

# The Wiskott-Aldrich syndrome: The actin cytoskeleton and immune cell function

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**Abstract.** Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive primary immunodeficiency characterised by immune dysregulation, microthrombocytopenia, eczema and lymphoid malignancies. Mutations in the WAS gene can lead to distinct syndrome variations which largely, although not exclusively, depend upon the mutation. Premature termination and deletions abrogate Wiskott-Aldrich syndrome protein (WASp) expression and lead to severe disease (WAS). Missense mutations usually result in reduced protein expression and the phenotypically milder X-linked thrombocytopenia (XLT) or attenuated WAS [1–3]. More recently however novel activating mutations have been described that give rise to X-linked neutropenia (XLN), a third syndrome defined by neutropenia with variable myelodysplasia [4–6]. WASp is key in transducing signals from the cell surface to the actin cytoskeleton, and a lack of WASp results in cytoskeletal defects that compromise multiple aspects of normal cellular activity including proliferation, phagocytosis, immune synapse formation, adhesion and directed migration.

**Keywords:** Wiskott-Aldrich syndrome, actin polymerization, lymphocytes, dendritic cells, migration, cell activation, immune cell function

## Abbreviations

AGM:	Aorto-gonad-mesonephros
ARPC:	aActin related protein complex component
CRIB:	Cdc42/Rac interactive binding
DC:	Dendritic cells
EVH1:	Ena-VASP homology domain 1
FRET:	Fluorescence Resonance Energy Transfer
GBD:	GTPase binding domain
GEF:	Guanine exchange factor
IS:	Immune synapse
MZB:	Marginal zone B cells
N-WASp:	Neuronal WASp
PH:	Pleckstrin homology
PIP <sub>2</sub> :	Phosphatidylinositol (4,5)-bisphosphate
PLC $\gamma$ :	Phospholipase C gamma
PSTPIP:	Proline, serine threonine-rich phosphatase interacting protein

SNX9:	Sorting nexin 9
TCR:	T cell receptor
Toca-1:	Transducer of Cdc42-dependent actin assembly-1
Treg:	Regulatory T cell
VCA:	Verprolin homology, central and acidic regions
WAS:	Wiskott-Aldrich syndrome
WAS KO:	WAS knockout
WASH:	WASp and SCAR homolog
WASp:	WAS protein
WAVE:	WASp family verprolin-homologous protein
WHAMM:	WASP homolog associated with actin, membranes, and microtubules
WIP:	WASp interacting protein
XLN:	X-linked neutropenia
XLT:	X-linked thrombocytopenia

## 1. Introduction

Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive primary immunodeficiency characterised by immune dysregulation, microthrombocytopenia, eczema and lymphoid malignancies. Mutations in the WAS gene can lead to distinct syndrome variations which largely, although not exclusively, depend upon the mutation. Premature termina-

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tion and deletions abrogate Wiskott-Aldrich syndrome protein (WASp) expression and lead to severe disease (WAS). Missense mutations usually result in reduced protein expression and the phenotypically milder X-linked thrombocytopenia (XLT) or attenuated WAS [1–3]. More recently however novel activating mutations have been described that give rise to X-linked neutropenia (XLN), a third syndrome defined by neutropenia with variable myelodysplasia [4–6]. WASP is key in transducing signals from the cell surface to the actin cytoskeleton, and a lack of WASp results in cytoskeletal defects that compromise multiple aspects of normal cellular activity including proliferation, phagocytosis, immune synapse formation, adhesion and directed migration.

## 2. Molecular structure

WASp is a multidomain protein that lacks intrinsic catalytic activity. It acts as an adaptor to bring together downstream mediators that facilitate Arp2/3-mediated actin polymerization. WASp consists of an N-terminal Ena-VASP homology domain 1 (EVH1), a basic domain, a GTPase binding domain (GBD), polyproline domain and the C-terminal domain comprising of a cluster of verprolin homology (V), central (C) and acidic regions (A) (the VCA domain) (Fig. 1). WASp was the first member of a family of proteins that shares the ability to regulate Arp2/3 activity through their VCA domains. The other mammalian members (Fig. 1) that share expression of the VCA domain include neural WASp (N-WASp), WASp family verprolin-homologous protein (WAVE) 1–3 and the more recently discovered Wiskott-Aldrich syndrome protein and SCAR homolog (WASH) and WASP homolog associated with actin, membranes, and microtubules (WHAMM) [7–9]. Many studies have investigated isolated protein domains to gain insight into the function of WASp family members and have extrapolated findings of one member to predict function of another. In particular, studies of N-WASp have been used to predict function of WASp. The overall protein sequence identity between WASp and N-WASp is around 50%, although sequence of the functional domain is much higher. Importantly, however, expression of WASp is restricted to haematopoietic cells while N-WASp is ubiquitously expressed [10,11].

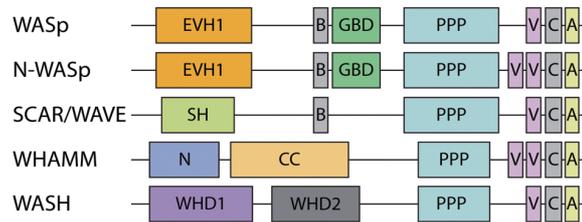


Fig. 1. Domain structure of WASp family protein members. Schematic representation of the protein domains of individual members of the WASp family. The C terminus, which is critical for regulation of actin polymerization, is highly conserved between the members, while the N terminus is divergent. EVH1, Ena Vasp homology domain; B, basic domain; GBD, GTPase binding domain; PPP, polyproline domain; V, verprolin homology domain; C, central domain; A, acidic domain; SH, Scar homology domain; N, N-terminal domain with insignificant homology to known protein; CC, coiled-coils domain; WHD1/2, WASH homology domain 1 and 2.

## 3. EVH1 and WIP

The EVH1 domain is a proline-rich region that has been implicated in actin remodelling. This domain is found in actin scaffold proteins, such as Mena, VASP and Evl. The best characterised EVH1 domain binding partners are members of the verprolin family of proteins. These include WASp-interacting protein (WIP), CR16 and WICH (WIP and CR16 homologous protein, also referred to as WIRE) [12]. Of these, WIP is probably the best characterized. It is a 503-amino acid long proline-rich protein, which is expressed in many tissues although most abundantly in haematopoietic cells [13]. While CR16 is known to be able to bind the WASp EVH1 domain, it is not expressed in haematopoietic cells; therefore, CR16/WASp interactions are not likely to be physiologically relevant [14]. WICH shows some ability to bind WASp, but this binding is much weaker compared to N-WASp binding. WICH is expressed at much lower levels in haematopoietic tissues compared to brain and gastrointestinal tissues, suggesting that N-WASp is the preferred binding partner to WICH [15].

Amino acids 416–488 of WIP were initially identified as being required for WASp binding [16]. The crystal structure of WIP bound to the EVH1 domain of N-WASp shows that a 25 amino acid fragment (aa 461–485) is minimally required for high affinity N-WASp binding [17]. Later the same group showed that for optimal binding, a larger sequence of WIP (aa 451–485) wraps around the EVH1 domain of N-WASp; they also identified three WIP epitopes that are involved in WIP/N-WASp binding [18]. Many studies have used N-WASp to study the interaction of WASp with WIP. It is likely that these interactions are similar, but that

the WIP/WASp interaction is more relevant *in vivo* given the similar tissue expression profiles. WIP inhibits N-WASp-mediated activation of Arp2/3 by direct interaction with N-WASp [19], and WIP maintains the autoinhibitory conformation of WASp *in vivo* [20]. A similar inhibitory mechanism exists for N-WASp, in which the WIP/N-WASp complex cannot initiate actin polymerization whereas free N-WASp can induce actin polymerization [21]. Toca-1 (transducer of Cdc42-dependent actin assembly) could release WIP binding, enabling Cdc42 to activate the protein and initiate actin polymerization [21].

Compelling evidence that WIP binding is important for WASp expression was provided by several groups who have shown that when WIP is absent, no or very low levels of WASp expression can be detected [22–24]. Furthermore, reduced levels of WASp expression in lymphocytes occur in WAS patients who carry mutations that abrogate WIP binding [23]. Interestingly, the majority of missense WASp mutations that give rise to clinical disease are located in the EVH1 domain. Several of these mutations (e.g. T45M, V75M, R86H, Y107C and A134T) result in reduced binding affinity for WIP [17,25,26] and are associated with reduced WASp expression, but normal mRNA levels. These observations suggest that WASp levels are low due to proteolysis as a consequence of altered WIP binding [1, 3,27,28]. WASp expression in lymphocytes of these patients could be partially restored by proteasome inhibition [23]. Together this evidence strongly suggests a role for WIP in protecting WASp from degradation. While N-WASp degradation appears mediated by ubiquitination [29], WASp degradation seems a complex mechanism, which may be dependent on phosphorylation or activation state. WASp degradation can be inhibited either by blocking the proteasome in unstimulated human and murine T cells [23] and murine splenocytes [30], or by blocking calpain proteases in stimulated human platelets [31,32], stimulated human T cells [23] and GM-CSF/TGF- $\beta$ -cultured splenic murine dendritic cells (DC) [22]. An attractive hypothesis is that the proteasome is responsible for background turnover of WASp degradation and that calpain proteases are responsible for WASp degradation in response to activation and cell surface signalling, but further mechanistic investigation is required.

In addition, WIP binding mediates recruitment of WASp to podosomes [22,33], the phagocytic cup [34] and to the T cell receptor signalling complex after T cell stimulation [23,35,36]. Similarly, WIP binding to N-WASp is required for the actin-based intracellular mo-

bility of vaccinia virus [37]. These observations suggest that by binding to WASp, WIP prevents degradation of WASp, keeps it in an inactive state and helps to achieve correct subcellular localization, thus providing an important spatial and temporal regulation of WASp activity.

#### 4. Basic domain and PIP<sub>2</sub>

The basic domain is found in all members of the WASp family, although the specific sequence is poorly conserved. In WASp, the basic domain consists of 10 amino acids, of which six are lysine residues. The basic domain of N-WASp binds phosphatidylinositol (4,5)-bisphosphate (PIP<sub>2</sub>) *in vitro* [38,39]. WASp can also bind PIP<sub>2</sub>, but the specific binding site remains unclear. Higgs and Pollard reported that a region encompassing the basic and GBD domains did not bind PIP<sub>2</sub>, in contrast to full length WASp [40]. Others have suggested that an N-terminal pleckstrin homology (PH) domain in WASp or N-WASp is responsible for PIP<sub>2</sub> binding [11,41]. However, the sequence of such a PH domain shows weak alignment with the consensus PH domain sequence and overlaps with the EVH1 domain, raising the question whether this domain should be called a PH domain [42]. Despite low sequence homology, PIP<sub>2</sub> binding and membrane localization were demonstrated for both WASp and N-WASp [11,41,43]. When the clinical C43W mutation was introduced into a truncated GST-WASp construct that contains the proposed PH domain, PIP<sub>2</sub> binding was diminished. This observation suggests functionality of the PH domain [41], although it should be noted that the C43W mutation likely affects binding of WIP as well [44]. Binding to WIP is important for N-WASp subcellular localization, and the corresponding C35W mutation in N-WASp abolished WIP-dependent recruitment of N-WASp to intracellular vaccinia virus [37].

Deletion of the basic domain of N-WASp diminished the ability of N-WASp to induce actin-driven motility of coated beads and actin polymerization in cell lysates. By contrast, activity in a purified protein system containing Arp2/3 complex and monomeric actin was increased, suggesting that additional binding partners mediate the basic domain regulation of N-WASp activity [45]. One of these binding partners is likely to be Cdc42. Positively charged residues of the basic domain contribute to Cdc42 binding, through an electrostatic steering mechanism that mediates active Cdc42 to bind the GBD domain [46]. In addition, the ba-

sic domain of N-WASp can bind the Arp2/3 complex, probably by contributing to the closed, autoinhibitory conformation of cytosolic N-WASp. PIP<sub>2</sub> binding could release this binding, enabling full activation by Cdc42 [38]. Whether this is also the case for WASp remains to be investigated.

## 5. GBD and activation by Cdc42 and phosphorylation

The GTPase binding domain (GBD), which spans from residue 235 to 288 in WASp, can bind Cdc42 and to a lesser degree Rac1 [12,47–49]. Contained within the GBD is the Cdc42/Rac interactive binding (CRIB) domain, a highly conserved sequence of 14 amino acid residues found in Rho type GTPase binding proteins [50,51]. The affinity of GTP-bound Cdc42 to WASp is at least 500-fold higher compared to the GDP-bound form [48] and its binding is important for WASp activation.

A second important binding partner of the GBD is the intrinsic WASp VCA domain. In its native, inactive state, residues 482–492 within the VCA domain bind to residues 242–310 at the carboxyl end of the GBD, as well as the adjacent sequence between GBD and the polyproline domain [52,53]. This intramolecular binding, which has also been observed for N-WASp [39], inhibits the binding of Arp2/3 to the VCA domain, thereby keeping the protein in an inactive, autoinhibited conformation that prevents actin polymerization. *In vivo* studies confirmed the autoinhibited conformation of WASp and N-WASp using Fluorescence Resonance Energy Transfer (FRET) techniques [20,54] and are facilitated by a recently developed antibody that specifically recognizes the open conformation [55,56]. Tyrosine residue 291, within the GBD (aa 256 in N-WASp), is thought to be important in stabilising the open molecular conformation. Y291 can be phosphorylated by a variety of kinases from the Src and Tec kinase families. Although the ability to induce Arp2/3-mediated actin polymerization increases upon phosphorylation, more potent actin polymerization is observed when either GTP-Cdc42 or SH2 and SH3-domain-containing proteins are bound. Full potential activity is achieved when Cdc42 and SH2/SH3 binding act synergistically on phosphorylated WASp [57]. A model has been proposed in which GTP-Cdc42 disrupts the autoinhibited conformation of WASp, allowing Y291 to be phosphorylated; this stabilises the opened molecular conformation and enables maximum actin polymerization [52,

53,57–59]. Other studies, however, have shown that partial deletion of the GBD (but not Y291) and consequently loss of GTP-Cdc42 binding, does not affect WASp activity, suggesting phosphorylation as an alternative Cdc42-independent mechanism of WASp activation [60,61].

Phosphorylation of WASp is important for many cell processes including TCR signalling, filopodia and podosome formation, migration and phagocytosis, and appears to be a key regulatory mechanism of WASp activation. Phosphorylation of N-WASp is thought to regulate protein activation in a similar fashion. Several studies have examined the importance of phosphorylation by mutating the WASp Y291 to a ‘phospho-dead’ phenylalanine (Y291F) or to a phospho-mimetic glutamic acid (Y291E). While variability exists depending on the experimental system, expression of WASp Y291F reduces the ability of WASp to induce actin polymerization and formation of phagocytic cups, filopodia or podosomes [34,56,60,62]. FRET showed that the autoinhibited molecular conformation of WASp Y291F is disrupted by Cdc42 activation, suggesting phosphorylation is not required for WASp activation, although the actin polymerization ability was not investigated [56]. Recently, knock-in mice were generated to express the murine equivalent (Y293F) of the human Y291F WASp mutant. These mice show a strikingly similar cellular phenotype to WASp deficient mice including reduced actin polymerization, podosome formation, migration and phagocytosis [30]. In contrast, introducing the phospho-mimetic Y291E showed normal cellular and protein function in some experimental systems, but enhanced actin polymerization and formation of filopodia and podosome structures in other systems [34,56,62]. Interestingly, mice expressing the phospho-mimetic Y293E phenotypically resemble WASp deficient animals due to decreased protein expression levels, which could be partly restored by inhibition of the proteasome [30]. Phosphorylation of Y256 by kinases such as Arg [63] is predicted to stabilise the ‘opened’ molecular conformation of N-WASp. Experiments using Y256 N-WASp mutants indeed show enhanced actin polymerization with a phospho-mimetic Y256E and reduced actin polymerization with a ‘phospho-dead’ Y256F mutant [64].

## 6. Polyproline domain

The polyproline domain is the largest of the functional domains of WASp. It contains multiple binding

Table 1  
WASp polyproline domain binding partners

Partner	Known function	References
Tyrosine kinases		
Btk	Binds and phosphorylates WASp.	[62,67,70–72]
Hck	Binds and phosphorylates WASp.	[62,74]
Fyn	Binds and phosphorylates WASp.	[60,68,69,73]
Lyn	Binds and phosphorylates WASp.	[72]
c-Src	Binding to WASp demonstrated.	[69,80,211]
c-Fgr	Binding to WASp demonstrated.	[69,73,80]
Tec	Binding to WASp demonstrated.	[70,212]
Itk	Binding to WASp demonstrated.	[70,212]
Sh2-SH3 adaptor molecules		
Nck	Binding and recruiting WASp to signalling complexes.	[73,75]
Grb2	Binding and recruiting WASp to signalling complexes.	[32,69–71,75]
p85 $\alpha$	Direct and indirect binding to WASp, recruitment to signalling complexes.	[69,80,81]
CrkL	Binding to WASp demonstrated.	[79]
Actin binding molecules		
profilin	Binds WASp and enhances Arp2/3-mediated actin polymerization.	[86]
cortactin	Binds WASp and enhances Arp2/3-mediated actin polymerization.	[213]
VASP	Binds WASp and enhances Arp2/3-mediated actin polymerization.	[65]
Other		
PLC $\gamma$	Binding to WASp demonstrated.	[69–71,75,80]
PSTPIP	Binds WASp and allows dephosphorylation by recruiting phosphatase.	[60,79,82–84,211]
SNX9	Binding and recruiting WASp to signalling complexes.	[81]

Btk, bruton's tyrosine kinase; Grb2, growth factor receptor-bound protein; Hck, haematopoietic cell kinase; Itk, IL-2-inducible T cell kinase; Nck, non-catalytic region of tyrosine kinase; PLC $\gamma$ , phospholipase C-gamma; PSTPIP, proline-serine-threonine phosphatase-interacting protein; SNX9, sorting nexin 9; Tec, tyrosine kinase expressed in hepatocellular carcinoma; VASP, vasodilator-stimulated phosphoprotein.

motifs for SH3 domain containing proteins (PXXP). Deletion of the polyproline domain abolishes the ability of WASp and N-WASp to induce actin polymerization [45,65,66]. Many molecules can bind WASp via their SH3 domains including non-receptor tyrosine kinases, actin binding molecules and adaptor molecules (see Table 1). Members of the Src and Tec family of kinase families can bind to the polyproline domain of WASp through their SH3 domains (see Table 1). Of these, Btk, Hck, Fyn and Lyn can all bind and phosphorylate WASp [60,62,67–74]. Many other kinases have the ability to bind WASp, but further investigations are required to elucidate their function and *in vivo* relevance (see Table 1). Whilst many kinases also contain SH2 domain binding motifs, no interaction with WASp through binding of SH2 domains with phosphorylated tyrosine residues have been reported. Phosphorylation of Y291 of WASp creates a potential SH2 binding motif, although this differs from the Src family SH2 consensus sequence (pYDFI instead of pYEED).

A second important group of binding partners for WASp are adaptor molecules that contain SH3 domains (Table 1). Nck and Grb2 are SH2-SH3 adaptor molecules, which were among the first identified to bind to WASp. They bind to the polyproline domain of WASp via their SH3 domains [73,75] and can re-

cruit other molecules through their SH2 domains. An example of this is the recruitment of WASp via Grb2 binding to the epidermal growth factor receptor after its stimulation [75]. Binding of Nck and Grb2 contributes to N-WASp activation and release of its autoinhibited conformation [76–78] and may function similarly for WASp. Two other SH2-SH3 adaptor molecules that can bind WASp are CrkL and the regulatory subunit p85 $\alpha$  of PI-3K. In platelets, CrkL binds WASp via its SH3 domain and recruits the kinase Syk to the complex, which has the potential to tyrosine phosphorylate WASp [79]. Direct binding of p85 $\alpha$  to WASp was shown in the lymphoblastoid Namalwa cell line and in monocytic U937 cells [69,80]. In T lymphocytes, p85 $\alpha$  binds CD28 and sorting nexin 9 (SNX9), with the latter binding WASp via its SH3 domain [81]. WASp function in platelets and the RAW macrophage cell line appears dependent on PI-3K activity, as inhibition of PI-3K by wortmannin or LY294002 inhibits WASp phosphorylation [32]. Further investigations are needed to define the role of p85 $\alpha$  in WASp activation and to determine whether its involvement could be cell type dependent.

Other SH3 domain containing molecules can bind the polyproline domain of WASp. Phospholipase C-gamma (PLC $\gamma$ ) is one such molecule, but its role in WASp function has not been studied in detail [69–71,

75,80]. PLC $\gamma$  also contains two SH2 domains, which could facilitate recruitment or localization of WASp to molecular complexes. The adaptor molecule PSTPIP (proline, serine, threonine-rich phosphatase interacting protein) acts as a scaffold to bind both WASp via its SH3 domain and the protein-tyrosine phosphatase PTP-PEST via its carboxyl-terminal domain [82,83]. This interaction enables PTP-PEST to dephosphorylate WASp and is important downstream of T cell receptor signalling and immunological synapse formation [60,82,84]. Binding of WASp through multiple SH3 domains of adaptor molecules induces dimerisation of WASp to further enhance its activity [85], which provides an additional way to regulate WASp activity.

A third group of molecules that bind WASp also binds actin monomers and enhances WASp-mediated actin polymerization. These include profilin, cortactin and VASP. The dual binding of profilin to monomeric actin and the polyproline domain of WASp enables profilin to recruit monomeric actin to the VCA domain. This step allows utilisation by the activated Arp2/3 complex and subsequent incorporation into nucleated actin filaments [86]. Cortactin contains an SH3 domain through which it can bind to WASp [19]. VASP can not only bind to WASp via its amino-terminal EVH1 domain, but also bind both monomeric actin and filamentous actin via its carboxyl-terminal EVH2 domain [65].

## 7. VCA domain

The carboxyl terminal VCA domain of WASp defines the business end of the protein and is characteristic of all the proteins in the WASp family. In WASp it contains three regions: the verprolin homology domain, central domain (which was originally thought to be a cofilin homology domain) and the terminal acidic domain. The VCA domain of cytosolic WASp is bound to the GBD, restricting recruitment of Arp2/3 and subsequent actin polymerization [52,53]. The Arp2/3 complex is a stable complex of seven different proteins that mediates reorganisation of actin monomers into filaments. Arp2 and 3 are structurally homologous to actin molecules while the other five proteins, called actin related protein complex component (ARPC) 1–5, have unique structures. Two models have been proposed to explain the mechanism of Arp2/3-mediated actin filament branching. In the dendritic nucleation model, Arp2/3 is bound to the mother filament and the daughter filament is formed by the Arp2, Arp3 and WASp-bound actin monomer. In this model nucle-

ation is initiated from the side of the pre-existing filament [87–89]. The barbed-end branching model proposes that Arp2/3 binds to the barbed end of an existing filament and incorporates one actin monomer (delivered by WASp) or either of the actin homologous Arp2 or Arp3 subunits onto the filament. The daughter filament then is formed by the remaining two units (WASP-delivered actin monomer or actin homologous units Arp2 or Arp3), and elongation of both daughter and mother filament occurs synchronously [90,91]. An activated ARP2/3 complex in the presence of monomeric actin, ATP and the VCA domain of any of the WASp family members are the minimal requirements for actin polymerization [38,92,93].

Upon release of the autoinhibited conformation of WASp, the CA region of the VCA domain is able to bind Arp2/3 via a conserved stretch of approximately 15 amino acids (465–483 in WASp). These form an amphipathic helix that exposes long-chain hydrophobic residues and a single positively charged arginine residue (aa 477) on one face of the helix, to which Arp2/3 is predicted to bind [94]. Constitutive phosphorylation by casein kinase 2 of two serine residues within the WASp VCA domain (S483 and S484) further enhances Arp2/3 binding [95]. Actin monomers are recruited by binding to amino acid residues within the V and C domains, which assists in delivering actin monomers to the activated Arp2/3 complex [96–98]. Binding of actin-binding molecules, such as profilin, to the polyproline domain likely also assists in the delivery of actin monomers to the V and C domain of WASp [86].

## 8. Cellular function

Activation of WASp occurs at multiple levels either through activation by Cdc42 or by phosphorylation of Y291. The large number of binding partners that interact with different domains of WASp provides additional ways to regulate function. In addition, WASp binding partners can be involved in dimerisation of WASp molecules, further enhancing WASp activity. Thus, WASp emerges as an important platform for regulating actin polymerization through activation of the Arp2/3 complex, which is key for cytoskeletal reorganisation. It is therefore not surprising that lack of WASp results in a wide range of defects of cellular function involving all haematopoietic cell lineages (see Fig. 2).

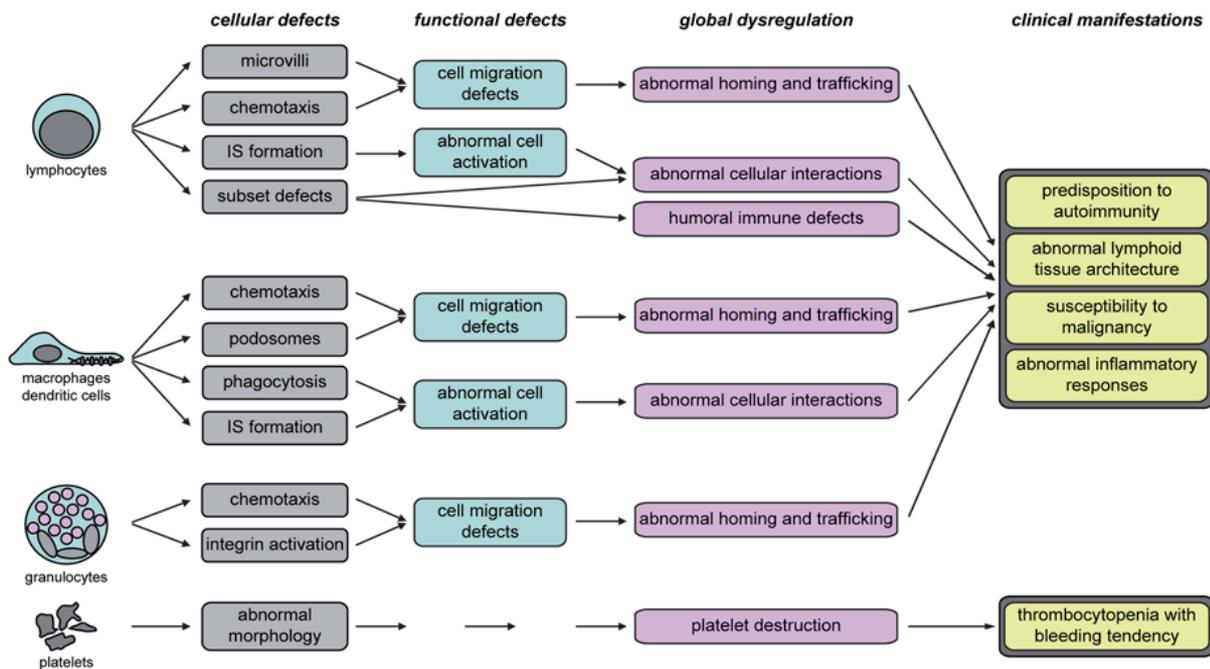


Fig. 2. Lack of WASp causes defective function of multiple haematopoietic cell lineages. WASp is required for many functions in haematopoietic cells and its absence results in defects of cellular function. The combination of defective function of multiple immune cell lineages culminates in global dysregulation of immune function, which manifests as abnormal inflammatory responses, autoimmunity and susceptibility to malignancy. In addition, lack of WASp causes a specific platelet defect, which results in increased platelet destruction and bleeding tendency.

## 9. WASp in the Haematopoietic system

Unlike the ubiquitously expressed N-WASp, WASp expression is restricted to the haematopoietic lineage of cells and is detected even in the earliest cells of the developing immune system in the aorto-gonad-mesonephros (AGM) region [99]. Despite the presence of WASp in cells of the early developing immune system, certain functions mediated by N-WASp or WAVE proteins may show some redundancy. In contrast, N-WASp and WAVE 2 KO mice are embryonic lethal [100,101] and WAVE 1 KO mice exhibit severe neuronal abnormalities [102]. The early expression and necessity for WASp was demonstrated by the apparent non-random inactivation of the X chromosome in the haematopoietic system of heterozygote females [103]. Where X inactivation has been skewed against the normal WAS allele, there have been cases of females with a WAS/XLT phenotype [104,105]. The requirement for WASp in the developing immune system is not fully understood. Wild type haematopoietic cells have a selective advantage in homing to the bone marrow as compared to WASp knock out (WAS KO) cells [106]. However once in this niche, there does not seem to be a critical role for WASp, although activating muta-

tions lead to severe neutropenia [107]. Haematopoietic progenitor levels and development of B cells in the bone marrow in particular both appear to be normal. This is in direct contrast to more mature cells of the lymphoid compartment, in which WASp confers a selective advantage [108,109]. In fact, as lymphocytes and myeloid lineage cells mature they seem to have a greater requirement for WASp in order to function. Several WAS patients have been described where secondary *de novo* mutations have resulted in a reversion to wild-type protein, leading to somatic mosaicism and suggesting a selective advantage for WASp positive cells [110–112]. In fact WAS seems to have a high prevalence of mosaicism even compared to other immunodeficiencies [113,114]. The selective advantage for wild-type protein expression is restricted to the lymphoid lineage, particularly in T cell and NK cell cells. Selective advantage depends upon where the reversion occurs, with some mutations showing only a T cell advantage [115,116], whereas other mutations confer advantage to both NK and T cell lineages [117–119]. In most cases T cell reversion is not sufficient to abrogate disease [116] even though T cells are one of the most affected lineages. This is probably because

only a limited repertoire is corrected, but also as a result of the multilineage nature of the disease. So far little selective advantage has been observed for B cells in mosaic patients [115].

## 10. T cell defects

Although human T cell development appears normal in WAS patients, the circulating lymphocyte number is reduced [120] and progressively declines throughout childhood [121,122]. T cells may exhibit morphological defects with reduced microvilli on the cell surface [123], which could affect normal migratory function, or reflect the activation state of the cell [124]. One of the major defects of WASp null T cells is their inability to proliferate in response to various stimuli [120]. They do not form an optimal immunological synapse (IS) [84,125], leading to defective receptor capping and subsequent IL-2 secretion upon CD3 $\epsilon$  cross linking. However, in some circumstances an IS can be formed [126]. It has also been proposed that it is not the initial IS formation but the re-formation of the synapse that is defective [127]. The mechanism leading to a poorly formed IS probably results from inefficient actin polymerization [128] or an inability to assemble signalling partners, such as CD2 associated protein, that are normally held in place by a scaffold involving WASp [84]. Upon ligation of the T cell receptor (TCR), WASp is recruited to lipid rafts which allow lateral movement of the TCR. In WAS patients and WAS KO mice, the reduced number and impaired upregulation of the rafts results in inefficient T cell activation in response to stimuli [125]. Although WASp mainly functions in actin polymerization, a WASp mutant with the VCA domain deleted, which is unable to polymerise actin, remains capable of enhancing NFAT translocation and transcriptional activation [129]. There is a proposed role for N-WASp and WASp in T cell development as dual WASp/N-WASp KO mice exhibit severe thymic hypocellularity [130]. Mice deficient in Vav-1, a guanine exchange factor (GEF) for Rho type GTPases that is implicated in actin cytoskeleton remodelling, have a similar blockage in thymic development at the DN3 stage [131]. Coronin-1 KO mice, which have increased actin polymerization, have T cell defects in the spleen and lymph nodes [132]. DOCK8 is reported to be a Rho-Rac GEF for Cdc42, and deficiency in DOCK8 would therefore be predicted to result in some phenotypic features in common with WAS. In fact, striking clinical and immunological similarities

have been observed, both in humans and in a recently reported mouse model of DOCK8 deficiency [133, 134]. The greater severity of DOCK8 deficiency as compared to WASp deficiency alone, may reflect GEF activity of DOCK8 that is not restricted to Cdc42, or Cdc42-mediated functions that do not involve WASp activity. Additionally, DOCK8 deficiency may compromise both N-WASp and WASp functions.

In addition to defects in CD4+ and CD8+ T cells, functional abnormalities in the regulatory T cell (Treg) populations are seen in both WASp deficient humans and mice [135–137]. Tregs are important in T cell homeostasis and control auto-reactive T cells. Treg abnormalities could partially explain the propensity for autoimmune dysregulation in WAS patients. The re-expression of WASp following a reversion mutation in a patient, demonstrated a selective advantage in the Treg population with a corresponding decrease in autoimmune disease and improved clinical condition [136].

## 11. B cell defects

Until recently it was thought that B cells were less severely affected by a lack of WASp compared to other lineages. Animal models initially revealed few defects in the B cell compartment, which were attributed in part to lack of T cell help [138,139]. WAS patients have increased prevalence of B cell malignancy [140], impaired antibody responses particularly to polysaccharide antigens [121,140], reduced B cell numbers and smaller germinal centres. B cells from WAS patients have defective transmembrane signalling [141] and reduced numbers of microvilli on their cell surface [138]. EBV transformed cell lines have defective actin polymerization [142]. B cells have defective polarisation, spreading, aggregation and migration in response to CXCL13 [143,144], and are defective in producing filopodia in response to bradykinin [145]. Specific subsets of B cells are also abnormal. Marginal Zone B cells (MZB) are reduced in WAS patients [146] and in WAS KO mice [144,147], consistent with a defect in response to polysaccharide antigens. Furthermore they fail to migrate in response to sphingosine-1-phosphate, which is crucial for MZB homing. Transplantation experiments further revealed a role for WASp in peripheral homeostasis. Although a lack of WASp does not affect development, WASp expression increases as B cells mature in the periphery. At this stage, there is a selective advantage of wild type over WAS null B cells. Similar results were observed in a revertant

patient where the mature B cell population expressed more WASp than immature B cells [108]. The defects in B cells are not as well defined compared to other lineages, but are consistent with abnormal regulation of the actin cytoskeleton and may be particularly important for development of autoimmunity.

## 12. NK Cell defects

NK cells are an important part of the innate immune system that removes virally infected cells, parasites and malignant cells [148]. They are predominantly found in the blood, but upon instigation of an inflammatory response rapidly migrate to the site of inflammation where they kill target cells and release cytokines to further stimulate the immune response [149]. As has been shown for T cells, NK cell cytotoxicity requires formation of an immunological synapse and lipid raft clustering, which in itself relies upon actin polymerization. Both WASp and actin are present in the IS [150]. WAS patient NK cells, although increased in number [151], have impaired cytotoxicity, which can be corrected by the addition of IL-2 [152]. This reversibility suggests that a lack of signal transduction is responsible for the defect. It is this lack of responsiveness to viruses (particularly those with oncogenic potential) that probably accounts for the increased incidence of malignancy in WAS. Furthermore, WASp is involved in the transcriptional regulation of cytokines via the translocation of NFAT into the nucleus and activation of transcriptional factors [153]. As in T cells, these events occur independently of WASp's actin polymerization function. More recently, migration of NK cells through ICAM-1 and VCAM-1 has been shown to rely upon WASp. WASp is rapidly phosphorylated following chemokine stimulation, and WAS KO NK cells have reduced ability to upregulate CD18 expression in response to CX-CL12 [154]. The importance of WASp in NK cells is demonstrated by the selective advantage conferred by spontaneous revertant mutations in WAS patients [119].

## 13. Invariant NKT cells

Invariant NKT cells are T cells that have both NK cell receptors and T cell receptors, but which recognize lipid antigens instead of peptide antigens via the non-classical class I MHC molecule CD1d [155]. By releasing a wide range of cytokines upon TCR engagement, they are thought to be particularly important in

initial responses to infection, as well as in protection from autoimmunity and clearance of malignant cells. iNKT cells in SAP KO mice are completely absent, suggesting a role in preventing EBV specific malignancies observed with SAP deficiency. Interestingly these same malignancies are often seen in WAS. In both WAS patients and KO murine models, iNKT cells are severely reduced [156,157] and secrete less cytokine when stimulated. This may contribute to the increased malignancy observed in WAS patients.

## 14. Myeloid defects

Many defects of the immune system in WAS have been discovered in cells of the myeloid lineage. The ability of neutrophils, DC and monocytes to respond appropriately to a stimulus, whether through migration, efficient antigen presentation or through phagocytosis, relies upon quick and regulated *de novo* actin polymerization.

Once stimulated, macrophages and DC have to migrate through tissues. They home either to sites of inflammation, or to secondary lymphoid tissues to initiate effector functions of other cells of the immune system. Cell locomotion is controlled by protrusions of the cell membrane and relies upon spatial and temporal regulation of actin polymerization. WAS macrophages have abrogated chemotactic responses to fMLP, MCP-1 [158], and CSF-1 [159], as compared to normal cells. DC from WAS patients also have intrinsic defects in their actin regulation. When stimulated *in vitro* with fMLP or RANTES, WAS DCs are non-motile. In addition, they do not polarise or extend dendritic processes [160]. Furthermore, Langerhans cell and DC migration are impaired *in vivo* [147,161]. Neutrophils do not assemble integrins properly either further suggesting migratory defects [162]. The defective migration coupled to impaired T cell activation probably contributes to a large proportion of the pathology observed in WAS [161,163].

Podosomes are thought to participate in cell motility through adhesion to extracellular matrix proteins and localized release of proteases. The latter allows the cells to migrate through the substratum; additionally release of MMPs occurs in osteoclasts [164] and vascular smooth muscle cells [165]. Podosomes are highly dynamic actin based structures that have a half-life of 2–12 minutes and are in close contact with the substratum. Podosomes have a basic actin core with actin regulatory proteins surrounded by a ring of vinculin and

other cellular adhesion molecules [166]. Podosome assembly and disassembly in both macrophages and DC is tightly regulated by WASp, WIP and Cdc42. Knock down of WASp using RNAi leads to reduced podosome numbers [167], and overexpression of the dominant negative form of Cdc42 (V12Cdc42) leads to podosome disassembly [168,169]. Macrophages [168] DCs [169] and osteoclasts [170] from WAS patients lack podosomes. In fact, similar to NK cells [154] WAS DC do not adhere as strongly to ICAM-1 and do not assemble integrins around podosomes [171]. Milder phenotype XLT patients have reduced podosome numbers, but correlation between podosome number and severity of clinical phenotype has not yet been established. WASp is recruited to podosomes as part of a complex of molecules including FBP17, WIP and dynamin2 [172]. WASp and WIP both associate in the podosome, and a lack of either results in loss of podosomes [22,33,173]. More recently, phosphorylation of WASp is thought to be crucial in the formation and turnover of podosomes [30,174].

Aberrant chemotaxis in macrophages and DC is therefore due to at least two defects. Firstly there is an abrogation in filopodia mediated by Cdc42 and WASp, which is crucial for chemotaxis [175,176]. Secondly abnormal polarisation and adherence to substrata leads to an inability to direct movement in a regulated manner. In deletion experiments, the minimal region of WASp required for chemotaxis is the VCA region. This is also the minimal region needed to stimulate actin nucleation, further linking the defects in WAS with dysregulation of actin polymerization [177]. Experiments where WASp is introduced back into WAS KO cells restore podosome formation [170,178–180] and partially restore defective migration [180].

WAS patient macrophages have a defect in IgG mediated phagocytosis and fail to efficiently assemble a phagocytic cup (normally comprised of F-actin and phosphorylated proteins including WASp [34]) although binding of IgG coated latex beads to the cell surface is normal [181]. Similarly when phagocytosis of apoptotic cells are investigated, WAS macrophages exhibit a defect *in vitro* and *in vivo* [182].

## 15. Platelet defects

Thrombocytopenia and small platelets are classical features of WAS [120] and bleeding is a major cause of morbidity and mortality [140]. However, the exact role of WASp in platelet development and function is

still not known. *In vitro* experiments have shown abnormal cytoarchitecture in WAS megakaryocytes, but the platelets formed in this system were of normal size [183]. As splenectomy can increase platelet numbers, in non-splenectomized patients the low numbers were thought to result from increased destruction. Indeed murine WAS KO platelets are destroyed quicker than wild-type platelets when transferred into wild-type recipients, suggesting an inherent defect. WAS patients are particularly susceptible to autoimmune thrombocytopenia, which is mediated by anti-platelet antibodies and in many patients likely compounds the splenic mediated platelet destruction [184]. Additionally WAS megakaryocytes prematurely form proplatelets, resulting in impaired thrombopoiesis [185]. Interestingly, WASp deficient platelets can activate Arp2/3 and make F-actin independent of WASp [90]. Together this evidence suggests that in most patients thrombocytopenia is mediated by a combination of several mechanisms, but further investigations are required to clarify this situation.

## 16. Autoimmunity and malignancy

The intrinsic defects in phagocytosis, chemotaxis, cytotoxicity, apoptosis and cell signalling appear to contribute to more than just the immunodeficiency observed in WAS. The high incidence of autoimmunity, inflammatory conditions and vasculitis seen in patients with WAS and XLT can develop even following bone marrow transplantation [186,187]. Management of these conditions continues to increase in importance as the management of the immunodeficiency and thrombocytopenia improves [140,188]. Abnormal Tregs have been implicated in the onset of autoimmunity by unbalancing T cell homeostasis [135–137]. Murine models are susceptible to colitis [139], which can also be induced upon transfer of WAS Tregs [189]. The peripheral tolerance that follows elimination of autoreactive T cells relies upon effective cell killing via FASL [190]. Even though FAS induced apoptosis is intact, secretion of active FASL is reduced, contributing to inefficient TCR-induced apoptosis and predisposition to autoimmunity [191]. Dysregulation of B cell tolerance is supported by the presence of autoantibodies in WAS patients and in both WASp null and phosphorylation mutant mice [30,136,191]. The ineffective phagocytosis of apoptotic cells could contribute to the aetiology of autoimmunity [182] by causing unchecked inflammatory responses or unbalanced

exposure to auto-antigens. In C1q deficient mice this has been linked to systemic lupus erythematosus [192]. Abnormal immune cell migration can lead to abnormal antigen presentation in the periphery instead of in lymphoid organs. This spatial dysregulation of antigen presentation and immune activation could initiate inflammation and cause the severe eczema observed in WAS patients.

Bypassing tolerance checkpoints for B and T cells has been proposed as a mechanism for lymphoid malignancy [193]. Chronic stimulation of autoreactive cells and ineffective clearance of virally infected cells could be a mechanism through which malignant lymphomas arise [194]. Because NK cell cytotoxicity is impaired in WAS patients [150,152], removal of virally infected cells is likely to be also impaired. The role of these cells in the development of malignancy is an area of future work. Recently, activating mutations in WASp (which give rise to XLN) have been found to lead to genetic instability through dysregulation of actin polymerization [107].

## 17. Treatment

The clinical variability, breadth of complications and unpredictable clinical course present a major challenge for the management of Wiskott Aldrich Syndrome. Early diagnosis is essential to reduce the likelihood of complications and subsequent long term morbidity. Management can then be planned according to clinical presentation, ongoing clinical course, gene mutation and level of WASp protein expression. Antibiotic prophylaxis (including *Pneumocystis jirovecii* prophylaxis), immunoglobulin replacement therapy, vaccination (live vaccine are not recommended) and platelet transfusions for severe or life threatening haemorrhage may all be required for patients with classical severe WAS. Aggressive treatment of eczema and infections (particularly Herpes viruses) is essential. Severe thrombocytopenia with recurrent haemorrhages is a particular problem. Rituximab (anti-CD20) can be effective when there is an autoimmune component to the thrombocytopenia. In resistant cases splenectomy may be necessary; however subsequent risk of severe sepsis (even following stem cell transplant) is significantly increased. Haematopoietic stem cell transplant, which corrects defects in all haematopoietic compartments, is the definitive treatment of choice [195–197]. Survival rates of over 85% following transplant are now obtained even with unrelated donors, but the procedure is

less successful with haploidentical donors [198–200]. Mixed chimerism may be associated with persistent autoimmunity [187]. One alternative treatment is somatic gene therapy. Initial experiments introducing WASp into null cells restored cytoskeletal abnormalities [169, 170,178,201] and cell signalling [202–204]. Moreover, transduction of murine haematopoietic stem cells with retroviral and lentiviral vectors, followed by engraftment of murine models, corrected multiple abnormalities of the immune system [179,180,205–207]. Further refinement has led to the use of endogenous human promoter sequences to express WASp at physiological levels [208–210]. Clinical trials using gammaretroviral vectors and lentiviral vectors are underway and show promise (Boztug et al, in press).

## 18. Concluding remarks

WASp function is regulated at several levels including release and stabilisation of an autoinhibitory conformation, oligomerisation and proteasomal degradation. Activated WASp provides an important scaffold for signalling molecules. As an adaptor molecule, WASp may be crucial for subcellular organization of signalling complexes to the actin cytoskeleton. While there might be a certain level of redundancy, WASp emerges as a significant regulator of the haematopoietic cytoskeletal system and is crucial for normal immune cell function. While insights into WASp activities have been instructive in our understanding of the actin cytoskeleton, they have also paved the way towards development of more effective therapies.

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