594 phalloidin. Both the WASP^{WH1} missense mutants expressing cells spread poorly compared to WASP_R-RFP expressing cells on anti-

CD3/anti-CD28 coated plates (Fig. 3.71B). Surprisingly, cell expressing $WASP_R^{L46P}$ -RFP had abundant membrane projections whereas $WASP_R^{A47D}$ -RFP expressing cells had almost negligible membrane projections compared to normal spreading and membrane projections of $WASP_R$ -RFP expressing cells (Fig. 3.71 A & C) Moreover, actin patches were observed inside the cells expressing $WASP_R^{L46P}$ -RFP (Fig. 3.71A). This abnormal actin cytoskeleton polymerization and reorganization could explain the abnormalities in Jurkat T-cells functions.

Figure 3.71 $WASP^{L46P}$ or $WASP^{A47D}$ were defective towards anti-CD3/anti-CD28 stimulated actin cytoskeleton organization. (A) $WASP^{KD}$ Jurkat T-cells expressing (1) RFP alone, (2) $WASP_R$ -RFP, (3) $WASP_R^{L46P}$ -RFP, (4) $WASP_R^{A47D}$ -RFP were plated on anti-CD3/anti-CD28 antibodies coated plate and incubated for 30 min. Cells after incubation were fixed and stained with Alexa 594 phalloidin. (B) The mean surface area of 30 randomly chosen cells in each three independent experiments was quantified using Metamorph software and plotted. (C) The average number of projections per cell was quantified for 30 cells in each set of experiment and experiment was repeated thrice. ** $P < 0.01$, *** $P < 0.001$ compared to $WASP_R$ -RFP expressing $WASP^{KD}$ Jurkat T-cells (unpaired student's t-test).

3.2.21 WASP^{L46P} or WASP^{A47D} does not affect WASP localization at TCR activation site

Besides WASP^{PRR} domain dependent localization of WASP to the immunological synapse [44], another mechanism of WASP recruitment to the immunological synapse has also been reported [190], which is dependent on the interaction through the WH1 domain of WASP. Therefore, the localization pattern of WASP^{WH1} missense mutants (affecting WASP-CIB interaction) were analyzed relative to polymerized actin after TCR induction.

Figure 3.72 WASP^{L46P} or WASP^{A47D} mutants could localize at TCR activation site. WT Jurkat T-cells expressing (1) RFP, (2) WASP-RFP, (3) WASP^{L46P}-RFP, (4) WASP^{A47D}-RFP were plated on stimulatory coverslip (anti-CD3 antibody coated) and incubated for 5.0 min. Cells were fixed and stained with Alexa 488 phalloidin.

Similar to WASP-RFP, both the WASP^{WH1} mutants tagged with RFP (WASP^{L46P}-RFP or WASP^{A47D}-RFP) were colocalized with actin ring at the periphery of the spreaded cells (Fig. 3.72). The colocalization of these

WASP^{WH1} mutants with polymerized actin ring could either taken place through the interaction with WIP or through the PRR domain of WASP. Therefore, no defect in colocalization pattern of WASP^{WH1} missense mutants was observed.

Table 3.7 List of constructs used and functional assays performed in this study

Constructs Assays	WASP	WASP^{S339Y}	WASP^{P373S}	WASP^{SP/YS}	WASP^{L46P}	WASP^{A47D}
Chemotaxis towards SDF-1α	Rescued	<u>Impaired</u>	<u>Impaired</u>	<u>Impaired</u>	<u>Impaired</u>	<u>Impaired</u>
Migration velocity	Rescued	Rescued	Rescued	Rescued	<u>Impaired</u>	<u>Impaired</u>
Adhesion to Fibronectin	Rescued	Rescued	<u>Reduced</u>	<u>Reduced</u>	Rescued	Rescued
IL-2 gene activation	Rescued	Rescued	<u>Impaired</u>	<u>Impaired</u>	<u>Impaired</u>	Rescued
Membrane projections	Rescued	<u>Less</u>	<u>Less</u>	<u>Less</u>	<u>More</u>	<u>Less</u>
Cell spreading on anti-CD3/anti-CD28 antibody	Normal	Normal	Normal	Normal	<u>Reduced</u>	<u>Reduced</u>
Colocalization with actin ring at TCR	Colocalized with actin at TCR	Small patches inside the cell	Diffused pattern of localization	Diffused pattern of localization	Colocalized with actin at TCR	Colocalized with actin at TCR

A summary of WASP and their missense mutants used in this study and their functional assays performed. All the assays except colocalization study were performed in WASP^{KD} Jurkat T-cells. The results obtained in respective assays are mentioned in this table. The WASP mutants in respective assays are compared with WT WASP.

The results obtained in this study provided deeper insight in to the effect of WASP^{PRR} missense mutations causing WAS. Both WASP^{PRR} missense mutations separately have their unique and common outcomes and together

have drastic effect on the WASP mediated Jurkat T-cell functions (summarised in table 3.7). Beside WASP^{PRR} missense mutations, WASP^{WH1} mutations also have some unique and common effects on Jurkat T-cell functions. By characterising WASP^{PRR} or WASP^{WH1} missense mutations in other hematopoietic cells system or animal model will provide a comprehensive knowledge which can be use to develop therapies.

4. Discussion

4.1 Redundant and unique functions of WASP and N-WASP

WASP and N-WASP are the actin cytoskeleton regulators necessary for many cellular processes such as cell motility, cell adhesion, cell division, vesicle transport, endocytosis, cell shape change and transcription regulation. Hematopoietic cells express both WASP and N-WASP, but N-WASP does not compensate for WASP deficiency completely giving rise to WAS. Although both unique as well as redundant roles of WASP and N-WASP have been identified in hematopoietic cells [60,97] the molecular differences in the proteins giving rise to the unique roles of WASP have not been characterized. In this study, the molecular and functional differences between WASP and N-WASP were characterized in Jurkat T-cells.

Initially, the expressions of WASP and N-WASP were compared at transcriptional level in the cell lines of hematopoietic origin (Jurkat T-cells, THP-1 cells, RAW264.7 cells and Raji B-cells) using WASP or N-WASP specific real time PCR primers. The expression of N-WASP (at mRNA level) was 40-66% lower compared to WASP expression (Fig. 3.1) in the cell lines of hematopoietic lineages, which is consistent with the previous studies [97,195]. Similarly, expression of N-WASP at transcriptional level was lower than WASP in primary T-cells either non-activated (18.7 fold lower) or activated with anti-CD3 alone (40 fold lower) or both anti-CD3/CD28 antibodies (32 fold lower) (Fig. 3.2). This indicates that the inability of N-WASP to compensate for the loss of WASP

completely could be due to insufficient expression of N-WASP compared to WASP in hematopoietic lineages.

To date, the expression pattern of WASP and N-WASP has not been compared in developmental stages of hematopoietic lineages. The development of B-cell is normal in WASP^{KO} mice [140]. However, it caused a severe reduction of marginal zone B-cells and their functional dysregulation [183,234,235]. Similarly, the initial developmental stages of T-cells appear to be normal in WASP^{KO} mice, but it is severely affected in WASP/N-WASP conditional double knockout mice suggesting the importance of N-WASP during developmental stages of T-cell [160]. It is possible that expression level of N-WASP is comparable to WASP expression during developmental stages and its expression is reduced once the developmental processes are over. Although, WASP plays a key role in functions of lymphocytes, the contribution of N-WASP in these cells cannot be discounted. In this study, the unique and redundant roles of WASP and N-WASP have been identified in Jurkat T-cells functions by knocking down of WASP expression followed by reconstitution of WASP or N-WASP expression. T-cells from WAS patients are defective in homing, chemotaxis towards chemokine SDF-1 α and IL-2 production in response to TCR stimulation [76,162,200,243,248]. Consistent with these studies, knocking down of WASP expression in Jurkat T-cells altered their chemotaxis towards chemokine SDF-1 α in Dunn chemotactic assay and in transwell migration assay (Fig. 3.6 & 3.7). This defective chemotaxis of WASP^{KD} Jurkat T-cells was rescued by exogenous WASP expression but not by N-WASP expression (Fig. 3.11A & 3.13). The directionality (cells within 40° arc facing the

chemokine source, determined by circular histograms) of WASP^{KD} Jurkat T-cells in SDF-1 α gradient was random, which was not rescued by N-WASP expression (Fig. 3.11B). However, N-WASP expression rescued the reduced migration velocity of WASP^{KD} Jurkat T-cells (Fig. 3.12A) suggesting that WASP plays a role in directional sensing required for directional migration of T-cells towards chemokine source. The inability of N-WASP in regulating directional migration of T-cells could be due to the defective polarization of T-cells expressing N-WASP or it might have affected the persistency of directional protrusions. Recently, it has been shown that WASP-deficient macrophages can form directional protrusions towards the chemokine (CSF-1) source, but the persistency of directional protrusions was reduced, resulting in random migration of macrophage cells [98]. Apart from chemotactic migration defects, knocking down of WASP expression enhanced the adhesion of Jurkat T-cells to fibronectin (Fig. 3.8A). T-cells express β 1 integrin which mediates T-cell adhesion to fibronectin [133]. There is one report stating that cell adhesion to fibronectin is not affected in WASP^{-/-} T-cells [76]. This could be due to higher (10 μ g/ml) amount of fibronectin used in the adhesion assay. It is possible that this concentration of fibronectin used may have saturated the receptors on the cell surface of both WT and WASP^{-/-} T-cells, masking any subtle difference in the binding of WASP^{-/-} T-cells. Consistent with their report, there was no significant difference observed in the adhesion of WT and WASP^{KD} Jurkat T-cells to fibronectin at the concentration of 10 μ g/ml (data not shown). By lowering the amount of fibronectin to 1.5 μ g/ml, a significant difference in adhesion was observed between WT and WASP^{KD} Jurkat T-cells

(Fig. 3.8A). This increased in adhesion of WASP-deficient Jurkat T-cells identified was not due to any change in $\beta 1$ integrin (ligand for fibronectin) expression (Fig. 3.8B). Cell-ECM adhesions are critical for cell migration and are mainly mediated by integrins [94]. In this coordinated process of cell migration and adhesion, cell regulates changes in actin cytoskeleton and assembly/disassembly of cell adhesion sites. Chemokine (SDF-1 α) induced DCs migration has been shown to require WASP activity for initiation of podosomes formation and polarization of cell towards the chemokine source [145]. Whereas, interaction of integrin to its ligand such as fibronectin promotes podosomes maturation and stabilization by promoting WASP activity in actin polymerization, which recruits other proteins required for podosome maturation and integrin's clustering. As a result, stabilization of leading edge in the direction of chemoattractant source takes place. For progression of leading edge during cell migration, podosomes need to release their attachments and this process requires cleavage of WASP and talin by the protease calpain [38]. Inhibition of calpain activity has been shown to stabilize podosomes, resulting in impaired migration of DCs [38]. Thus, suggesting that WASP is essential for both podosomes assembly and disassembly which is required for myeloid cells migration. Expression of exogenous WASP but not N-WASP significantly reduced the cell adhesion of WASP^{KD} Jurkat T-cells to fibronectin, which was comparable to the adhesion of WT Jurkat T-cells (Fig. 3.14). WASP and N-WASP have differing sensitivities to calpain proteases. N-WASP, unlike WASP is not cleaved by calpain [195]. Calpain cleavage of WASP is important for regulation of WASP mediated cell

adhesion and migration. Thus, it is possible that inability of N-WASP to rescue the chemotaxis and adhesion of WASP^{KD} Jurkat T-cells is due to its calpain insensitivity.

Dunn chemotactic assay performed in this study provide both chemokine signalling as well as integrins mediated signalling to Jurkat T-cells, because cells were first allowed to attach to fibronectin before inducing them in chemokine gradient. Chemotaxis of Jurkat T-cells was not observed when cells were not attached to fibronectin coated surface (data not shown). Thus, suggesting that chemokine signalling and integrins mediated adhesions are dynamically regulated during chemotaxis of T-cells.

Besides playing role in chemotaxis and adhesion to ECM, WASP is required for formation of actin rich membrane projections (microvilli). Lymphocytes from WAS patients have been examined by scanning electron microscopy and found to have fewer microvilli, although it did not affect the expression of various surface receptors [109,143]. Similarly, WASP-deficient B-cells are defective in filopodia extension in response to bradykinin stimuli [6]. All these studies suggest that WASP plays an important role in regulating dynamics of membrane projections in hematopoietic cells.

Microvilli on T lymphocyte have been shown to contain receptors for diverse signalling molecules, which help in sensing the cell's surroundings and acting as sites for signal transduction [138]. Similarly, microvilli on B-cells have been shown to be enriched in MHC class II molecules and ICAM-1 [80] required for T-

cell activation. These microvilli on immune cells plays a critical role in first two steps of cell adhesion during trans-endothelium migration, a process essential for recruiting lymphocytes to the site of inflammation. The first step is tethering, which is mediated by interactions through selectins present on the tip of microvilli [4,176]. The second step is triggering, which is regulated by chemokine receptors such as CXCR4 or CCR5 are mainly present on microvilli [198]. Once the lymphocytes get activated by chemokine, it quickly losses their microvilli and polarized towards the increasing concentration of chemokine source in order to penetrate endothelium barrier. The dynamic formation and resorption of membrane projections on Jurkat T-cells were not observed when stimulated with SDF-1 α . It could be because of direct contact of Jurkat T-cells with chemokine source without through endothelium layer. However, cells formed pseudopod towards SDF-1 α gradient (Data not shown) indicating that these microvilli are not required during the chemotaxis of T-cells. This resorption of T-cells microvilli is mediated by SDF-1 α induced Rac1 activation [153]. Similarly, active Rac1 and Cdc42 have been shown to induce lamellipodia and filopodia in B-cells respectively, suggesting the role of Rac1 in lymphocytes chemotaxis [154,233]. From all these observations, it can be concluded that WASP activity in microvilli formation (which is essential for initiation of chemotaxis) and in chemotaxis (dependent on cell polarization, pseudopod formation and cell-ECM adhesion) is regulated by different WASP interacting proteins.

The functions of T-cell microvilli have been well studied, but WASP dependent regulation of actin cytoskeleton architecture during T-cell activation has not been

studied well. In this study, the role of WASP in cytoskeleton architecture changes have been analysed during TCR stimulation; a process necessary for T-cell activation. WT Jurkat T-cells when stimulated with surface coated anti-CD3/anti-CD28 antibodies, formed membrane projections, which were significantly less in number in WASP^{KD} Jurkat T-cells (Fig. 3.9). No membrane projections were detected even in WT Jurkat T-cells when cells were plated on anti-CD3 coated coverslip suggesting that signals from CD28 costimulatory receptor are essential for optimal actin cytoskeleton changes (Data not shown). Expression of exogenous WASP rescued the membrane projections defect of WASP^{KD} Jurkat T-cells. Although, N-WASP has been shown to induce filopodia formation in NIH3T3 fibroblast cells [136], its expression did not rescue the membrane projections defect of WASP^{KD} Jurkat T-cells (Fig. 3.15). However, spreading of WASP^{KD} Jurkat T-cells on stimulatory surface (determined by average surface area) was normal (Fig. 3.9), which could be mediated by endogenous N-WASP as marked reduction in spreading of WASP/N-WASP double knockout B-cells was observed in comparison to WASP^{KO} B-cells [236].

WASP has been shown to be important for IL-2 production in response to TCR stimulation [97,248]. Consistent with previous reports, WASP^{KD} Jurkat T-cells were defective in IL-2 gene transcription after TCR ligation, which was rescued by exogenous WASP expression (Fig. 3.16). The ectopic expression of N-WASP was unable to fully rescue the chemotactic response of WASP^{KD} Jurkat T-cells, which might be due to the defective actin cytoskeleton rearrangement by N-WASP.

However, expression of N-WASP rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells (Fig. 3.16) suggesting that the endogenous N-WASP expression may not sufficient to rescue IL-2 production defect of WASP^{KD} Jurkat T-cells. It has been reported that WASP regulates nuclear translocation of NFAT2 which is independent of its role in actin polymerization and reorganization [93]. NFAT is a transcription factor, which has been shown to regulate IL-2 expression [53]. The role of WASP in regulating IL-2 production was found to be independent of its role in immunological synapse formation [43]. Moreover, the N-terminal region of WASP has been shown to be important for NFAT activation as WASP mutant with deletion in C-terminal region (that was defective in actin polymerization) potentiated NFAT transcription following TCR activation by anti-CD3/anti-CD28 antibodies [197]. All these studies suggest that WASP has two independent functions: first the actin cytoskeleton rearrangement, which regulates chemotactic migration and cell adhesion; second is IL-2 production. N-WASP is able to rescue the IL-2 gene transcription defect but not the chemotactic migration defect of WASP^{KD} Jurkat T-cells suggesting that N-WASP plays a redundant role with WASP in IL-2 production.

Jurkat T-cell when dropped on to the stimulatory surface (anti-CD3 coated coverslip), it start forming initial filopodial contacts with the stimulatory surface, which are later converted into lamellipodial structures. These lamellipodial structures provide more contacts for T-cell with the stimulatory surface. As these contacts thickened, a circumferential actin ring develops that moves outward and define the periphery of the cell (a region of strongest contact with the stimulatory

surface) [24]. This whole process start from TCR engagement to T-cell spreading is quite rapid (within 5.0 mins) and localization of WASP during this process changes over time, moving from initial TCR contact site to the periphery of the cell. In this study, the localization pattern of WASP or its mutants relative to actin were analysed when cells were fully spread and formed a circumferential actin ring at the periphery of the cell. Once the cells were dropped on to the stimulatory surface, they have sufficient time (5.0 to 10 mins) to make maximum contact with the stimulatory surface. In order to study the localization of WASP or its mutants relative to actin, WT Jurkat T-cells were used, because WASP^{KD} Jurkat T-cells were stably expressing GFP (due to S1 shRNA) and cannot be used further for colocalization study. Moreover, WASP or its mutants were expressed under the CMV promoter as WASP expression under the WASP promoter is quite weak to observe in fluorescence microscope. Following these conditions, differential localization of WASP and N-WASP after stimulation with anti-CD3 coated surface was observed (Fig. 3.17). WASP was mainly colocalized with actin ring at the periphery of the membrane, whereas most of the N-WASP was located at the centre (nuclear localization) of the spread cell suggesting that differential localization of N-WASP in T-cells could be one of the reasons of functional differences between WASP and N-WASP.

In conclusion, WASP has a unique role in directional migration of T-cells and has a redundant role with N-WASP in TCR mediated IL-2 production. Figure 4.1

summarized the redundant and non-redundant functions of WASP and N-WASP in Jurkat T-cell functions.

Figure 4.1 Unique and redundant functions of WASP and N-WASP in Jurkat T-cell.

WASP has some unique functions which are not rescued by N-WASP expression in WASP^{KD} Jurkat T-cells. Chemotaxis of N-WASP expressing cells was random towards SDF-1α gradient (1), which could be due to defective polarization or increased adhesion of cells (2). N-WASP expression does not rescue the membrane projection defect of WASP^{KD} Jurkat T-cells although, cells' spreading on anti-CD3/anti-CD28 coated surface is not affected (3). N-WASP can rescue the IL-2 gene transcription defect suggest its redundant role with WASP (4).

4.2 I30 region of WASP: a molecular difference between WASP and N-WASP

4.2.1 WASP has a unique 30 amino acid insertion compared to N-WASP

An internal 30 amino acid region has been identified (a.a.158-187) (referred to as I30 region) in WASP, which is present between BR and WH1 domain of WASP and this region of WASP is absent in N-WASP (Fig. 3.18). The I30 region of WASP contains 9 proline residues out of 30 amino acid (30% Proline), suggesting that beside proline rich region of WASP (PRR: a.a.312-417), I30 region also mediate interaction with SH3 domain containing proteins. A peptide of WASP comprising I30 region (WASP₁₃₈₋₂₆₅) interacted with eight SH3 domain containing tyrosine kinases (Fyn, Hck, Lyn, Fgr, Src) and adaptor proteins (Grb2, Nck1, Toca1) tested in yeast two hybrid assay (Table 3.1). Recently, Fyn has been reported to interact via its SH3 domain with N-terminal region of WASP (a.a 1-171) [191] which comprising 14 amino acids of I30 region of WASP. The interaction of WASP₁₃₈₋₂₆₅ peptide with Btk was not observed which is contrary to a previous study that reported interaction between WASP₁₇₀₋₁₈₅ peptide with Btk. [59]. It is possible that the Btk interaction with WASP might be indirect and mediated by adaptor proteins. All these studies point to the importance of WASP-I30 region in regulation of WASP activity.

Beside interaction with SH3 domain containing proteins (Table: 3.1), it was possible that I30 region of WASP may also interact with unknown interactors, which leads to functional difference between WASP and N-WASP. Therefore, a

GST pull down assay was performed using GST tagged smaller peptides of WASP (GST-WASP₁₃₈₋₃₂₀ or GST-WASP₁₃₈₋₃₂₀^{ΔI30}). These small peptides of WASP were expressed in *E.coli*, purified and purified peptides of WASP were used to identify the novel interactors from Jurkat T-cells lysate. Proteins interacted with these smaller peptides of WASP were eluted and separated on 10% SDS-PAGE. However, there was no difference observed in the protein profile (Data not shown). There could be few possible explanations attributing to this result. Firstly, WASP₁₃₈₋₃₂₀ is a smaller peptide and some of the proteins may interact at two different positions on a WASP molecule. Therefore, smaller peptides of WASP may not be sufficient for this kind of interaction. Secondly, only strong interactions can be observed by *in vitro* pull down assay as weak interactors might have been washed away due to extensive washing steps in GST pull down assay. To rule out the second possibility, library screening was also performed using monocyte cDNA library and WASP₁₋₃₂₀ peptide (comprising I30 region) as bait. However, no novel WASP interactors were identified (Data not shown).

A point mutation in I30 region of WASP (G187C) has been reported to cause XLT [96] affected WASP-Toca1 interaction (Table 3.2) which was further confirmed by His tag pull down assay (Fig. 3.21). Moreover, results from His tag pull down assay suggest that WASP contain two binding sites for Toca1 interaction; I30 region and PRR of WASP. Binding of Toca1 at two different places on WASP may have its synergistic role in Cdc42 dependent WASP activation and loss of one of these WASP-Toca1 interactions either due to deletion of I30 region or a point mutation in I30 region of WASP (G187C) might affect Cdc42-Toca1 dependent

WASP activation. Thus, these studies further suggest the importance of I30 region in regulation of WASP activity.

4.2.2 Conformation analysis of WASP^{ΔI30}

Bi-molecular fluorescence complementation assay (BiFC)

Interactions and colocalization of proteins have been studied using fluorescence resonance energy transfer (FRET) based approaches. FRET occurs when two fluorophores are separated in distances that are less than 10nm. By quantification of the quenching of donor fluorescence, protein-protein interactions can be detected in which proteins are tagged by fluorophores [110]. However, FRET suffers from several drawbacks like spectral bleed-through, photobleaching and low background to noise ratio. New methods have since been developed which do not have the shortcomings of FRET assays. Hu and coworkers [92] have developed Bi-molecular Fluorescence Complementation (BiFC) assay, where fragments of fluorescent molecules are fused to separate proteins. Upon interactions of these proteins, the fluorescence would be reconstituted. Jeong and coworkers [102] utilized the same method in which the conformation of maltose binding protein was studied using the fluorescence complementation assay. In this study, the BiFC assay was adopted to study the conformational changes in WASP using *S. cerevisiae* cells, because they do not contain any of known interactors of WASP. Thus, the effect of WASP interactors on the conformational changes of WASP can be studied by expressing WASP together with its known interactors in yeast cells. Using BiFC assay, Lim et al [125] have shown that WASP sensor

adopts an auto-inhibited closed conformation, which was further stabilized by WIP as fluorescence signal of WASP sensor was increased in the presence of WIP.

In this study, conformations of WASP^{Δ130} and WASP^{G187C} sensors were analysed using BiFC assay. The fluorescence intensities of WASP^{Δ130} and WASP^{G187C} sensors were reduced and comparable to fluorescence intensity of WASP sensor in the absence of WIP suggesting that all the three WASP sensors adopted open conformation in the absence of WIP (Fig. 3.23A & B). The YFP fluorescence intensity of WASP sensor or WASP^{Δ130} sensor or WASP^{G187C} sensor was increased in the presence of WIP. However, this increased in fluorescence intensity of WASP^{Δ130} sensor was significantly lower compared to WASP or WASP^{G187C} sensors suggesting that WASP^{Δ130} sensor exists in a partially open conformation even in the presence of WIP (Fig. 3.23A & B). The reduced fluorescence intensity of WASP^{Δ130} sensor compared to WASP sensor was neither due to its poor expression in yeast cells (Fig. 3.23C) nor due to its inability to form a functional WASP^{Δ130}-WIP complex (Fig. 3.29A) as WASP^{Δ130} was able to suppress the growth defects of *las17Δ* yeast strain. Binding of WIP has been shown to protect WASP from proteases [64,192] and this interaction stabilizes the closed conformation of WASP. WIP has been shown to inhibit the N-WASP/Cdc42 activated actin polymerization mediated by Arp2/3 complex [136]. These observations raised a question: Does WASP molecule in complex with WIP activates Arp2/3 complex and perform actin polymerization? WIP has been shown to be critical for localization of WASP in the cortical patches in *S. cerevisiae* cells. Furthermore, the VCA domain of WASP and the V domain of WIP are crucial for

suppression of the growth defect suggesting that WASP and WIP have to form a functional complex for actin polymerization in order to suppress the growth defects of *las17Δ* cells [180]. Thus, WASP can mediate Arp2/3 complex activation when WASP still exists in its closed conformation. Many groups have shown that Rho family of GTPase such as Cdc42 binds to the GBD of WASP and relieve WASP from its closed conformation [111,187]. Later, SH3 domain containing protein Toca1 was identified that can interact with both Cdc42 and N-WASP and is required for activation of N-WASP-WIP complex, leading to Arp2/3 mediated actin polymerization [89]. Another SH3 domain containing protein Nck1 has been shown to activate WASP independent to Cdc42 via its three SH3 domains and promote WASP mediated actin nucleation [167]. Toca1 and Nck1 have been shown to open up the auto-inhibitory closed conformation of WASP even in the presence of WIP [125].

Both Toca1 and Nck1 failed to relieve partially closed conformation of WASP^{ΔI30} sensor in the presence of WIP as there was no further reduction in YFP fluorescence intensity of WASP^{ΔI30} sensor observed (Fig. 3.24A & B). Although, Toca1 or Nck1 interacted with WASP^{ΔI30} (through PRR of WASP) (Fig. 3.21) but failed to alter the conformation of WASP^{ΔI30}. It is possible that Toca1 or Nck1 need to interact with both I30 region as well as PRR of WASP together at the same time in order to relieve the auto-inhibitory conformation of WASP. Nck1 but not Toca1 was able to open up the closed conformation of WASP^{G187C} sensor in the presence of WIP (Fig. 3.25A & B) which could be due to the loss of WASP-Toca1

interaction at second binding site of WASP (Table. 3.2) suggesting that Gly¹⁸⁷ is critical for WASP^{I30}-Toca1 interaction. This could explain the ability of Nck1 and failure of Toca1 to open up the closed conformation of WASP^{G187C} sensor. This result confirms the involvement of both I30 region and PRR of WASP together at the same time in the conformational changes of WASP mediated by Toca1 or Nck1. Consequences of partially opened conformation of WASP^{ΔI30} on Hck mediated WASP-Tyr²⁹¹ phosphorylation was analysed. Phosphorylation of WASP has been shown to regulate WASP activity both *in vitro* and *in vivo* [58,69]. Similar to Toca1, Hck interacts with both I30 region and PRR of WASP (Fig. 3.27) and deletion of I30 region increased Tyr²⁹¹ phosphorylation of WASP^{ΔI30} (Fig. 3.28). This might be due to partial open conformation WASP^{ΔI30} had increased the accessibility of kinases to Tyr²⁹¹ residues. This result is in concert with the previously reported WASP activating mutation L270P (identified in patients with severe congenital neutropenia) that stabilizes WASP in an open conformation [68], resulted in increased Tyr²⁹¹ phosphorylation of WASP [170]. From all these results, it can be concluded that the I30 region of WASP behaves as a regulatory region for altering WASP conformation via interacting with SH3 domain containing proteins.

4.2.3 Role of WASP^{I30} region in Jurkat T-cells functions

Deletion of I30 region of WASP or a point mutation in I30 region (WASP^{G187C}) do not affect interaction between WASP and WIP (mediated by the WH1 domain of WASP) and function of VCA domain of WASP (which is still able to bind and activate the Arp2/3 complex) determined by growth rescue experiment (Fig. 3.29).

Most importantly this result shows that both the N-terminus and C-terminus of WASP in WASP^{ΔI30} or WASP^{G187C} are still folded in the conformation required for their activity. WASP is crucial for reorganization of the actin cytoskeleton in T-lymphocytes and it plays an essential role together with WIP in T-cell chemotaxis and homing [76]. Expression of WASP^{ΔI30} did not rescue the chemotaxis of WASP^{KD} Jurkat T-cells determined by Dunn chamber assay and further confirmed with transwell migration assay (Fig. 3.30 & 3.32). Most importantly, WASP after deletion of I30 region, behaved similar to N-WASP in chemotaxis of Jurkat T-cells (defective directionality of migration without affecting migration velocity) (Fig. 3.30B & 3.31A). However, WASP^{ΔI30} expression significantly reduced the adhesion of cells to fibronectin compared to WASP or N-WASP expressing cells (Fig. 3.33). This defective chemotaxis of WASP^{ΔI30} expressing cells could be due to defective polarization of cells towards the chemokine SDF-1 α gradient. Another possible explanation for the defective chemotaxis of WASP^{ΔI30} expressing cells could be due to increase in turnover of WASP^{ΔI30} at adhesion site (leading edge), that resulted in reduced cell adhesion and random motility. The rate of WASP turnover at adhesion sites is dependent on Tyr²⁹¹ phosphorylation of WASP [132]. Deletion of I30 region destabilized the closed conformation of WASP, resulted in increased in Hck mediated Tyr²⁹¹ phosphorylation of WASP and thus, might have increased its turnover rate at adhesion sites. Perhaps, the open conformation of WASP^{ΔI30} had allowed the binding of other partners involved in WASP turnover and dissolution of adhesion sites. Podosomes in macrophages from XLN patients (due to point mutation I294T in the GTPase binding domain of WASP) are highly

dynamic in nature with high rate of turnover [5] resulted in defective migration. This was due to an open conformation of WASP^{I294T} mutant that had increased WASP-Tyr²⁹¹ phosphorylation and promoted WASP degradation, which resulted in increase in podosomes disassembly [5]. This implies that a right balance between WASP activation and degradation is necessary for regulated actin polymerization, which is essential for directional migration. Compared to WASP expressing cells, expression of WASP^{G187C} had its minimal effect on directional migration of Jurkat T-cells (Fig. 3.31A). However, this mutation has pronounced effect on adhesion of cells to fibronectin. Adhesion of WASP^{G187C} expressing cells was significantly reduced compared to WASP expressing cells (Fig. 3.33). As this mutation is associated with XLT, which has its profound effects on platelets adhesion and aggregation, therefore this mutation has minimal effects on T-cell functions. Both the WASP mutants (WASP_R^{ΔI30}-RFP or WASP_R^{G187C}-RFP) were colocalised with actin ring at the periphery of anti-CD3 (TCR) stimulated Jurkat T-cells (Fig. 3.36). But WASP^{ΔI30} expressing cells did not rescue the TCR/CD28 induced membrane projections formation defect of WASP^{KD} Jurkat T-cells (Fig. 3.35). This observation could be due to defective regulation of actin polymerization as deletion of I30 region partially open up the closed conformation of WASP or due to loss of interaction of WASP^{ΔI30} with other unknown proteins required for membrane projections formation.

Apart from defects observed in T-cell chemotaxis and adhesion to fibronectin, expression of WASP^{ΔI30} was unable to rescue the anti-CD3/anti-CD28 stimulated IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells. Surprisingly, WASP^{KD}

Jurkat T-cells expressing WASP^{G187C} were also impaired in IL-2 gene transcription (Fig. 3.34). This defect in IL-2 expression was not due to the poor adhesion and spreading of Jurkat T-cells expressing WASP^{ΔI30} or WASP^{G187C} to the plate bound anti-CD3/anti-CD28 antibodies. Abnormal IL-2 expression in WASP^{G187C} expressing cells indicating that glycine at 187 position or region comprising the Gly¹⁸⁷ is crucial for activating IL-2 gene transcription, although it able rescue the chemotaxis of WASP^{KD} Jurkat T-cells. Thus, explaining a dual role of I30 region which are independent of each other; actin cytoskeleton remodelling (which is important for chemotaxis) and IL-2 gene transcription. Role of WASP has been shown to be important for the dephosphorylation and nuclear translocation of NFATp, p-Erk nuclear translocation, Elk1 phosphorylation, and c-fos expression after TCR engagement [54,158]. The abnormalities in any of these WASP dependent processes are likely to affect IL-2 gene expression. The DNA binding activity of NFAT was found to be severely impaired in WASP^{-/-} T-cells [54]. WASP is known for its actin polymerization function in the cytoplasm. However, localization of WASP in the nucleus of human primary T_H1 differentiating cells has also been observed [212]. Nuclear WASP has been shown to bind to a subset of immune function genes such as genomic loci of *T-BET* (primary regulator of T_H1 cell fate), *RUNX3* (secondary regulator of T_H1 cell fate) and *IFNG* (T_H1 signature cytokine gene) that regulates T_H1 cell fate and T_H1-differentiating cytokine milieu [212]. All these studies suggest that WASP is regulated by many cytoplasmic factors and WASP regulates many effector molecules. Therefore, deletion of I30 region or point mutation in I30 region of

WASP (G187C) may have affected the WASP mediated effectors functions or nuclear localization of WASP effectors, which could be responsible for defective IL-2 gene expression.

Involvement of I30 region in chemotaxis was confirmed by inserting I30 region of WASP in N-WASP between its WH1 domain and basic region (Fig. 3.37). Expression of N-WASP-I30 rescued the defective chemotaxis of WASP^{KD} Jurkat T-cells towards SDF-1 α analyzed by Dunn chemotactic assay (Fig. 3.38 & 3.39) and transwell migration assay (Fig. 3.40A). Furthermore adhesion of N-WASP-I30 expressing cells to fibronectin was comparable to WASP expressing cells and significantly reduced than N-WASP expressing cells (Fig. 3.41). Thus, suggesting that I30 region of WASP alone is sufficient to mediate interactions required for chemotaxis of T-cells. Although, N-WASP is insensitive to calpain and expressed well (exogenous N-WASP) in comparison to WASP in Jurkat T-cells, expression of N-WASP-I30 was found to be slightly reduced compared to N-WASP expression (determined by immunoblotting with common His-tag added to the C-terminus of WASP or N-WASP or N-WASP-I30 gene) (Fig. 3.40B). Similar to N-WASP, N-WASP-I30 is also insensitive to calpain determined by calpain activator (CaCl₂ + Ionomycin treatment) or calpain inhibitor (calpeptin) treatment of HEK293T cells expressing WASP-His or N-WASP-His or N-WASP-I30-His respectively (Data not shown). Thus, reduced expression of N-WASP-I30 observed in Jurkat T-cells could be due to its proteasome mediated degradation, which is essential for maintaining coordination between cell migration and adhesion. Expression of N-WASP-I30 rescued the IL-2 gene transcription defect

of WASP^{KD} Jurkat T-cells (Fig. 3.42). Thus, suggesting that the region of N-WASP essential for TCR induced T-cell activation is not affected by I30 insertion and it has been inserted at the right place in N-WASP. TCR/CD28 induced actin cytoskeleton architecture changes in N-WASP-I30 expressing cells was normal (cells spread well and formed membrane projection after stimulation with anti-CD3/anti-CD28 antibodies) and behaved similar to WASP expressing cells (Fig. 3.43). Most importantly, the cellular localization pattern of N-WASP-I30-RFP after TCR stimulation was similar to WASP-RFP localization pattern (N-WASP-I30-RFP was mainly located to the actin ring at the periphery of the spread cell). In contrast, N-WASP-RFP was mainly located at the centre (nuclear) of the spread cell (Fig. 3.44). This could be due to the presence of leucine rich NES element (LPPLPLHP) in I30 region of WASP, which may have kept the N-WASP-I30 in the cytoplasm of the cell. Another possible reason for this observation may be because of binding of cytoplasmic molecules with I30 region, which may have targeted N-WASP-I30 to the periphery of the stimulated cell. Cytoplasmic localization of N-WASP-I30-RFP or WASP-RFP was observed compared to cytoplasmic and a strong nuclear localization of N-WASP-RFP when expressed in mouse embryonic myoblast cells (C2C12 cells) (Data not shown) suggesting the possibility of presence of NES element in the I30 region of WASP. Deletion of I30 region did not affect WASP^{ΔI30} localization at TCR stimulation sites, which could be mediated by PRR of WASP. As interactions through SH3 domain of tyrosine kinases and adaptor proteins has been shown to be important for localization of WASP to the TCR: APC contact site [249].

In conclusion, results from this study suggest that I30 region of WASP plays a regulatory role in WASP activation and tyrosine phosphorylation, which determines the Jurkat T-cell functions such as chemotaxis, adhesion to ECM, TCR induced activation, actin cytoskeleton remodelling and cellular localization (Fig. 4.2). Thus, it can be proposed that I30 region of WASP is one of the molecular differences between WASP and N-WASP.

Figure 4.2 Role of I30 region of WASP in Jurkat T-cell functions. WASP has a unique region of 30 amino acids which is absent in N-WASP. Deletion of I30 region altered WASP conformation and affected WASP functions in chemotaxis and IL-2 production. However, insertion of WASP-I30 region in N-WASP rescued all the functional defects of WASP Δ I30 Jurkat T-cells. Thus, I30 region of WASP provides one of the molecular differences between WASP and N-WASP.

4.3 Molecular characterization of WASP^{PRR} missense mutations causing WAS

Proline rich region of WASP (PRR) (a.a.312-417) has been shown to be important for interaction with SH3 domain containing proteins [96], which targets WASP to the immunological synapses [44] and promotes WASP activation [249]. Two point mutations at amino acid position 339 (S339Y) and 373 (P373S) have been reported to occur together on the same gene in WAS patients (WASPbase). In this study, the molecular defects of these two WASP^{PRR} missense mutations were characterized individually (WASP^{S339Y} or WASP^{P373S}) or together (WASP^{SP/YS}) for their effects on WASP mediated Jurkat T-cell functions.

WASP^{PRR} mutations either individually or together do not abolish WASP interaction with SH3 domain containing tyrosine kinases and adaptor proteins tested by yeast two hybrid assay (Table 3.4). The possible explanation of this result is that the SH3 domain containing proteins may binds to two different domains in WASP (for example Toca1; Fig. 3.21B and Hck; Fig. 3.27) or to the different motifs within the PRR of WASP. The point mutations (WASP^{SP/YS}) might affect one of these binding sites. Recently, the N-terminal region of WASP (a.a 1-170) has been shown to interact with SH3 domain of Fyn [191]. In addition, Fyn has also been shown to interact with PRR of WASP as WASP mutant lacking PRR (WASP^{ΔPro}) failed to interact with Fyn [21]. All these results suggest that WASP likely to have more than one binding sites for interaction with SH3 domain containing proteins.

Although, many pathways for WASP recruitment to the T-cell activation sites have been proposed [190.249], the recruitment of WASP via Nck1 has been well characterized. Localization of WASP^{PRR} mutants were analysed in Jurkat T-cells after TCR stimulation (anti-CD3 coated surface). WASP^{PRR} mutants do not affect the interaction of WASP with Nck1; however, localization of WASP at the TCR contact sites were disrupted (Fig. 3.61). WASP^{S339Y}-RFP is localized in the form of small clusters which might be the initial TCR contact sites, even though the cell had spread well and formed actin ring at the periphery. Thus, indicate that WASP^{S339Y} mutant can localise to the initial TCR contact sites but unable to move to the periphery of the cell, the region where cell makes maximum contacts with TCR. However, its expression rescued the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cell (Fig. 3.59) indicating that WASP^{S339Y} mutant can form functional contacts with TCR in the form of initial clusters, which has recruited and activated all the downstream effectors required for T-cell activation. The localization of WASP^{P373S}-RFP was diffused throughout the spread cell (Fig. 3.61) and this mutant was unable to rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells. WASP^{SP/YS}-RFP behaved similar to WASP^{P373S}-RFP in terms of its localization pattern at TCR contact sites and T-cell activation, which suggest the critical and dominating effect of WASP^{P373S} mutant over the WASP^{S339Y} mutant (Fig. 3.59). This might be due to the altered conformation of these mutants which have affected the WASP interactions required for localization of WASP molecules at the correct place during TCR activation.

Expression of WASP^{PRR} mutants did not rescue the fewer membrane projections defects of WASP^{KD} Jurkat T-cells when stimulated with anti-CD3/anti-CD28 antibodies coated surface (Fig. 3.60). This could be due to the defect in CD28 signalling mediated cytoskeleton architecture changes in T-cells. As engagement of CD28 without TCR stimulation can induce filopodia formation [189] and cytokines gene transcription [179]. However, to date, the correlation between these CD28 induced filopodia formation and cytokines gene transcription has not been identified. Therefore, it is difficult to determine how WASP mutants can rescue the IL-2 gene transcription defect but not the membrane projections defect (or vice versa) of WASP^{KD} Jurkat T-cells?

Conformation analysis of WASP^{PRR} mutants revealed that WASP^{S339Y} or WASP^{SP/YS} mutant adopts partially open conformation compared to WT WASP or WASP^{P373S} mutant in the presence of WIP, suggesting the involvement S339Y mutation in opening of closed conformation of WASP (Fig. 3.47 & 3.48A). Unlike WT WASP, the closed conformation of WASP^{P373S} mutant was not altered either in the presence of Toca1 or Nck1 suggesting that the mutation has affected the ability of WASP to undergo conformational change (Fig. 3.49). In contrast to WASP^{P373S} mutant, the fluorescence intensity of WASP^{S339Y} was further reduced in the presence of either Toca1 or Nck1 (Fig. 3.51 & 3.52A). The fluorescence intensity of WASP^{SP/YS} sensor was not reduced either in the presence of Toca1 or Nck1, which could be the effect of WASP^{P373S} mutation in opening of partially closed conformation of WASP^{SP/YS} sensor. Thus, suggesting that proline at

position 373 of WASP is crucial for WASP conformation changes. The altered conformation of WASP^{PRR} mutants resulted in reduced Hck mediated Tyr²⁹¹ phosphorylation (Fig. 3.53), though WASP^{PRR} mutants do not affect interaction of WASP with Hck shown by yeast two hybrid assay and pull down assay. Phosphorylation of WASP plays a critical role in WASP activation, which is indispensable for many WASP dependent processes such as proliferation, chemotaxis, assembly and disassembly of adhesion structures [30]. The consequences of decreased Tyr²⁹¹ phosphorylation of WASP^{PRR} mutants on WASP mediated Jurkat T-cell functions were analysed. All three WASP^{PRR} mutants did not rescue the chemotaxis of WASP^{KD} Jurkat T-cells towards chemokine SDF-1 α (Fig. 3.54). In addition, the directionality of migration of cells expressing WASP^{PRR} mutants was random (Fig. 3.55). The chemotactic defects observed in WASP^{PRR} mutants expressing WASP^{KD} Jurkat T-cells were neither due to loss of formation of functional WASP-WIP complex nor due to instability of WASP mutants. As WASP^{PRR} mutants were able to rescue the growth defect of *las17* Δ yeast strain (Fig. 3.46A). Impaired chemotaxis of monocytes has been observed when WASP binding to WIP was blocked using a small C-terminal region of WIP (residue 321-503) [21]. Apart from chemotactic defect, adhesion of WASP^{P373S} or WASP^{SP/YS} expressing cells was significantly reduced compared to normal adhesion of WASP or WASP^{S339Y} mutant expressing cells. Thus, suggesting the critical effect of WASP^{P373S} mutant in regulation of WASP functions (Fig. 3.58).

Figure 4.3 Effects of WASP^{PRR} mutations in regulation of WASP functions. WASP^{PRR} mutations either together or individually do not affect WASP interaction with SH3 domain containing proteins but affect formation of functional complex. These two mutations individually have their distinct or common effects on WASP mediated functions but have profound or cumulative effect when present together. Both mutants either individually or together do not rescue the chemotaxis of WASP^{KD} Jurkat T-cells which either could be due to decreased Tyr²⁹¹ phosphorylation or their altered conformation, but not due to their inability to form functional WASP-WIP complex.

From all these observations, it can be conclude that WASP^{PRR} mutants can interact with SH3 domain containing proteins, but unable to form functional complex required for WASP activities. Both the mutations (S339Y, P373S) have their individual effects on WASP mediated functions though, P373S has more severe effects. However, both mutations together have profound effects on WASP mediated T-cell functions causing WAS (Fig. 4.3).

4.4 Characterization of WASP^{WH1} missense mutations L46P and A47D in Jurkat T-cells chemotaxis

WASP is a multi-domain protein which regulates interactions with many cytoplasmic molecules leading to polymerization and reorganization of actin machinery. In 1997, Ramesh et al had identified WIP as a WASP^{WH1} domain interacting protein and later this protein has been characterized as an important regulator of WASP activity [64]. In 2006, calcium and integrin binding protein-1 (CIB1) was identified as another WASP^{WH1} domain interacting protein, its role in α Ib β 3 mediated platelet adhesion and aggregation has been well defined [225]. WH1 domain of WASP has been reported as a highly mutable region in WAS/XLT patients [96,103]. In one report, 52 WASP^{WH1} missense mutations were characterized in terms of their effect on WASP-WIP interactions, WASP stability in yeast cells and ability to rescue the growth defect of *las17* Δ yeast strain. Out of these 52 mutations analysed, 13 of them abolished formation of functional WASP-WIP complex [181]. But still it is not clear how these WASP^{WH1} missense mutations affect WASP functions leading to WAS/XLT. Therefore, library screening was performed in order to identify novel WASP^{WH1} interacting partners, but beside WIP and CIB1 no other WASP^{WH1} domain interacting partner was identified. CIB1 is a 22 kDa EF hand containing protein which is expressed ubiquitously. In this study, twenty-five WASP missense mutations were screened for their interaction with CIB1 and six missense mutations (G40V, T45M, L46P, A47D, P58L, G70W) were found to abolish WASP-CIB1 interaction (Fig. 3.63), but do not abolish WASP-WIP interaction (Fig. 3.64). The stability of these six

missense mutations were tested in WASP^{KD} Jurkat T-cells and four of them (WASP^{G40V}, WASP^{T45M}, WASP^{P58L}, WASP^{G70W}) were found to affect stability of WASP (Fig. 3.65) suggesting that WIP is not the only chaperone for WASP. The expressions of other two missense mutations (WASP^{L46P} and WASP^{A47D}) were readily detected. Recently, it has been reported that WASP is ubiquitinated at lysine 76 and 81 [184] which is in the region of WASP critical for WASP-CIB1 interaction. It is possible that both WIP and CIB1 binds to different regions in the WH1 domain of WASP and protect WASP from proteolysis.

WASP^{KD} Jurkat T-cells stably expressing WASP_R^{L46P} or WASP_R^{A47D} were impaired in chemotaxis (Fig. 3.66A & 3.68). Moreover, their migration velocities (Fig. 3.66B) and directionality were also impaired (Fig. 3.67). However, their adhesion to fibronectin was comparable to WT WASP expressing cells (Fig. 3.69) suggesting that the defect in chemotaxis observed in mutants expressing cells was not due to their altered adhesion to fibronectin. Over expression of CIB1 has been shown to decrease migration of REF52 cells on fibronectin as a result of PAK1-LIMK dependent cofilin phosphorylation [123]. In contrast, in another study, the role of CIB1 as a positive regulator of megakaryocyte migration towards SDF-1 α has been identified [116]. The expression of CIB1 has been reported to be upregulated in breast cancer tissue compared to normal tissue [149] suggesting the regulatory role of CIB1 in cell migration. Thus, the loss of interaction of WASP^{L46P} or WASP^{A47D} with CIB1 could be the possible reason for defective Jurkat T-cell chemotaxis. Binding of CIB1 to WASP relieves the auto-inhibition

suggesting that CIB1 may regulate the activity of WASP by altering WASP conformation (Data not shown). Thus, loss of WASP interaction with CIB1 (due to mutations) may have affected the CIB1 mediated WASP activation which is required for chemotaxis. Although, both mutants colocalised with actin ring at the periphery of spread cell after TCR stimulation (Fig. 3.72), expression of WASP^{A47D} but not the expression of WASP^{L46P} rescued the T-cell activation defect of WASP^{KD} Jurkat T-cells indicating that IL-2 production is not regulated by WASP-CIB1 interaction (Fig. 3.70).

Analysis of actin cytoskeleton organization revealed that both the mutants (WASP^{L46P} or WASP^{A47D}) expressing cells were impaired in actin cytoskeleton organization after stimulation with anti-CD3/anti-CD28 antibodies (Fig. 3.71). Mutants expressing cells did not spread well on anti-CD3/anti-CD28 coated coverslip compared to WASP expressing cells suggesting their dominant negative effect on cell spreading. Abundant membrane projections were observed in WASP^{L46P} expressing cells. Whereas, almost negligible membrane projections were observed in WASP^{A47D} expressing cells compared to WASP expressing cells. It could be due to the altered conformation adopted by WASP mutants which have affected CD28 signaling required for actin cytoskeleton remodeling. The role of WASP in T-cell activation is independent of its role in actin polymerization [197]. The abundant membrane projections in cells expressing WASP^{L46P} could have compromised the ability of the mutant to rescue the T-cell activation defect of WASP^{KD} Jurkat T-cells.

On the basis of degree of severity of disease, the mutations on WASP gene have been characterized in to three different categories: WAS, XLT, XLN. Patients with mutations in their WASP gene that allowed the expression of normal size WASP but in reduced amount were grouped in XLT category (mainly affect platelets functions, milder form). While the patients whose lymphocytes express either truncated WASP or no WASP expression were grouped in to WAS category (severe form). Although, this genotype-phenotype correlation is clear but is not absolute, it is difficult to predict the clinical course in individual cases based solely on the type of WASP mutation. Different patients with same mutations in their WASP gene can have different levels of WASP protein expression. In addition, somatic mosaicism has been found in more than 11% of Wiskott Aldrich Syndrome patients, in which reversion mutations restore the expression of WASP [11,62]. Thus, it is possible that a WASP mutation which has been reported to cause XLT in one patient may have severe effects in another patient. Because of the diverse phenotypes manifested in WAS, XLN and XLT patients, treatment of these patients should vary according to the molecular defect or the cause of the disease. By characterising WASP mutations in hematopoietic cells system or animal model will provide a deeper understanding of the molecular mechanism of WASP regulation which will give rise the way for individualized or personalised treatment for WAS, XLT and XLN patients.

4.5 Future Directions

In this study, experiments have been performed to characterize the WASP mutants, which are first based on the identification of molecular effects caused by mutations. Second, they are based on functional defects observed in WAS/XLT patients. Finally, experiments are performed to identify how one functional defect correlates with other. But further studies need to be performed to identify the molecular mechanism affected by the WASP mutations. For example, how WASP mutants or N-WASP affect directionality of migration without affecting the migration velocity and role of cell adhesion in chemotaxis? It is possible that cell polarization towards the gradient of chemokine source has been affected. Therefore, Jurkat T-cells expressing WASP mutants can be studied for their effects in cell polarization and recruitment of cell adhesion molecules during chemotaxis. For this assay, SDF-1 α presented by fibronectin can be used to induce the chemotaxis as described by Pelletier et al., 2000 [173]. It is possible that WASP activity in membrane projections formation and SDF-1 α induced chemotaxis may be regulated by different WASP activators. Rac1 has been shown to induce lamellipodia whereas Cdc42 induces filopodia formation. Expression of dominant negative form of Rac1 or Cdc42 along with WASP mutant in WASP^{KD} Jurkat T-cells will give rise to identify the molecules involved in regulating WASP activity during membrane projections formation or chemotaxis.

Engagement of CD28 independent of TCR signalling can induce filopodia formation and cytokines production in T-cells [179]. For optimal T-cell activation,

signals from both TCR and coreceptor (CD28) are required. Therefore, membrane projections formation in this study has been performed on anti-CD3/anti-CD28 antibodies coated coverslip. However, whether signals from CD28 receptor is enough for WASP mediated membrane projections formation and the role of these membrane projections in IL-2 production has not been identified. The role of Rho family of GTPase Cdc42 has been observed in CD28 mediated filopodia formation [189]. Defect in membrane projections formation observed in WASP^{KD} Jurkat T-cells indicate that Cdc42 could have targeted WASP (no other WASP family member) for membrane projection formation. This can be confirmed by blocking WASP-Cdc42 interaction (by expressing a small peptide of WASP comprising GBD) in Jurkat T-cells followed by stimulation with anti-CD28 antibody. The importance of WASP Tyr²⁹¹ phosphorylation in CD28 signalling can be further studied.

I30 region in WASP is one of the molecular differences between WASP and N-WASP which determines WASP specific functions in Jurkat T-cells. The importance of I30 region in other hematopoietic system or mouse model can be studied. N-WASP has been shown to rescue many cytoskeleton defects of WASP-deficient macrophages, but it was unable to rescue the defect in Fcγ-R mediated phagocytosis [97]. By expressing N-WASP-I30 in WASP-deficient macrophages, the role in I30 region in Fcγ-R mediated phagocytosis can be determined. Expression of N-WASP-I30 was lower than N-WASP in T-cells, which suggests the role of I30 region in the proteasome mediated degradation. Thus, it would be interesting to identify the role of I30 region in proteasome mediated proteolysis of

N-WASP-I30 and its influence on cell migration can be further characterized. I30 region of WASP has been shown to interact with number of tyrosine kinases and adaptor proteins. Therefore, further studies need to be done in order to determine the importance of I30-SH3 domain containing proteins interactions in T-cell functions. It is possible that beside interaction with SH3 domain containing proteins, there are other unknown proteins which might interact with I30 region of WASP and participate in signalling pathways. Similarly, it is possible that beside WIP and CIB1, the other unidentified proteins may interact with the WH1 domain of WASP and loss of these interactions due to mutations in WASP may be responsible for WAS/XLT. Although library screening has been performed in this study but, it may not be sufficient to identify the unknown interactors of WASP which either bind to WH1 domain or I30 region of WASP. Therefore, a large scale library screening need to be performed using two or more than two different cDNA libraries.

There is more than 50% sequence divergence in the PRR of WASP and N-WASP suggesting that these two proteins may interact with different sets of SH3 domain containing proteins. Thus, to elucidate the specific binding partners of WASP and N-WASP, side by side comparison of binding affinities and/or interactions with SH3 domain containing proteins can be performed.

Although, the role of CIB1 in migration of megakaryocytes towards SDF-1 α [116] and migration of CHO cells [151] have been reported, its role in T-cell chemotaxis has not been determined. The influence of WASP-CIB1 interaction is direct or

indirect on cell migration can be further studied. Thus, it would be interesting to identify the importance of WASP-CIB1 interaction in T-cell migration, either by knocking down of CIB1 expression or by blocking WASP-CIB1 interaction (by expressing dominant negative mutant of CIB1) in T-cells. Out of six WASP^{WH1} mutations, four of them (G40V, T45M, P58L, G70W) affected WASP stability however, two of them (L46P, A47D) expressed well in Jurkat T-cells. It could be possible that the four mutants due to loss of WASP-CIB1 interaction are unstable. If this is the case, then how other two mutations (L46P, A47D) which also did not bind to CIB1 escape from proteolysis? Expression of four mutations affecting WASP stability can be analysed in the presence of proteasome inhibitor.

Three point mutations (L46P, G187C, P373S) individually at three different locations (WH1 domain, basic region, PRR) in WASP are unable to rescue the IL-2 gene transcription defect of WASP^{KD} Jurkat T-cells. Are these three point mutations affecting the activity of common WASP effectors which are responsible for IL-2 production? Therefore, the activity of immediate downstream effectors or upstream regulator (responsible for IL-2 production) of WASP in WASP mutant expressing cells can be analysed.

Finally, the ability of N-WASP-I30 in rescuing the WASP-deficiency can be further studied in WASP^{KO} mice. WASP^{-/-} HSC expressing WASP or N-WASP-I30 can be transplanted in to WASP^{KO} mice or mice irradiated with γ -rays. The ability of transplanted HSCs to differentiate in to different hematopoietic lineages

can be studied. Moreover, T-cells or dendritic cells or B-cells can be isolated from HSCs transplanted mice and different functional assays (chemotaxis, activation, adhesion assay, actin cytoskeleton reorganization, cell polarization assay, podosomes formation by dendritic cells or macrophages) can be performed. WASP^{-/-} T-cells are defective in homing [76]. Thus, WASP-deficient T-cells expressing exogenous WT WASP or N-WASP-I30 can be labelled with TRITC or Alexa-488 and injected intravenously in to the WT recipient. After one hour of injection, the homing of WT WASP or N-WASP-I30 expressing T-cells to the various lymphoid organs can be determined. Similarly, other WASP mutants (WASP^{PRR} mutants or WASP^{WH1} mutants or deletion mutants) can be expressed in WASP^{-/-} HSCs. These WASP mutants expressing HSCs can be transplanted in to WASP^{KO} mice or mice irradiated with γ -rays and similar studies can be performed.

5.0 References

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WASPbase: <http://pidj.rcai.riken.jp/wasppbase/missense.htm>

Appendix 1

Plasmids used in this study

Plasmid	Description	Expression host	Reference
YEplac112	TRP1 2 μ m ORI plasmid	Yeast	Gietz et. al., 1988
YEplac181	LEU2 2 μ m ORI plasmid	Yeast	Gietz et. al., 1988
YEplac195	URA3 2 μ m ORI plasmid	Yeast	Gietz et. al., 1988
pFIV	pFIV-CMV-cop GFP	Mammalian	Results 3.0 & 4.0
PEPCG-WP-int	PEPCG-Cop-Neo with WASP promoter-SV40 intron	Mammalian	Results 3.0 & 4.0
pACT2	LEU2 vector with Gal4p DNA activation domain	Yeast	Clontech
pAS2-1	TRP1 vector with Gal4p DNA binding domain	Yeast	Clontech
WASP sensor	YEplac112 with NLS-YFP ₁₋₁₅₄ -WASP-YFP ₁₅₅₋₂₃₈	Yeast	Results 3.0 & 4.0
NLS-WIP	YEplac195 with NLS-WIP	Yeast	Results 3.0 & 4.0
NLS-Toca1	YEplac181 with NLS- Toca1	Yeast	Results 3.0 & 4.0
NLS-Nck1	YEplac181 with NLS- Nck1	Yeast	Results 3.0 & 4.0
WASP ^{Δ130} sensor	YEplac112 with NLS-YFP ₁₋₁₅₄ -WASP ^{Δ130} -YFP ₁₅₅₋₂₃₈	Yeast	Results 3.0
WASP ^{G187C} sensor	YEplac112 with NLS-YFP ₁₋₁₅₄ -WASP ^{G187C} -YFP ₁₅₅₋₂₃₈	Yeast	Results 3.0
WASP ^{S339Y} sensor	YEplac112 with NLS-YFP ₁₋₁₅₄ -WASP ^{S339Y} -YFP ₁₅₅₋₂₃₈	Yeast	Results 4.0
WASP ^{P373S} sensor	YEplac112 with NLS-YFP ₁₋₁₅₄ -WASP ^{P373S} -YFP ₁₅₅₋₂₃₈	Yeast	Results 4.0
WASP ^{SP/YS} sensor	YEplac112 with NLS-YFP ₁₋₁₅₄ -WASP ^{SP/YS} -YFP ₁₅₅₋₂₃₈	Yeast	Results 4.0
WASP-His	pFIV with WASP-His	Mammalian	Results 3.0
WASP ^{Δ130} -His	pFIV with WASP ^{Δ130} -His	Mammalian/ HEK293T	Results 3.0
WASP ₁₃₈₋₃₂₀ -His	pFIV with WASP ₁₃₈₋₃₂₀ -His	Mammalian/ HEK293T	Results 3.0
WASP ₁₃₈₋₃₂₀ ^{Δ130} -His	pFIV with WASP ₁₃₈₋₃₂₀ ^{Δ130} -His	Mammalian	Results 3.0

WASP ^{S339Y} -His	pFIV with WASP ^{S339Y} -His	Mammalian/ HEK293T	Results 4.0
WASP ^{P373S} -His	pFIV with WASP ^{P373S} -His	Mammalian/ HEK293T	Results 4.0
WASP ^{SP/YS} -His	pFIV with WASP ^{SP/YS} -His	Mammalian/ HEK293T	Results 4.0
Toca1	pFIV with Toca1	Mammalian/ HEK293T	Results 3.0
HCK-GFP	pFIV with HCK-GFP	Mammalian/ HEK293T	Results 3.0 & 4.0
WASP	YEplac112 with WASP	Yeast	Results 3.0 & 4.0
WASP ^{Δ130}	YEplac112 with WASP ^{Δ130}	Yeast	Results 3.0
WASP ^{G187C}	YEplac112 with WASP ^{G187C}	Yeast	Results 3.0
WASP ^{S339Y}	YEplac112 with WASP ^{S339Y}	Yeast	Results 4.0
WASP ^{P373S}	YEplac112 with WASP ^{P373S}	Yeast	Results 4.0
WASP ^{SP/YS}	YEplac112 with WASP ^{SP/YS}	Yeast	Results 4.0
WIP	YEplac181 with WIP	Yeast	Results 4.0
W7si (lentiviral)	pFIV-H1/U6-W7	Mammalian/ Jurkat	Results 3.0
W8si (lentiviral)	pFIV-H1/U6-W8	Mammalian/ Jurkat	Results 3.0
W7 (lentiviral)	pFIV-H1-W7	Mammalian/ Jurkat	Results 3.0
W8 (lentiviral)	pFIV-H1-W8	Mammalian/ Jurkat	Results 3.0
W7-W7 (lentiviral)	pFIV-H1-W7-W7	Mammalian/ Jurkat	Results 3.0
W8-W8 (lentiviral)	pFIV-H1-W8-W8	Mammalian/ Jurkat	Results 3.0
S1-S1 (lentiviral)	pFIV-H1-S1-S1	Mammalian/ Jurkat	Results 3.0
EBV-W7 (lentiviral)	pFIV-EBV-W7	Mammalian/ Jurkat	Results 3.0
EBV-W8	pFIV-EBV-W8	Mammalian	Results 3.0
S1 (retroviral)	pSIR-U6-S1	Mammalian/ Jurkat	Results 3.0
RFP	PEPCG-WP-int with RFP	Mammalian/ Jurkat	Results 3.0 & 4.0

WASP _R -RFP	PEPCG-WP-int with WASP _R -RFP	Mammalian/Jurkat	Results 3.0
WASP _R ^{Δ130} -RFP	PEPCG-WP-int with WASP _R ^{Δ130} -RFP	Mammalian/Jurkat	Results 3.0
WASP _R ^{G187C} -RFP	PEPCG-WP-int with WASP _R ^{G187C} -RFP	Mammalian/Jurkat	Results 3.0
WASP _R ^{S339Y} -RFP	PEPCG-WP-int with WASP _R ^{S339Y} -RFP	Mammalian/Jurkat	Results 4.0
WASP _R ^{P373S} -RFP	PEPCG-WP-int with WASP _R ^{P373S} -RFP	Mammalian/Jurkat	Results 4.0
WASP _R ^{SP/YS} -RFP	PEPCG-WP-int with WASP _R ^{SP/YS} -RFP	Mammalian/Jurkat	Results 4.0
WASP _R ^{G40V} -RFP	PEPCG-WP-int with WASP _R ^{G40V} -RFP	Mammalian/Jurkat	Results 4.0
WASP _R ^{T45M} -RFP	PEPCG-WP-int with WASP _R ^{T45M} -RFP	Mammalian/Jurkat	Results 4.0
WASP _R ^{L46P} -RFP	PEPCG-WP-int with WASP _R ^{L46P} -RFP	Mammalian/Jurkat	Results 4.0
WASP _R ^{A47D} -RFP	PEPCG-WP-int with WASP _R ^{A47D} -RFP	Mammalian/Jurkat	Results 4.0
WASP _R ^{P58L} -RFP	PEPCG-WP-int with WASP _R ^{P58L} -RFP	Mammalian/Jurkat	Results 4.0
WASP _R ^{G70W} -RFP	PEPCG-WP-int with WASP _R ^{G70W} -RFP	Mammalian/Jurkat	Results 4.0
PEPCG-WP-int-His	PEPCG-WP-int with His tag	Mammalian/Jurkat	Results 3.0
WASP _R -His	PEPCG-WP-int with WASP _R -His	Mammalian/Jurkat	Results 3.0
N-WASP-His	PEPCG-WP-int with N-WASP-His	Mammalian/Jurkat	Results 3.0
WASP _R ^{N-WASP-WH1} -His	PEPCG-WP-int with WASP _R ^{N-WASP-WH1} -His	Mammalian/Jurkat	Results 3.0
WASP ^{N-WASP-PVVCA} -His	PEPCG-WP-int with WASP ^{N-WASP-PVVCA} -His	Mammalian/Jurkat	Results 3.0
WASP _R ^{N-WASP-WH1/Δ130} -His	PEPCG-WP-int with WASP _R ^{N-WASP-WH1/Δ130} -His	Mammalian/Jurkat	Results 3.0
N-WASP ^{WASP-130} -His	PEPCG-WP-int with N-WASP ^{WASP-130} -His	Mammalian/Jurkat	Results 3.0
BD-Vector	pAS2-1	Yeast	Results 3.0 & 4.0
BD-WASP ₁₃₈₋₂₆₅	pAS2-1 with WASP ₁₃₈₋₂₆₅	Yeast	Results 3.0

BD-WASP ₁₃₈₋₂₆₅ ^{Δ130}	pAS2-1 with WASP ₁₃₈₋₂₆₅ ^{Δ130}	Yeast	Results 3.0
BD-WASP ₁₃₈₋₂₆₅ ^{G187C}	pAS2-1 with WASP ₁₃₈₋₂₆₅ ^{G187C}	Yeast	Results 3.0
BD-WASP	pAS2-1 with WASP	Yeast	Results 4.0
BD-WASP ^{S339Y}	pAS2-1 with WASP ^{S339Y}	Yeast	Results 4.0
BD-WASP ^{P373S}	pAS2-1 with WASP ^{P373S}	Yeast	Results 4.0
BD-WASP ^{SP/YS}	pAS2-1 with WASP ^{SP/YS}	Yeast	Results 4.0
BD-WASP ₃₀₆₋₄₀₇	pAS2-1 with WASP ₃₀₆₋₄₀₇	Yeast	Results 4.0
BD-WASP ₃₀₆₋₄₀₇ ^{SP/YS}	pAS2-1 with WASP ₃₀₆₋₄₀₇ ^{SP/YS}	Yeast	Results 4.0
BD-WASP ₁₋₃₂₀	pAS2-1 with WASP ₁₋₃₂₀	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅	pAS2-1 with WASP ₁₋₂₆₅	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{M6I}	pAS2-1 with WASP ₁₋₂₆₅ ^{M6I}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{L27F}	pAS2-1 with WASP ₁₋₂₆₅ ^{L27F}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{E31K}	pAS2-1 with WASP ₁₋₂₆₅ ^{E31K}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{L35H}	pAS2-1 with WASP ₁₋₂₆₅ ^{L35H}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{L39P}	pAS2-1 with WASP ₁₋₂₆₅ ^{L39P}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{G40V}	pAS2-1 with WASP ₁₋₂₆₅ ^{G40V}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{R41G}	pAS2-1 with WASP ₁₋₂₆₅ ^{R41G}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{C43W}	pAS2-1 with WASP ₁₋₂₆₅ ^{C43W}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{T45M}	pAS2-1 with WASP ₁₋₂₆₅ ^{T45M}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{L46P}	pAS2-1 with WASP ₁₋₂₆₅ ^{L46P}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{A47D}	pAS2-1 with WASP ₁₋₂₆₅ ^{A47D}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{Q52H}	pAS2-1 with WASP ₁₋₂₆₅ ^{Q52H}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{A56V}	pAS2-1 with WASP ₁₋₂₆₅ ^{A56V}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{P58L}	pAS2-1 with WASP ₁₋₂₆₅ ^{P58L}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{W64R}	pAS2-1 with WASP ₁₋₂₆₅ ^{W64R}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{G70W}	pAS2-1 with WASP ₁₋₂₆₅ ^{G70W}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{C73Y}	pAS2-1 with WASP ₁₋₂₆₅ ^{C73Y}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{V75M}	pAS2-1 with WASP ₁₋₂₆₅ ^{V75M}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{Q77G}	pAS2-1 with WASP ₁₋₂₆₅ ^{Q77G}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{S82P}	pAS2-1 with WASP ₁₋₂₆₅ ^{S82P}	Yeast	Results 4.0

BD-WASP ₁₋₂₆₅ ^{F84L}	pAS2-1 with WASP ₁₋₂₆₅ ^{F84L}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{I85T}	pAS2-1 with WASP ₁₋₂₆₅ ^{I85T}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{R86H}	pAS2-1 with WASP ₁₋₂₆₅ ^{R86H}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{Q99R}	pAS2-1 with WASP ₁₋₂₆₅ ^{Q99R}	Yeast	Results 4.0
BD-WASP ₁₋₂₆₅ ^{L105P}	pAS2-1 with WASP ₁₋₂₆₅ ^{L105P}	Yeast	Results 4.0
AD-Vector	pACT2	Yeast	Results 3.0 & 4.0
AD-WIP	pACT2 with WIP	Yeast	Results 4.0
AD-CIB	pACT2 with CIB	Yeast	Results 4.0
AD-Fyn	pACT2 with SH3 domain of Fyn	Yeast	Results 3.0 & 4.0
AD-Fgr	pACT2 with SH3 domain of Fgr	Yeast	Results 3.0 & 4.0
AD-Lyn	pACT2 with SH3 domain of Lyn	Yeast	Results 3.0 & 4.0
AD-Hck	pACT2 with SH3 domain of Hck	Yeast	Results 3.0 & 4.0
AD-Toca1	pACT2 with Toca1	Yeast	Results 3.0 & 4.0
AD-Nck1	pACT2 with Nck	Yeast	Results 3.0 & 4.0
AD-Grb2	pACT2 with Grb2	Yeast	Results 3.0 & 4.0
AD-Src	pACT2 with SH3 domain of Src	Yeast	Results 3.0 & 4.0
AD-PIK	pACT2 with SH3 domain of PIK	Yeast	Results 3.0 & 4.0
AD-Tec	pACT2 with SH3 domain of Tec	Yeast	Results 3.0 & 4.0
AD-VASP	pACT2 with VASP	Yeast	Results 3.0 & 4.0
AD-PSTPIP	pACT2 with SH3 domain of PSTPIP	Yeast	Results 3.0 & 4.0
AD-Btk	pACT2 with SH3 domain of Btk	Yeast	Results 3.0 & 4.0
AD-Lck	pACT2 with SH3 domain of Lck	Yeast	Results 3.0 & 4.0
AD-Cortactin	pACT2 with SH3 domain of Cortactin	Yeast	Results 3.0 & 4.0
AD-Abl	pACT2 with SH3 domain of Abl	Yeast	Results 3.0 & 4.0
AD-IRSp53	pACT2 with IRSp53	Yeast	Results 3.0 & 4.0
GST-WASP ₁₋₃₂₀	pGEX with GST-WASP ₁₋₃₂₀	Bacteria	Data not shown
GST-WASP ₁₋₃₂₀ ^{ΔB30}	pGEX with GST-WASP ₁₋₃₂₀ ^{ΔB30}	Bacteria	Data not shown

Appendix 2

Primers used in this study:

Primers	Sequence 5'-3'	Remarks	Reference
WAS-F	5'CAT GCC ATG GGA ATG AGT GGG GGC CCA ATG GGA G 3'	1-22 of WASP cDNA	Results 3.0 & 4.0
WAS-R	5'CGC GGA TCC GTC ATC CCA TTC ATC ATC TTC ATC TTC 3'	1480-1506 of WASP cDNA	Results 3.0 & 4.0
YFP ¹⁻¹⁵⁴ -F	5'GTCGACCTCTAGAATGGTG AGCAAGGGCGAGGAGCTG 3'	Forward primer YFP ¹⁻¹⁵⁴	Results 3.0 & 4.0
YFP ¹⁻¹⁵⁴ -R	5'CATATGTCCCATGGCTCCTC CTCCTCCGGCCATGATATAG ACGTTGTGGCTG 3'	Reverse primer YFP ¹⁻¹⁵⁴	Results 3.0 & 4.0
YFP ¹⁵⁵⁻²³⁸ -F	5'CTCGAGTGGATCCGGAGGA GGAGGAGACAAGCAGAAGA ACGGCATCAAGG	Forward primer YFP ¹⁵⁵⁻²³⁸	Results 3.0 & 4.0
YFP ¹⁵⁵⁻²³⁸ -R	5'GCGATTAAGTTGGGTAACG CCAGG 3'	Reverse primer YFP ¹⁵⁵⁻²³⁸	Results 3.0 & 4.0
WAS ^{G187C} -F	5'GGT GGA GAC CAA GGA TGC CCT CCA GTG GGT CCG 3'	Forward primer (Mutation Gly 187 Cys)	Results 3.0
WAS ^{G187C} -R	5'CGG ACC CAC TGG AGG GCA TCC TTG GTC TCC ACC 3'	Reverse primer (Mutation Gly 187 Cys)	Results 3.0
WAS ^{P373S} -F	5'CCA CCA CCA CCC CCT TCA GCT ACT GGA CGT TCT 3'	Forward primer (Mutation Pro 373 Ser)	Results 4.0
WAS ^{P373S} -R	5'AGA ACG TCC AGT AGC TGA AGG GGG TGG TGG TGG 3'	Reverse primer (Mutation Pro 373 Ser)	Results 4.0
W7si-F	5'AAAG TGA GAT GCT TGG ACG AAAA 3'	Forward primer W7 siRNA	Results 3.0 ([164])
W7si-R	5'AAAA TTTT CGT CCA AGC ATC TCA 3'	Reverse primer W7 siRNA	Results 3.0 ([164])
W8si-F	5'AAAG TCT CAG TTC TCT TCA CTCA 3'	Forward primer W8 siRNA	Results 3.0 ([164])
W8si-R	5'AAAA TGAG TGA AGA GAA CTG AGA 3'	Reverse primer W8 siRNA	Results 3.0 ([164])
W7-F	5'GAT CCC CTG AGA TGC	Forward primer W7	Results 3.0

	TTG GAC GAA AAT TCA AGA GAT TTT CGT CCA AGC ATC TCA TTT TTG 3'	shRNA	([164])
W7-R	5'AAT TCA AAA ATG AGA TGC TTG GAC GAA AAT CTC TTG AAT TTT CGT CCA AGC ATC TCA CG 3'	Reverse primer W7 shRNA	Results 3.0 ([164])
S1	5'CAA TCT CTT GAA TTG TTC AGC TGA ATT CCC TGC TTT TTA GAG TGG TCT CAT ACA G 3'	Forward primer S1 shRNA	Results 3.0
WASP _R	5'GAT CAA ATC CGG CAA GGT ATC CAA CTGA ACA AGA CCC CT 3'	1306-1344 of WASP cDNA	Results 3.0
Grb2-F	5'CATGCCATGGAAGCCATCG CCAAATATGAC 3'	Forward primer, cDNA	Results 3.0 & 4.0
Grb2-R	5'CGCGGATCCGGGTGACATA ATTGCGGGGAAAC 3'	Reverse primer, cDNA	Results 3.0 & 4.0
Lck SH-F	5'CATGCCATGGTAACTCCAG GCTTCCTGCCGA 3'	Forward primer, cDNA, SH3 domain	Results 3.0 & 4.0
Lck SH-R	5'CGCGGATCCGCTCAGGTTC TTGAAGAACCAGG 3'	Reverse primer, cDNA, SH3 domain	Results 3.0 & 4.0
Tec SH-F	5'CATGCCATGGAAGATAATA GTGAAGAAATCGTTGTAG 3'	Forward primer , cDNA, SH3 domain	Results 3.0 & 4.0
Tec SH-R	5'CGCGGATCCAACCTGGGTAC CGAAGCCTGGTG 3'	Reverse primer, cDNA, SH3 domain	Results 3.0 & 4.0
VASP-F	5'CATGCCATGGTAAGCAGCG AGACGGTCATCTGTTC	Forward primer, cDNA	Results 3.0 & 4.0
VASP-R	5'CGCGGATCCGGGAGAACCC CGCTTCCTCAG 3'	Reverse primer, cDNA	Results 3.0 & 4.0
Hck SH-F	5'CATGCCATGGGGCCTAATA GCCACAACAGCAAC 3'	Forward primer, cDNA, SH3 domain	Results 3.0 & 4.0
Hck SH-R	5'CGCGGATCCTTGAAAAACC ACTCCTCTGTCTC 3'	Reverse primer, cDNA, SH3	Results 3.0 & 4.0
Src SH-F	5'CATGCCATGGGCTTCAACT CCTCGGACACCG 3'	Forward primer, cDNA, SH3 domain	Results 3.0 & 4.0
Src SH-R	5'CGCGGATCCTCCTCAGCCT GGATGGAGTCG 3'	Reverse primer, cDNA, SH3 domain	Results 3.0 & 4.0
Fyn SH-F	5'CATGCCATGGGGACCTTGC GTACGAGAGGAG 3'	Forward primer, cDNA, SH3 domain	Results 3.0 & 4.0
Fyn SH-R	5'CGCGGATCCGTACCACTCT TCTGCCTGGATAG 3'	Reverse primer, cDNA, SH3 domain	Results 3.0 & 4.0
Btk SH-F	5'CGCGGATCCATGCCAGCAG	Forward primer,	Results 3.0 &

	CAGCACCAGTCTCC 3'	cDNA, SH3 domain	4.0
Btk SH-R	5'CCGGAATTCTCAGCCTGAC TCCGAGTCATG 3'	Reverse primer, cDNA, SH3 domain	Results 3.0 & 4.0
Abl SH-F	5'CGCGGATCCATGAATGACC CCAACCTTTTCGTTGC 3'	Forward primer, cDNA, SH3 domain	Results 3.0 & 4.0
Abl SH-R	5'CCGGAATTCTGCTGCTCAGC AGATACTCAGCG 3'	Reverse primer, cDNA, SH3 domain	Results 3.0 & 4.0
PIK SH-F	5'CATGCCATGGCTGAGGGGT ACCAGTACAGAG 3'	Forward primer, cDNA, SH3 domain	Results 3.0 & 4.0
PIK SH-R	5'CGCGGATCCGGGAGGCGAG ATTTTTTTCCTTCC 3'	Reverse primer, cDNA, SH3 domain	Results 3.0 & 4.0
Fgr SH-F	5'CATGCCATGGATAGTGGCA CCATCAGGGGTG 3'	Forward primer, cDNA, SH3 domain	Results 3.0 & 4.0
Fgr SH-R	5'CGCGGATCCAAAGTACCAC TCTTCAGCTTG 3'	Reverse primer, cDNA, SH3 domain	Results 3.0 & 4.0
Lyn SH-F	5'CATGCCATGGGACAGAGGT TTCAAATAAGATC 3'	Forward primer, cDNA, SH3 domain	Results 3.0 & 4.0
Lyn SH-R	5'CGCGGATCCGAAAACCAC TCTTCTGTTTCTAAGG 3'	Reverse primer, cDNA, SH3 domain	Results 3.0 & 4.0
N-WASP-F	5'CATGCCATGGGACATATGA GCTCCGTCCAGCAGCAGC 3'	Forward primer, N- WASP cDNA	Results 3.0
N-WASP-R	5'CCGGAATTCTCAACTCGAG TCTTCCCACTCATCATCCTC TC 3'	Reverse primer, N- WASP cDNA	Results 3.0
WaWh-F	5'GGGCCGTCGAAATCAGAGG CAAAGTGGAGACAGAC 3'	Forward primer, WASP ^{N-WASP-WH1}	Results 3.0
WaWh-R	5'TTTGCCTCTGATTTGACGG CCCAAAGGTCTGTAAC 3'	Reverse primer, WASP ^{N-WASP-WH1}	Results 3.0
WaPV-F	5'GCGCCAGGAGCCACCACCT CCACCACCATCAAG 3'	Forward primer, WASP ^{N-WASP-PVCA}	Results 3.0
WaPV-R	5'GGAGGTGGTGGCTCCTGGC GCCTCATCTCCTGC 3'	Reverse primer, WASP ^{N-WASP-PVCA}	Results 3.0
WASL-I30-R1	TCTTCATTGGCTGGTGTGGT GGTGGGGTAGCTGACCATT TGGGGGATCTCGTCTTTTC	First reverse primer, N-WASP ^{WASP-I30}	Results 3.0
WASL-I30-R2	5'GATGCAGGGGCAGGGGTG GGAGCCCTCCTCTTCTCTCTT CATTGGCTGGTGTGGTGGT 3'	Second reverse primer, N- WASP ^{WASP-I30}	Results 3.0
WASL-I30-R3	5'CATGCCATGGGTAGATTAG GGCCTCCTTGGTCTCCACCTG GATGCAGGGGCAGGGGTGGG A 3'	Third reverse primer, N- WASP ^{WASP-I30}	Results 3.0

CIB-F	5'CATGCCATGGGGGGCTCGG GCAGTCG 3'	Forward primer, CIB cDNA	Results 4.0
CIB-R	5'CGCGGATCCCAGGACAATC TTAAAGGAGCTGGC 3'	Reverse primer, CIB cDNA	Results 3.0
WAS ^{M6I} -F	5' CATG CC ATG GGA AGT GGG GGC CCA ATC GGA GGA AGG CCC GGG 3'	Forward primer (Mutation Met 6 Phe)	Results 4.0
WAS ^{L27F} -F	5' ATA CCC TCC ACC CTC TTC CAG GAC CAC GAG AAC 3'	Forward primer (Mutation Leu 27 Phe)	Results 4.0
WAS ^{L27F} -R	5' GTT CTC GTG GTC CTG GAA GAG GGT GGA GGG TAT 3'	Reverse primer (Mutation Leu 27 Phe)	Results 4.0
WAS ^{E31K} -F	5' CTC CTC CAG GAC CAC AAG AAC CAG CGA CTC TTT 3'	Forward primer (Mutation Glu 31 Lys)	Results 4.0
WAS ^{E31K} -R	5' AAA GAG TCG CTG GTT CTT GTG GTC CTG GAG GAG 3'	Reverse primer (Mutation Glu 31 Lys)	Results 4.0
WAS ^{L35H} -F	5' CAC GAG AAC CAG CGA CAC TTT GAG ATG CTT GGA 3'	Forward primer (Mutation Leu 35 His)	Results 4.0
WAS ^{L35H} -R	5' TCC AAG CAT CTC AAA GTG TCG CTG GTT CTC GTG 3'	Reverse primer (Mutation Leu 35 His)	Results 4.0
WAS ^{L39P} -F	5' CGA CTC TTT GAG ATG CCT GGA CGA AAA TGC TTG 3'	Forward primer (Mutation Leu 39 Pro)	Results 4.0
WAS ^{L39P} -R	5' CAA GCA TTT TCG TCC AGG CAT CTC AAA GAG TCG 3'	Reverse primer (Mutation Leu 39 Pro)	Results 4.0
WAS ^{G40V} -F	5' CTC TTT GAG ATG CTT GTA CGA AAA TGC TTG ACG 3'	Forward primer (Mutation Gly 40 Val)	Results 4.0
WAS ^{G40V} -R	5' CGT CAA GCA TTT TCG TAC AAG CAT CTC AAA GAG 3'	Reverse primer (Mutation Gly 40 Val)	Results 4.0
WAS ^{R41G} -F	5' TTT GAG ATG CTT GGA GGA AAA TGC TTG ACG CTG 3'	Forward primer (Mutation Arg 41 Gly)	Results 4.0
WAS ^{R41G} -R	5' CAG CGT CAA GCA TTT TCC TCC AAG CAT CTC AAA 3'	Reverse primer (Mutation Arg 41 Gly)	Results 4.0
WAS ^{C43W} -F	5' ATG CTT GGA CGA AAA TGG TTG ACG CTG GCC ACT 3'	Forward primer (Mutation Cys 43 Trp)	Results 4.0

WAS ^{C43W} -R	5' AGT GGC CAG CGT CAA CCA TTT TCG TCC AAG CAT 3'	Reverse primer (Mutation Cys 43 Trp)	Results 4.0
WAS ^{T45M} -F	5' GGA CGA AAA TGC TTG ATG CTG GCC ACT GCA GTT 3'	Forward primer (Mutation Thr 45 Met)	Results 4.0
WAS ^{T45M} -R	5' AAC TGC AGT GGC CAG CAT CAA GCA TTT TCG TCC 3'	Reverse primer (Mutation Thr 45 Met)	Results 4.0
WAS ^{L46P} -F	5' CGA AAA TGC TTG ACG CCG GCC ACT GCA GTT GTT 3'	Forward primer (Mutation Leu 46 Pro)	Results 4.0
WAS ^{L46P} -R	5' AAC AAC TGC AGT GGC CGG CGT CAA GCA TTT TCG 3'	Reverse primer (Mutation Leu 46 Pro)	Results 4.0
WAS ^{A47D} -F	5' AAA TGC TTG ACG CTG GAC ACT GCA GTT GTT CAG 3'	Forward primer (Mutation Ala 47 Asp)	Results 4.0
WAS ^{A47D} -R	5' CTG AAC AAC TGC AGT GTC CAG CGT CAA GCA TTT 3'	Reverse primer (Mutation Ala 47 Asp)	Results 4.0
WAS ^{Q52H} -F	5' GCC ACT GCA GTT GTT CAC CTG TAC CTG GCG CTG 3'	Forward primer (Mutation Gln 52 His)	Results 4.0
WAS ^{Q52H} -R	5' CAG CGC CAG GTA CAG GTG AAC AAC TGC AGT GGC 3'	Reverse primer (Mutation Gln 52 His)	Results 4.0
WAS ^{A56V} -F	5' GTT CAG CTG TAC CTG GTG CTG CCC CCT GGA GCT 3'	Forward primer (Mutation Ala 56 Val)	Results 4.0
WAS ^{A56V} -R	5' AGC TCC AGG GGG CAG CAC CAG GTA CAG CTG AAC 3'	Reverse primer (Mutation Ala 56 Val)	Results 4.0
WAS ^{P58L} -F	5' CTG TAC CTG GCG CTG CTC CCT GGA GCT GAG CAC 3'	Forward primer (Mutation Pro 58 Leu)	Results 4.0
WAS ^{P58L} -R	5' GTG CTC AGC TCC AGG GAG CAG CGC CAG GTA CAG 3'	Reverse primer (Mutation Pro 58 Leu)	Results 4.0
WAS ^{W64R} -F	5' CCT GGA GCT GAG CAC AGG ACC AAG GAG CAT TGT 3'	Forward primer (Mutation Trp 64 Arg)	Results 4.0
WAS ^{W64R} -R	5' ACA ATG CTC CTT GGT CCT GTG CTC AGC TCC AGG 3'	Reverse primer (Mutation Trp 64 Arg)	Results 4.0
WAS ^{G70W} -	5' ACC AAG GAG CAT TGT	Forward primer	Results 4.0

F	TGG GCT GTG TGC TTC GTG 3'	(Mutation Gly 70 Trp)	
WAS ^{G70W} -R	5' CAC GAA GCA CAC AGC CCA ACA ATG CTC CTT GGT 3'	Reverse primer (Mutation Gly 70 Trp)	Results 4.0
WAS ^{C73Y} -F	5' CAT TGT GGG GCT GTG TAC TTC GTG AAG GAT AAC 3'	Forward primer (Mutation Cys 73 Tyr)	Results 4.0
WAS ^{C73Y} -R	5' ATT ATC CTT CAC GAA GTA CAC AGC CCC ACA ATG 3'	Reverse primer (Mutation Cys 73 Tyr)	Results 4.0
WAS ^{V75M} -F	5' GGG GCT GTG TGC TTC ATG AAG GAT AAC CCC CAG 3'	Forward primer (Mutation Val 75 Met)	Results 4.0
WAS ^{V75M} -R	5' CTG GGG GTT ATC CTT CAT GAA GCA CAC AGC CCC 3'	Reverse primer (Mutation Val 75 Met)	Results 4.0
WAS ^{D77G} -F	5' GTG TGC TTC GTG AAG GGT AAC CCC CAG AAG TCC 3'	Forward primer (Mutation Asp 77 Gly)	Results 4.0
WAS ^{D77G} -R	5' GGA CTT CTG GGG GTT ACC CTT CAC GAA GCA CAC 3'	Reverse primer (Mutation Asp 77 Gly)	Results 4.0
WAS ^{S82P} -F	5' GAT AAC CCC CAG AAG CCC TAC TTC ATC CGC CTT 3'	Forward primer (Mutation Ser 82 Pro)	Results 4.0
WAS ^{S82P} -R	5' AAG GCG GAT GAA GTA GGG CTT CTG GGG GTT ATC 3'	Reverse primer (Mutation Ser 82 Pro)	Results 4.0
WAS ^{F84L} -F	5' CCC CAG AAG TCC TAC CTC ATC CGC CTT TAC GGC 3'	Forward primer (Mutation Phe 84 Leu)	Results 4.0
WAS ^{F84L} -R	5' GCC GTA AAG GCG GAT GAG GTA GGA CTT CTG GGG 3'	Reverse primer (Mutation Phe 84 Leu)	Results 4.0
WAS ^{I85T} -F	5' CAG AAG TCC TAC TTC ACC CGC CTT TAC GGC CTT 3'	Forward primer (Mutation Ile 85 Thr)	Results 4.0
WAS ^{I85T} -R	5' AAG GCC GTA AAG GCG GGT GAA GTA GGA CTT CTG 3'	Reverse primer (Mutation Ile 85 Thr)	Results 4.0
WAS ^{R86H} -F	5' AAG TCC TAC TTC ATC CAC CTT TAC GGC CTT CAG 3'	Forward primer (Mutation Arg 86 His)	Results 4.0
WAS ^{R86H} -R	5' CTG AAG GCC GTA AAG GTG GAT GAA GTA GGA CTT	Reverse primer (Mutation R 86 H)	Results 4.0

WAS ^{Q99R} -F	5' CGG CTG CTC TGG GAA CGG GAG CTG TAC TCA CAG 3'	Forward primer (Mutation Gln 99 Arg)	Results 4.0
WAS ^{Q99R} -R	5' CTG TGA GTA CAG CTC CCG TTC CCA GAG CAG CCG 3'	Reverse primer (Mutation Gln 99 Arg)	Results 4.0
WAS ^{L105P} -F	5' GAG CTG TAC TCA CAG CCT GTC TAC TCC ACC CCC 3'	Forward primer (Mutation Leu 105 Pro)	Results 4.0
WAS ^{L105P} -R	5' GGG GGT GGA GTA GAC AGG CTG TGA GTA CAG CTC 3'	Reverse primer (Mutation Leu 105 Pro)	Results 4.0
IL-2 F	5'CAAACCTCTGGAGGAAGTG CT 3'	Real time PCR	Results 3.0 & 4.0
IL-2 R	5'- AATGGTTGCTGTCTCATCAGC 3'	Real time PCR	Results 3.0 & 4.0
MRPL27-F	5'CTGGTGGCTGGAATTGACC GCTA 3'	Real time PCR	Results 3.0 & 4.0
MRPL27-R	5'CAAGGGGATATCCACAGAG TACCTTG 3'	Real time PCR	Results 3.0 & 4.0
hWASrt-F	5'CTGTGTGCTTCGTGAAGGA TAA 3'	Real time PCR	Results 3.0
hWASrt-R	5'TCGTCTGCAAAGTTCAGCC C 3'	Real time PCR	Results 3.0
h-NWASPrF	5'AAGGATGGGAAACTATTGT GGGA 3'	Real time PCR	Results 3.0
h-NWASPrF	5'GACGGCCCAAAAGGTCTGT AA 3'	Real time PCR	Results 3.0
m-GAPDHrt-F	5'CCCTCACAATTTCCATCCCA 3'	Real time PCR	Results 3.0
m-GAPDHrt-F	5'TCCCTAGGCCCTCCTGTTA 3'	Real time PCR	Results 3.0
mWASrt-F	5'CCAGCCGTTTCAGCAGAACA T 3'	Real time PCR	Results 3.0
mWASrt-R	5'GGTTATCCTTCACGAAGCA CA 3'	Real time PCR	Results 3.0

Appendix 3

Protein sequence of WASP

```
1  msggpmggrp  ggrgapavqq  nipstllqdh  enqrlfemlg  rkcltlatav  vqlylalppg
61  aehwtkehcg  avcfvkdnpg  ksyfirlygl  qagrllweqe  lysqlvystp  tpffhtfagd
121  dcqaglnfad  edeaqafral  vqekiqrnrq  rqsqdrqlp  ppptpaneer  rgglpplplh
181  pggdqqgppv  gpplslglatv  diqnpditss  ryrqlpapgp  spadkkrsqk  kkiskadiga
241  psgfkvhshv  gwdpqngfdv  nnldpdlrsl  fsragiseaq  ltaetskli  ydfiedqggl
301  eavrqemrrq  eplpppppps  rggnqlprpp  ivggnkgrsg  plppvplgia  pppptprgpp
361  ppgrggpppp  pppatgrsgp  lpppppgagg  ppmppppppp  ppppssgngp  appplppalv
421  pagglapggg  rgalldqirg  giqlnktpga  pessalqppp  qsseglvgal  mhvmqkrsra
481  ihssdegedq  agdededew  dd
```

cDNA sequence of WASP

```
1  atgagtgggg  gccaatggg  aggaaggccc  gggggccgag  gagcaccagc  ggttcagcag
61  aacataccct  ccaccctcct  ccaggaccac  gagaaccagc  gactctttga  gatgcttgga
121  cgaaaatgct  tgacgctggc  cactgcagtt  gttcagctgt  acctggcgct  gccccctgga
181  gctgagcact  ggaccaagga  gcattgtggg  gctgtgtgct  tcgtgaagga  taacccccag
241  aagtccctact  tcatccgcct  ttacggccct  caggctggtc  ggctgctctg  ggaacaggag
301  ctgtactcac  agcttgtcta  ctccaccccc  acccccttct  tccacacctt  cgctggagat
361  gactgccaag  cggggctgaa  ctttgacagc  gaggacgagg  cccaggcctt  ccgggcccctc
421  gtgcaggaga  agatacaaaa  aaggaatcag  aggcaaagtg  gagacagacg  ccagctaccc
481  ccaccaccaa  caccagccaa  tgaagagaga  agaggagggc  tcccacccct  gcccctgcat
541  ccagggtggg  accaaggagg  ccctccagtg  ggtccgctct  ccctggggct  ggcgacagtg
601  gacatccaga  accctgacat  cacgagttca  cgataccgtg  ggctcccagc  acctggacct
661  agcccagctg  ataagaaacg  ctccagggaag  aagaagatca  gcaaagctga  tattggtgca
721  cccagtggat  tcaagcatgt  cagccacgtg  ggggtgggacc  cccagaatgg  atttgacgtg
781  aacaacctcg  acccagatct  gcggagtctg  ttctccaggg  caggaatcag  cgaggcccag
841  ctccacgacg  ccgagacctc  taaacttata  tacgacttca  ttgaggacca  ggggtgggctg
901  gaggtgtgtc  ggcaggagat  gagggccag  gagccacttc  cgccgcccc  accgccatct
961  cgaggaggga  accagctccc  ccggccccc  attgtggggg  gtaacaagg  tcgttctggt
1021  ccactgcccc  ctgtaccttt  ggggattgcc  ccacccccac  caacaccccg  gggacccccca
1081  cccccaggcc  gagggggccc  tccaccacca  cccctccag  ctactggacg  ttctggacca
1141  ctgccccctc  cccccctgg  agctggtggg  ccacccatgc  caccaccacc  gccaccaccg
1201  ccaccgccc  ccagctccgg  gaatggacca  gcccctccc  cactccctcc  tgcctctggtg
1261  cctgcccggg  gcctggcccc  tgggtggggg  cggggagcgc  ttttgatga  aatccggcag
1321  ggaattcagc  tgaacaagac  ccctggggcc  ccagagagct  cagcgctgca  gccaccacct
1381  cagagctcag  agggactggt  gggggccctg  atgcacgtga  tgcagaagag  aagcagagcc
1441  atccactcct  ccgacgaagg  ggaggaccag  gctggcgatg  aagatgaaga  tgatgaatgg
1501  gatgactga
```