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Review

WIP: more than a WASp-interacting protein

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ABSTRACT

WIP plays an important role in the remodeling of the actin cytoskeleton, which controls cellular activation, proliferation, and function. WIP regulates actin polymerization by linking the actin machinery to signaling cascades. WIP binding to WASp and to its homolog, N-WASp, which are central activators of the actin-nucleating complex Arp2/3, regulates their cellular distribution, function, and stability. By binding to WASp, WIP protects it from degradation and thus, is crucial for WASp retention. Indeed, most mutations that result in WAS, an X-linked immunodeficiency caused by defective/absent WASp activity, are located in the WIP-binding region of WASp. In addition, by binding directly to actin, WIP promotes the formation and stabilization of actin filaments. WASp-independent activities of WIP constitute a new research frontier and are discussed extensively in this article. Here, we review the current information on WIP in human and mouse systems, focusing on its associated proteins, its molecular-regulatory mechanisms, and its role as a key regulator of actin-based processes in the immune system. *J. Leukoc. Biol.* 96: 713–727; 2014.

Introduction

The WAS is an X-linked disorder associated with microthrombocytopenia, skin conditions, and immune dysfunction [1]. Patients suffering from classic WAS commonly exhibit immunodeficiency, eczema, increased susceptibility to infections, as well as a heightened risk of developing autoimmune disorders and hematopoietic malignancies. A milder WAS variant, called XLT, is characterized by minimal immunodeficiency [2, 3].

Mutations causing WAS were found to be located on a single gene, coding for an actin nucleation promoting factor, named the WASp [4, 5]. Interestingly, most mutations resulting in WAS occur in the binding site for the WASp partner, named WIP. Some of these mutations have been shown to impair the interaction between these two proteins and lead to WASp degradation [6, 7], making the interaction between WIP and WASp the subject of intense research. WIP was first identified as a binding partner of WASp using the yeast two-hybrid system [8] and was later established as a chaperone of WASp [9, 10] and a regulator of WASp/N-WASp activity [11, 12]. Moreover, a recent study showed that a stop codon mutation in the WIP gene causes a human immunodeficiency with similar features to WAS and characterized by undetectable levels of WASp [13]. These findings highlight the importance of the WIP-WASp interaction for immune function.

WIP is crucial for WASp-dependent actin polymerization. Actin remodeling is essential for the creation of several actin-rich cellular structures, such as filopodia (spike-shaped cellular projections) and lamellipodia (thin, sheet-shaped cellular protrusions), and for the formation and stabilization of the interface area between lymphocytes and APCs, termed the IS. In migrating leukocytes, the creation and stabilization of podosomes, short actin-rich adhesion structures crucial for cellular motility and invasion, are dependent on the interactions of WIP with WASp [14, 15]. A podosome consists of an actin core surrounded by a ring of adaptor proteins, such as talin, vinculin, fimbrin, gelsolin, and paxillin, which may serve as a link to integrins [16]. WASp, WIP, and the actin-nucleator protein complex Arp2/3 are present inside of the podosome actin core [14, 17]. Recently, we found in T cells that WIP-WASp interaction not only controls WASp stability but also actin homeostasis throughout TCR activation [18].

Abbreviations: $^{-/-}$=null, aa= amino acid, ABM-2=actin-based motility-2, Arp2/3=actin-related protein 2/3, Blow=blown fuse, BMMC=bone marrow-derived mast cell, CR16=corticosteroids and regional expression 16, CrkL=Crk-like, cSMAC=central supramolecular activation cluster, DC=dendritic cell, FRET=fluorescence resonance energy transfer, G-actin=globular-actin, GBD=GTPase-binding domain, GPVI=platelet glycoprotein VI, IS=immunological synapse, KO=knockout, MMP=matrix metalloproteinase, N-WASp=neuronal Wiskott-Aldrich syndrome protein, NF-AT=NF of activated T cells, NKIS=NK immunological synapse, nWIP=nano-Wiskott-Aldrich syndrome protein-interacting protein, PKC=protein kinase C, PRD=proline-rich domain, pSMAC=peripheral supramolecular activation cluster, PTK=protein tyrosine kinase, SH3=Src homology 3, Sltr=solitary, SMAC=supramolecular activation cluster, V-domain=verprolin homology domain, VCA=verprolin homology/cofilin homology/acidic region, WAS=Wiskott-Aldrich syndrome, WASp=Wiskott-Aldrich syndrome protein, WAVE=Wiskott-Aldrich syndrome protein family verprolin homolog, WH1=Wiskott-Aldrich syndrome protein homology domain 1, WH2=Wiskott-Aldrich syndrome protein homology 2, WICH/WIRE=Wiskott-Aldrich syndrome protein-interacting protein related and corticosteroids and regional expression 16 homolog/Wiskott-Aldrich syndrome protein-interacting protein related, WIP=Wiskott-Aldrich syndrome protein-interacting protein, XLT=X-linked thrombocytopenia, ZAP-70=ζ-chain-associated protein kinase of 70 kDa

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Several studies provide evidence indicating that WIP might also promote actin polymerization independently of WASp. It was shown that overexpression of WIP in BJAB cells, a human Burkitt lymphoma B cell line, results in the elevation of cellular F-actin levels [8]. In addition, WIP was shown to bind actin. This binding stabilizes actin filaments, as WIP decelerates F-actin disassembly, as was demonstrated by in vitro assays [12]. Strikingly, a comparison of defects in proliferation, in the increase of F-actin content, and in cellular polarization in T lymphocytes in $WASp^{-/-}$ versus $WIP^{-/-}$ mice revealed a more severe phenotype in the $WIP^{-/-}$ mice (see Table 1). This indicates that indeed, WIP plays a critical role in actin regulation that goes beyond its role as a chaperone of WASp [19, 20]. However, these data describing the unique role of WIP are limited and require further examination.

The WASp-independent functions of WIP can be explained by its interactions with other proteins involved in the actin polymerization machinery and by its regulatory activity on multiple proteins with key roles in the activation and function of various immune cells. Besides WIP association with G- and F-actin [12], WIP also binds to profilin [8], an actin-sequestering protein involved in the dynamic turnover and restructuring of the actin cytoskeleton [21]. Profilin is essential for processes, such as organ development, wound healing, control of cellular shape, and cell motility. In addition, WIP supports the creation of podosomes by its interaction with cortactin, an activator of the Arp2/3 complex, thereby promoting the formation and stabilization of actin branches [22, 23]. All of these interactions are essential for functions, such as cytotoxicity of NK cells [24, 25], mast cell signaling [26], and cellular motility and tissue infiltration mediated by podosome formation in DCs [10] and macrophages [15].

In this review, we provide an overview of WIP, describing its structure and functions in various immune cell types, and compare the outcomes of WIP deficiency in the human and mouse systems. We also introduce the various family members of WIP. Additional emphasis will be given to recent data revealing the novel interactions between WIP and its binding partners and their importance for WIP function in leukocytes.

STRUCTURE OF WIP AND ITS HOMOLOGS

Human WIP is a 503-aa protein expressed in multiple cell types. The WIP protein family, also called the verprolin family, includes WIP, CR16, and WICH/WIRE. CR16 was discovered as a brain protein regulated by glucocorticoids, and its expression was also reported in the heart, lung, and testis [27, 28]. CR16 binds to N-WASp in brain cells [29], and recently, this complex was also discovered in the human testis. Indeed, decreased levels of these proteins are present in the testes of men with idiopathic azoospermia, suggesting that CR16 and N-WASp may sometimes play a role in the pathogenesis of this condition [30]. The WICH/WIRE protein is present in multiple organs, participates in receptor-mediated endocytosis [31], and translocates to sites of actin assembly in the cell periphery in the presence of platelet-derived growth factor. WICH/WIRE binds preferentially to N-WASp rather than WASp [32], thereby promoting the formation of cross-linked actin filaments [33]. Recently, WICH/WIRE was found to also induce filopodia independently of N-WASp [34].

WIP was first described in cells of hematopoietic lineages. Analysis of WIP mRNA levels revealed that it is more abundant in PBMCs than in nonhematopoietic lineage cells [8]; however, a comprehensive analysis of the protein levels of WIP in various cell types is missing. To unravel the overlapping and/or distinct roles of WIP and its homologs in the regulation of leukocyte function, characterization of the relative distributions of WIP, WICH/WIRE, and CR16 is required.

WIP is characterized by a multidomain structure that allows it to bind to various proteins participating in the regulation of actin organization and polymerization (**Fig. 1**). The N-terminus of WIP displays a high degree of homology to verprolin (Vrp1p), an actin-binding protein present in yeast and important for actin organization, cell polarity, and endocytosis [35, 36]. This region, called the V-domain, is present in all WIP family proteins [37]. Indeed, WIP was shown to compensate for defects in cytoskeletal organization observed in $verprolin^{-/-}$ yeast, whereas a truncated form of WIP, lacking its N-terminal proximate region, is unable to restore these functions [38]. The V-domain of WIP is comprised of its first 112 aa and contains two WH2 domains [39] that were recently identified by us as novel binding sites of WASp [18]. The first WH2 domain contains the actin-binding KLKK sequence, which is involved in WIP binding to G-actin and in the stabilization of F-actin; removal of the WIP V-domain abolishes this activity [12]. Similar to WIP, CR16 and WICH/WIRE were shown to bind both G- and F-actin [37] (Fig. 1).

The N-terminal region of WIP also encompasses a glycine-rich stretch that may contribute to the structural flexibility of this domain. CR16 and WICH/WIRE, however, lack this polyglycine region [40].

All members of the WIP family contain ABM-2 motifs. WIP contains three ABM-2 motifs, located at aa 8–13, aa 379–384, and aa 427–432 [8, 39]; CR16 also contains three ABM-2 motifs, and WIRE contains six ABM-2 motifs. The three ABM-2 motifs of WIP constitute potential profilin-binding sites [37]. Profilin fulfills two distinct and somewhat dichotomous functions important for the maintenance of the actin pool: catalyzing the exchange of actin-bound ADP to ATP, thereby converting poorly polymerizable ADP-actin monomers into readily polymerizable ATP-actin monomers and sequestering these monomers from the pool of polymerizable actin, helping to prevent spontaneous, uncontrolled actin nucleation and polymerization [41].

The N-terminus of WIP is followed by a central region that contains proline-rich stretches, which serve as docking sites for the SH3-containing proteins, CrkL and Nck, adaptor proteins that are essential components of signal transduction pathways in lymphocytes (described below). These proteins bind to aa 321–415 of WIP [11, 42], whereas Nck also binds to aa 247–261 [43]. An additional SH3-containing protein, cortactin, binds the PRD domain via aa 136–205 of WIP.

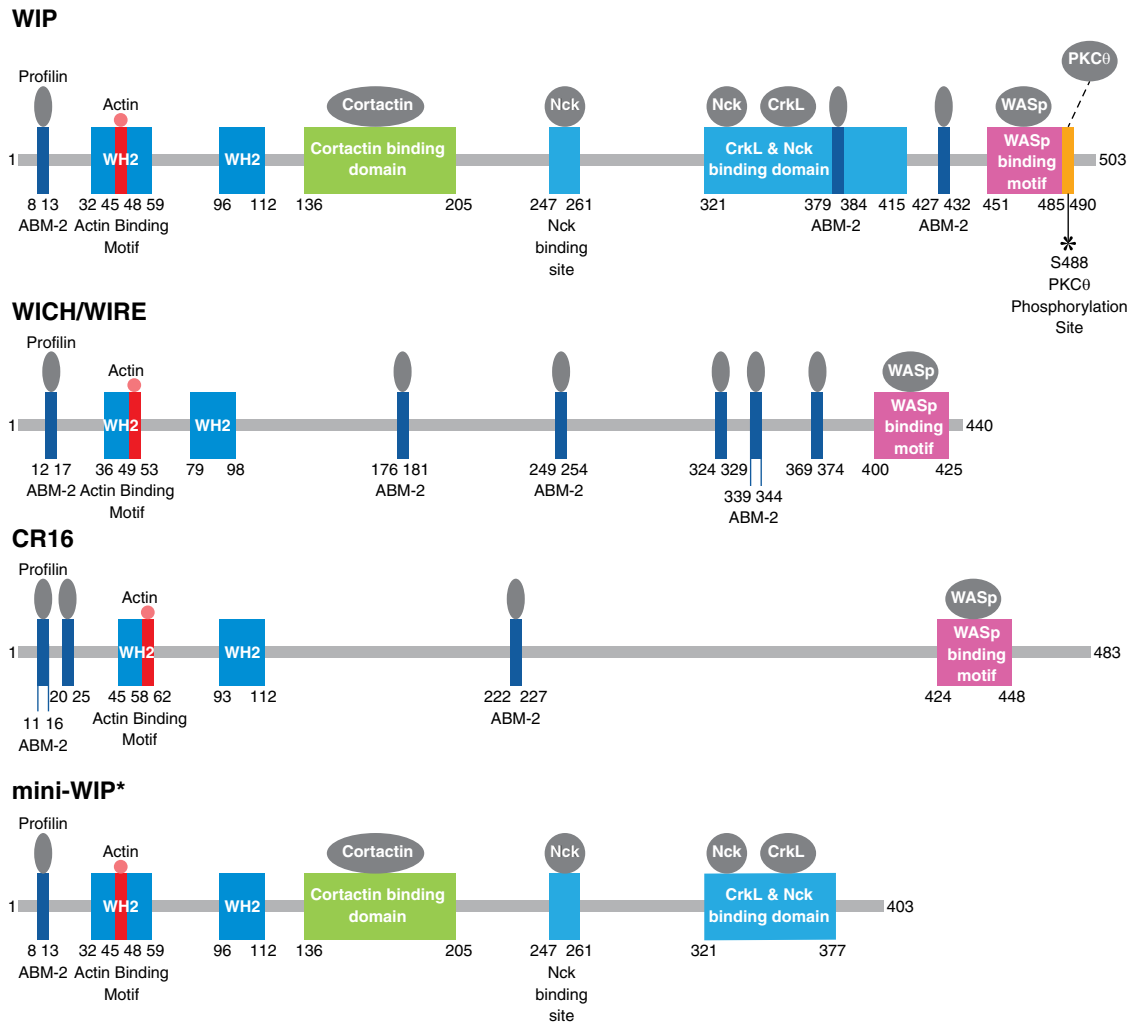

Figure 1. Structural models of WIP family members, WIP, WICH/WIRE, CR16, and mini-WIP. The various domains are depicted, with residue numbers and binding sites indicated for interacting proteins. *The data describing the binding partners of mini-WIP are based on the known WIP interactions and on the homology between WIP and mini-WIP. Please note that the dark blue rectangles represent ABM-2 (actin-based motility) sites.

The C-terminal region of WIP binds to WASp and N-WASp via their N-terminal WH1, which is structurally similar to the enabled-vasodilator-stimulated phosphoprotein homology domain 1; however, it differs in its binding ligands [44]. This region also fulfills a similar function in other WIP family members, allowing WICH/WIRE to bind to WASp and N-WASp [32] and CR16 to bind to N-WASp [29].

The region of WIP/WASp interaction has been mapped to aa 451–485 of WIP by nuclear magnetic resonance studies [45, 46], showing that residues 454–459, 461–468, and 476–485 come into direct contact with WASp/N-WASp during binding. The WH1 domain of WASp serves as the WIP-binding site. These findings were corroborated by studies showing that rat N-WASp aa 26–147 and human WASp aa 34–149 are important for WIP binding [45].

Another protein related to WIP is mini-WIP, a short, 403-aa long WIP isoform that lacks the C-terminal WASp-binding region (Fig. 1) [47]. Mini-WIP, whose function is as yet unknown, was found in the peripheral blood, as well as in mouse splenocytes and several transformed human immune cell lines. This truncated version of WIP was suggested to participate in the regulation of actin dynamics in a WASp-independent manner.

INTERACTIONS OF WIP WITH WASP AND N-WASP

WASp belongs to a larger family of proteins capable of activating the Arp2/3 complex. Other members of this family include N-WASp [48], as well as the WAVE subfamily [49, 50], WASp and suppressor of cyclic amp receptor homolog [51], junction-mediating regulatory protein, and WASp homolog associated with actin membranes and microtubules [52, 53]. WASp and N-WASp share a high degree of similarity in structure and in mechanism of activity. WASp is expressed exclusively in hematopoietic cell lineages and precursor cells,

whereas N-WASp is widely expressed in multiple tissues [48]. It was shown that WASp and N-WASp are involved in the development of a normal T cell, as double KO of these proteins impaired the transition of T cells from the double-negative to the double-positive stage during development [54]. However, as WAS patients possess a normal level of N-WASp [55], it is clear that N-WASp cannot compensate for WASp-impaired or -lacking functions, and thus, WASp/N-WASp are not entirely redundant.

In resting cells, WASp/N-WASp is present in an autoinhibited conformation induced by the interaction of its C-terminal VCA domain with a hydrophobic patch located within the GBD. This autoinhibitory interaction masks the binding sites for the Arp2/3 complex. Following T cell activation, Cdc42, a Rho family GTPase, binds to the WASp GBD site. This, together with the phosphorylation of the Y291 site by PTK, induces a dramatic conformational change in WASp, releasing the VCA domain and enabling its interaction with the Arp2/3 complex, thereby promoting actin polymerization [56, 57]. An in vitro study has shown that WIP inhibits Cdc42-induced activation of N-WASp and regulates N-WASp-mediated actin polymerization [12]. Another study using the xenopus oocyte system has shown that WIP suppresses N-WASp activity [58]. It was therefore suggested that WIP binding stabilizes the closed, inactive conformation of WASp/N-WASp.

WIP plays an important role in the recruitment of WASp to areas of active actin assembly, such as the IS [11], and in the formation of podosomes [10] following antigen receptor or chemokine receptor signaling. A recent study in DCs demonstrated that the formation of the WIP-WASp complex may not be necessary for the initial localization but is required for the full retention of WASp at the podosomes [59].

In T cells, more than 80% of WIP and WASp exists in WIP-WASp complexes [60]. Interestingly, most missense mutations in WASp, which result in defective or absent WASp activity and that were found to cause WAS, are located in the WIP-binding region of WASp [6]. Indeed, some of these mutations disrupt the WIP-WASp interaction, leading to WASp degradation [6, 7]. However, the mechanism of WASp degradation was not known until recently.

The protease, calpain, is known to degrade unbound WASp. Yet, the calpain inhibitor only partially restores WASp levels in activated T cells from WAS patients [9]. In addition, proteasome inhibitors do not fully restore WASp levels in T cells from $WIP^{-/-}$ mice [9], indicating that unbound WASp degradation is mediated by calpain and an additional degradative process.

We have demonstrated recently that WASp is ubiquitylated following TCR activation. WASp ubiquitylation is dependent on the phosphorylation of tyrosine 291, which results in the recruitment of the E3 ligases Cbl-b and c-Cbl, which ubiquitylate WASp. This study also mapped WASp ubiquitylation sites to lysine residues 76 and 81, located in the WASp WH1 domain. Mutations at both ubiquitylation sites greatly decreased ubiquitylation, resulting in WASp accumulation. Deletion of WASp WH1 domain was found to alter the interaction between WIP and WASp, as evident by FRET analysis, as well as modifying actin rearrangement dynamics. These data imply that the WH1 domain, crucial for WASp binding to WIP, plays an essential role in the regulation of WASp stability and are in agreement with the evidence that most WAS-causing mutations are located in this domain [7] (**Fig. 2**).

Investigation of the fate of the WIP-WASp complex following leukocyte activation raised the hypothesis that WIP disassociates from WASp to enable WASp to induce Arp2/3-mediated actin polymerization. It was found that following T cell activation, WIP is phosphorylated at a single-consensus PKC phosphorylation motif (RxxSxR, residues 485–490) [11] and that WIP phosphorylation on S488 causes dissociation of the WIP-WASp complex. More recent studies demonstrated that in T cells, stimulation of the TCR complex induces phosphorylation of WIP but does not result in WIP-WASp dissociation following this phosphorylation event nor is dissociation necessary for WASp activation or function [61]. This discrepancy is potentially attributed to a difference in the antibodies used in the two studies. The initial study used antibodies specific for a WASp-binding proximate site on WIP, possibly resulting in WIP-WASp disassociation caused by the binding of the antibody. In a subsequent study, using a new mAb, no disassociation was observed [47]. These data emphasize the lack of information regarding the molecular nature of WIP-WASp/N-WASp interactions and the manner by which WIP shields WASp from degradation. These issues are the subjects of intense debate.

We have recently conducted an in-depth study of the nature of WIP-WASp molecular complex, revealing the mechanism through which WIP protects WASp from degradation in resting versus activated T cells [18]. We showed that the WIP-WASp interaction occurs via two distinct molecular interfaces. The first interface comprises the WIP C-terminal domain and the WASp WH1 domain, which is regulated by WIP phosphorylation on S488 by PKCθ following T cell activation. The second interface comprises the WIP N-terminal domain and WASp VCA domain. The binding of WIP to WASp via this site is dependent on actin, which was shown to stabilize the WIP-WASp complex. With the use of a novel triple-color FRET technology [62], we demonstrated that TCR engagement results in a partial disassociation of the WIP-WASp complex. This mechanism of activation causes the disassociation of only the first binding site, involving the WIP C-terminal and WASp WH1 domains, while retaining the second interaction between WIP N-terminal and WASp VCA domains intact. Strikingly, these conformational changes within the WIP-WASp complex result in the exposure of WASp ubiquitylation sites located in the WH1 domain, leading to WASp degradation. We also found that WIP phosphorylation is responsible for the destabilization of the first interaction, and therefore, it is crucial for the activation and degradation of WASp and for the regulation of actin-dependent processes [18].

These findings, revealing a novel site for the interaction between WIP and WASp, are in agreement with a recent study of lymphocytes derived from XLT patients that demonstrated restoration of WASp levels by the expression of a synthesized 41-aa long peptide that includes the C-terminal WASp-binding domain of WIP [63]. The ex vivo overexpression of this peptide, termed nWIP, in lymphocytes derived from WAS pa-

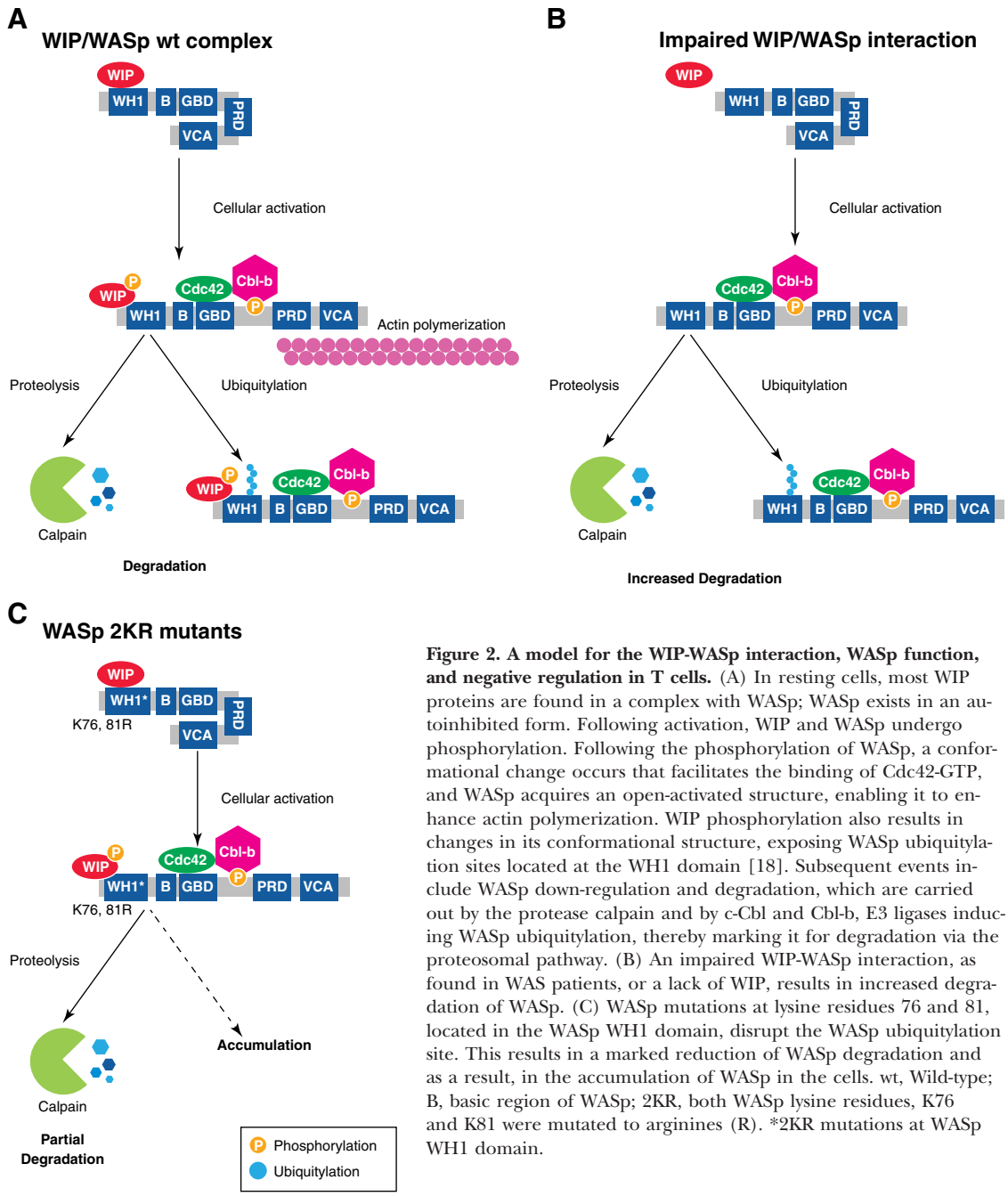

Figure 2. A model for the WIP-WASp interaction, WASp function, and negative regulation in T cells. (A) In resting cells, most WIP proteins are found in a complex with WASp; WASp exists in an autoinhibited form. Following activation, WIP and WASp undergo phosphorylation. Following the phosphorylation of WASp, a conformational change occurs that facilitates the binding of Cdc42-GTP, and WASp acquires an open-activated structure, enabling it to enhance actin polymerization. WIP phosphorylation also results in changes in its conformational structure, exposing WASp ubiquitylation sites located at the WH1 domain [18]. Subsequent events include WASp down-regulation and degradation, which are carried out by the protease calpain and by c-Cbl and Cbl-b, E3 ligases inducing WASp ubiquitylation, thereby marking it for degradation via the proteosomal pathway. (B) An impaired WIP-WASp interaction, as found in WAS patients, or a lack of WIP, results in increased degradation of WASp. (C) WASp mutations at lysine residues 76 and 81, located in the WASp WH1 domain, disrupt the WASp ubiquitylation site. This results in a marked reduction of WASp degradation and as a result, in the accumulation of WASp in the cells. wt, Wild-type; B, basic region of WASp; 2KR, both WASp lysine residues, K76 and K81 were mutated to arginines (R). *2KR mutations at WASp WH1 domain.

tients, not only restored WASp levels but also corrected the defects in actin-cytoskeleton reorganization. However, nWIP, although expressed at much higher levels, did not coprecipitate more WASp protein than full-length WIP [63]. This suggests that nWIP binds to WASp with lower affinity than full-length WIP, and additional regions of WIP may be required to stabilize and regulate the WIP-WASp interaction in resting and activated immune cells.

It was found that whereas WIP is necessary for protecting WASp from degradation, the levels of N-WASp in WIP$^{-/-}$ fibroblasts are normal [9]. These findings suggest that the stability of N-WASp is not dependent exclusively on WIP. Indeed, N-WASp was shown to be susceptible to degradation by the proteasomal pathway [64] but not by the calcium-dependent cysteine protease, calpain. This could be an intrinsic characteristic of N-WASp, differentiating it from WASp; in accordance with this option, WIP overexpression results in increased WASp but not N-WASp levels in T cells [9]. However, to date, there are no sufficient data regarding whether WIP interacts with similar or differentiated affinity with WASP or N-WASp, and additional studies are required to elucidate whether WIP is involved in mediating the differences and similarities between WASp and N-WASp function.

The specific susceptibility of N-WASp to proteasomal degradation may also depend on the effect of WIP family proteins CR16 and WICH/WIRE, which may supplement the protective activity of WIP. Indeed, it was shown in a recent study that WICH/WIRE and WIP are interchangeable in the case of vaccinia-induced actin polymerization and actin-based motility [43]. In addition, WIP and WICH/WIRE are involved in monocyte chemotaxis [65]. This suggest that WIP family proteins might also substitute for WIP in the protection of WASp/N-WASp from degradation in hematopoietic cells and perhaps in mediating other cellular functions. The overlapping activity of the WIP family members in the regulation of numerous cellular phenotypes is unknown. A comparison of the affinity of each member of the WIP family to WASp/N-WASp could provide a better understanding of the mechanism through which WIP protects WASp/N-WASp from degradation and those controlling the immune response.

An additional mechanism that might regulate the WIP-WASp complex involves the competition between WASp and other proteins for their binding to WIP. Recently, it was found that in drosophila, the protein Blow, required for myoblast fusion, competes with WASp for binding the drosophila ortholog of WIP (Sltr) [66]. This study suggested a mechanism by which Blow dissociates WASp from Sltr and promotes the formation of F-actin barbed ends, facilitating additional actin polymerization and branching. An ortholog or a homolog of Blow in mammals has not been revealed yet by bioinformatic analyses. The finding of a human Blow ortholog regulating WIP-WASp dynamics might shed light on the function of this complex in the regulation of actin polymerization.

WIP, WASP, PKC, AND THE IS

During the interaction between T lymphocytes and APCs, multiple signaling molecules are recruited to the IS, which consists of three major regions, referred to as SMACs. The cSMAC is formed from TCR-MHC complexes and is surrounded by the pSMAC, formed from LFA-1 complexed with its ligands. The pSMAC is, in turn, encircled by the distal SMAC, comprised mainly of CD45, a receptor tyrosine phosphatase [67, 68]. The formation and stabilization of the IS depend on F-actin polymerization and its movement toward the cSMAC [69].

PKCθ, a serine/threonine kinase, is a member of the Ca^{2+}-independent, novel PKC subfamily, expressed mainly in T cells, skeletal muscle cells, kidney cells, and lung cells [70]. It is required for NF-κB, NF-AT, and AP-1 transcriptional activity and for the production of IL-2 [71, 72]. PKCθ is considered a major regulator of T-lymphocyte survival, and in leukemic T cells, it was found to offer protection from Fas- or UV-induced apoptosis [73, 74]. PKCθ is recruited to the IS [75], following the activation of the costimulatory receptor, CD28 [76], localizing in between the cSMAC and the pSMAC.

Recently, it was found that PKCϵ and PKCη are also recruited to the T cell IS [77] and that they are required for the accumulation of PKCθ at this site. These data suggest that PKCϵ and PKCη are upstream regulators of PKCθ. However, whereas only a combined suppression of PKCϵ and PKCη resulted in the inhibition of the microtubule-organizing center polarization, suppression of PKCθ alone efficiently impaired this response, emphasizing its key role in T cell signaling. In agreement with this, we have clearly demonstrated that F-actin reorganization, following T cell activation, is regulated by WIP phosphorylation by PKCθ [18].

The radial symmetry of the IS plays a major role in stabilizing the intercellular contact by balancing and canceling out motile forces. The breaking of the radial symmetry of the immune synapse disrupts intercellular contact and allows T cell migration. The formation, disruption, and reformation of IS symmetry thus participate in the regulation of T cell activation and migration.

Interestingly, PKCθ negatively regulates the stability of the IS by causing periodic symmetry breaking of the pSMAC, whereas WASp is important for the stabilization of the IS and its symmetry [78]. These two opposing effects create cycles of IS formation, breaking of symmetry, and reformation. These cycles might be important for enhancing antigen sensing, leading to prolonged signaling [79].

Whereas it is currently unclear how PKCθ induces IS symmetry breakage in T cells, experiments performed in other systems suggest that this process involves cooperative myosin II activation [80]. A study performed on NK cells showed that upon cellular activation, myosin IIA binds to phosphorylated WIP, and a complex is formed consisting of WIP, WASp, myosin IIA, and actin [24]. It was suggested that the inclusion of WASp in this complex leads to the inhibition of myosin IIA. As myosin IIA contributes to the generation of contractile forces that destabilize the IS of T cells [81], its inhibition allows the reformation of pSMAC symmetry after the periodic symmetry breaking [78]. If this model is correct, it provides an interesting feedback mechanism of PKCθ activity in IS symmetry regulation, promoting WIP phosphorylation and the subsequent binding and inhibition of myosin IIA, thereby restoring IS symmetry. Further study is required to clarify the role of this molecular complex in the lifetime of the IS and the delicate balance between T cell/APC interaction, T cell spreading, and T cell migration.

WIP FUNCTIONS WITHIN MOLECULAR SIGNALING COMPLEXES

WIP regulates a wide range of cellular processes that control leukocyte activation (**Fig. 3**). These processes are mediated by the binding of WIP to various signaling proteins via its diverse molecular domains that control its molecular function. WIP possesses actin-regulatory properties by interacting with the actin machinery or by participating in the formation of actin-regulatory complexes.

WIP facilitates efficient actin polymerization independently of WASp by its interaction with multiple actin regulators

Following the engagement of leukocyte-activating receptors, WIP binds to several molecular elements, which, in turn, control actin dynamics and homoeostasis crucial for the immune

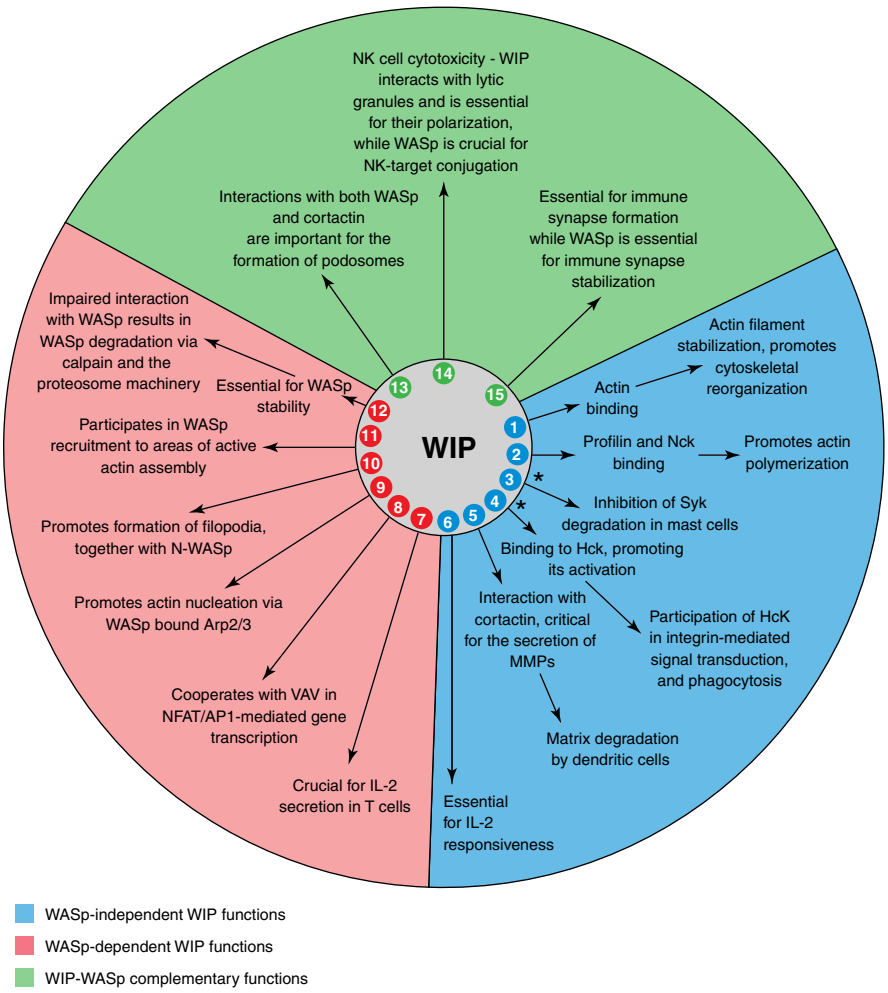

Figure 3. Model for WIP functions classified as WASp-independent, WASp-dependent, or those executed by complementary WIP and WASp mechanisms. *WASp-independent WIP functions (blue)*: 1. WIP binds to G- and F-actin and reduces the rate of actin disassembly [12]. 2. WIP was found to bind to profilin [8]. WIP may serve as a link between G-actin bound to profilin and various WIP-binding partners involved in actin regulation. This helps to provide new G-actin monomers to the actin polymerization machinery. 3. Syk is a tyrosine kinase that participates in mast cell signaling essential for degranulation. WIP protects Syk from degradation by the proteosomal pathway [26]. 4. WIP serves as an activator of Hck, a Src protein kinase responsible for mediating outside-in integrin signaling and cellular function of various immune cells, among them, monocytes [82]. 5. Cortactin associates with sites of dynamic actin reorganization at the actin core of forming podosomes [83, 84] and participates in MMP secretion [85]. WIP-cortactin interaction is essential for the maturation of podosomes and for the secretion of MMP [23]. 6. WIP plays a critical role in IL-2 responsiveness, CD25 up-regulation, and IL-2R signaling in T cells, independently of WASp [20]. *The importance of WASp in mediating WIP interaction with these proteins has not been fully determined. *WASp-dependent WIP functions (pink)*: 7. WIP KO T cells fail to secrete IL-2 following TCR activation, similar to T cells of WASp patients [19]. 8. The cooperation between WIP and VAV in promoting IL-2 production is dependent on WASp binding by WIP [86]. 9. WASp binds to Arp2/3, an actin nucleation factor, via its VCA domain, and activates it, thus enhancing actin branching [57]. 10. WIP participates in the bradykinin-Cdc42-N-WASp pathway, leading to the formation of filopodia [12]. 11. WIP-CrkL binding enables the recruitment of the CrkL-WIP-WASp complex to the IS [11]. 12. WIP is crucial for the retention of WASp; some mutations observed in WAS patients disrupt the interaction between WIP and WASp [6]. *WIP-WASp complementary functions (green)*: 13. DCs and macrophages of WAS patients completely lack podosomes [87]. When WIP binding to WASp is blocked, podosome formation is similarly impaired [15]. The WIP-cortactin interaction is important for the development and maturation of podosome structure. 14. WIP and WASp both play a role in NK cell cytotoxicity; whereas WAS patients have impaired conjugate formation of NK target cells [88], WIP is essential for lytic granule polarization and secretion [25]. 15. WIP is required for immune-synapse formation [19], whereas WASp is essential for IS stabilization and reformation after symmetry break events [78].

cell response. The proline-rich regions of WIP serve as binding sites for multiple binding partners, among them Nck, an adaptor protein responsible for coupling receptor tyrosine kinase activation to downstream signaling molecules. In T cells, Nck participates in the signaling cascade, starting from TCR ligation and leading to actin polymerization [89–91]. Nck contains three SH3 domains (SH3.1, SH3.2, and SH3.3) that interact selectively with target proteins. Interaction between the second SH3 domain of Nck and WIP PRD domain, aa 321–415, was initially reported to mediate Nck binding to WIP [42]. However, a more recent study provides a detailed analysis of the WIP-Nck interaction, demonstrating two WIP-Nck-binding sites at positions 247–261 aa and 328–342 aa sharing a common PXXPXRXL motif, which interacts exclusively with the second SH3 domain of Nck [43]. The third SH3 domain (SH3.3) of Nck, located at the C-terminus, mediates its interaction with WASp [92]. Both of these interactions lead to actin polymerization.

Importantly, most studies have focused on WIP recruitment to this complex, in parallel to Nck, to inhibit WASp/N-WASp activation and also to protect the former from degradation. However, two studies [43, 93] have shown that WIP provides an essential link between Nck and N-WASp, which in turn, activates Arp2/3 to induce actin polymerization. These data clearly demonstrate that WIP is an essential mediator in this signaling network.

WIP contains three ABM-2 motifs that can facilitate its binding to profilin. The cooperation between profilin and WIP is an important pathway promoting actin polymerization. Indeed, the first profilin-binding ABM-2 site is located in proximity to the G-actin-binding motif, suggesting that the simultaneous binding of actin and profilin to WIP may be required for actin polymerization, as in the case of the enabled/vasodilator-stimulated phosphoprotein conserved family of actin-regulatory proteins [8, 39, 94]. Moreover, WIP may serve as a link between profilin-bound G-actin with various WIP-binding part-

ners involved in actin regulation. Indeed, Nck was shown to coprecipitate with profilin in pull-down assays, although it does not contain a profilin-binding site, indicating that the two proteins rely on a mediator for their interaction. This suggestion is also corroborated by the lack of direct Nck-profilin interaction, as demonstrated in a yeast two-hybrid system [42], suggesting that WIP may mediate the interaction of Nck and profilin.

Actin remodeling can be also regulated by WIP association with cortactin [95], a protein that recruits the Arp2/3 complex to F-actin and promotes actin polymerization and rearrangement [22]. Study of WIP mutants by the yeast two-hybrid system suggests that WIP binds to cortactin via its PRD domain and the fourth SH3 domain (SH3.4) of cortactin. Indeed, the deletion of WIP aa 110–170 was found to prevent cortactin binding [95]. As WIP binds cortactin and G-actin monomers, it may serve as an interface between G-actin and cortactin-bound Arp2/3, thus enhancing actin nucleation and branching.

WASp-independent role of WIP in the regulation of leukocyte migration, motility, and adhesion

Migration and motility of leukocytes are crucial for appropriate antigen recognition and for the production of an efficient immune response. In addition, the recognition of pathogens is dependent on the adhesion of leukocytes to their targets following invasion into the site of inflammation. Cortactin activity is also associated with dynamic actin assembly at the leading edges of migrating cells, contributing to cellular motility [96] and the formation of podosomes [97], thereby enhancing adhesion. Cortactin was also suggested as participating in MMP secretion [85], a crucial step in the migration of various immune cells. The WIP-cortactin interaction was recently shown to be critical for the maturation of podosomes and for extracellular matrix degradation by murine DCs [23]. These findings suggested that the binding of WIP to cortactin is crucial for MMP secretion and localized matrix degradation. However, the exact mechanism by which WIP regulates MMP secretion is still unclear.

Integrin-mediated signal transduction involves the activity of the Src-family kinase Hck, which is expressed predominantly in hematopoietic cell types and is also implicated in phagocytosis, podosome formation [98], and promotion of the IFN-γ-independent activation pathway in monocytes (also known as the alternative activation pathway) in response to IL-13 stimulation [99]. WIP was found to bind Hck [82], but the exact mechanisms of the interaction are yet to be described. It is possible that the PRD of WIP facilitates this binding, as the SH3 domain of Hck is required for this interaction [82]. WIP was suggested to activate Hck, implying that WIP might play a role in the regulation of leukocyte integrin-mediated adhesion and migration. Interestingly, additional studies investigating the link between WIP and the Src family kinases, Src and Fyn, suggest that they are involved in signaling complexes that regulate processes of actin polymerization, NF-AT nuclear translocation, and IL-2 synthesis [100, 101]. However, further investigation is required to elucidate the cooperation between WIP and the Src family kinases in the regulation of immune cell function.

WIP-WASp-containing complexes are crucial for T-lymphocyte function

Another mechanism that links WIP activity to actin polymerization in T lymphocytes is its binding to the adaptor protein CrkL, forming a CrkL-WIP-WASp complex. This binding is mediated by the N-terminal SH3 domain of CrkL (SH3.1) and the proline-rich region of WIP. Following T cell activation, this complex is recruited to lipid rafts and to the IS by CrkL association with ZAP-70, a PTK that is recruited to the TCR upon T cell activation. This ZAP-70-CrkL-WIP-WASp complex, together with the phosphorylation of WIP by PKCθ, was suggested to couple TCR engagement to WASp activation and therefore, to the initiation of actin polymerization [11].

In T cells, the adaptor protein Nck binds directly to the Rho family guanine nucleotide exchange factor, Vav1 [102], forming a triple-molecular complex together with Src homology 2 domain-containing leukocyte protein of 76 kDa. Nck recruits WASp to the TCR site, whereas Vav1, in turn, activates Cdc42, a Rho family GTPase responsible for WASp activation [103]. Vav1 is a regulator of the IL-2 promoter and of NF-AT/AP-1-mediated gene transcription [104, 105]. WIP was shown to cooperate with Vav1 in the regulation of T cell activation and in the induction of NF-AT/AP-1 activation via a region of WIP, encompassing aa 112–237. Moreover, with the use of a deletion mutant of WIP lacking its WASp-binding domain, it was shown that the ability of WIP to enhance NF-AT/AP-1 activation and IL-2 production in the presence of Vav1 depends on WASp binding to WIP [86]. Indeed, similar defects in actin polymerization and cellular proliferation were observed in WASp and $Vav1^{-/-}$ T cells [106, 107], a similarity that can be explained by the fact that both proteins cooperate with WIP in promoting these central T cell functions. However, the precise role of the WIP-WASp-Vav1 cooperation in the regulation of these processes is unknown.

To date, most studies examine the impact of WASp or N-WASp on Arp2/3-mediated actin polymerization outside of the context of WIP. To distinguish between WIP functions that are WASp dependent from those that are WASp independent, WIP-WASp or N-WASp complexes need to be investigated specifically. In our recently published study, we have examined the interplay between WIP and WASp and followed the dynamics of their molecular complex throughout TCR activation. We have demonstrated a tight connection among WIP phosphorylation, WIP binding to actin, and cellular actin rearrangement. We showed that WIP phosphorylation, following TCR stimulation, is crucial for the interaction of WIP with actin, which is crucial for the stabilization of the WIP-WASp complex. FRET-based analysis revealed that a WIP mutant mimicking its unphosphorylated form displayed a decreased binding of actin compared with wild-type WIP or with a mutant mimicking constitutively phosphorylated WIP. In addition, expression of the "unphosphorylated" WIP impaired T cell spreading significantly, clearly demonstrating the importance of WIP phosphorylation for proper actin rearrangement [18].

Altogether, these data support the hypothesis that the multiple functions of WIP are dictated by its involvement in the formation of multiple molecular complexes involving a large range of associated proteins.

WIP FUNCTION IN THE VARIOUS IMMUNE CELL TYPES

Human and mouse systems have been used for studying the role of WIP and WASp in immune cells. In several cases, these organisms differ in the effects of WIP and WASp deficiencies on the immune system, resulting in greater defects in the human system compared with the mouse. Therefore, the study of the role of WIP in the regulation of immune response using the mouse model needs to be considered with appropriate concern. Various effects of WIP and WASp deficiencies on immune cell function in the mouse and human systems are summarized in Table 1. One conclusion that could be drawn from these studies is that WIP deficiency results in greater immune cell defects (at least in some cases) compared with WASp-deficient cells. This strongly indicates that immune cell regulation by WIP may involve several signaling pathways that go beyond WASp activity.

A major problem that we encountered while gathering these data was to separate WIP functions that are WASp dependent from those that are WASp independent. The reason for this is the key role of WIP in the stabilization and function of WASp, preventing the specific KO of WIP without down-regulating WASp activity. A future challenge for the field will be to generate WIP-deficient models that express intact WASp levels, perhaps by the expression of the WIP portion that protects WASp from proteolysis. Besides the ability to distinguish between WASp-dependent and -independent function of WIP, the identification of this WASp-stabilizing peptide will have far-reaching implications for treating WAS and XLT patients. An additional approach that might assist in addressing this challenge is to express the ubiquitylation-resistant variant of WASp [7], in which stability is not dependent on WIP, in combination with WIP suppression or expression of WIP mutants with impaired activity. The generation of this system could significantly advance our understanding of WIP unique properties that are independent of WASp.

Lymphocytes

Isolated lymphocytes from WAS patients exhibit a relatively smooth surface and fail to spread and proliferate in response to external stimulation. These phenomena are a result of WASp deficiency, leading to impaired formation of membrane structures [108]. To define the role of WIP in the immune response and specifically in the development of WAS immune dysfunctions, $WIP^{-/-}$ mice were generated. WIP-null mice display severe lymphopenia, reduction of the white pulp, and a reduced number of B lymphocytes [109]. $WIP^{-/-}$ T cells fail to proliferate (see Table 1), to secrete IL-2, and to increase their F-actin content following TCR ligation [19]. These cells spread very poorly on an anti-CD3-activating surface, displaying a reduced ability to polarize and to form cortical protrusions. Interestingly, $WIP^{-/-}$ in T cells results in impaired IS formation, whereas IS formation is intact in $WASp^{-/-}$ cells [78]. These results might suggest that the role of WIP in the IS is independent of that of WASp.

Proliferation of T cells in response to stimulating antibodies was studied in human WAS patients, as well as in transgenic mice models. It was found previously that T cells taken from WASp KO mice [107, 110] or patients [111] proliferate poorly in response to immobilized anti-CD3. However, in response to soluble anti-CD3, introduced by APCs, WASp KO cells proliferate normally. Interestingly, WIP/WASp double-KO eliminates T cell proliferation in response to immobilized or soluble anti-CD3 and also results in a defective IL-2 response, failing to up-regulate CD25 and induce STAT5-dependent gene expression [20]. These findings suggest that the defects in the immune response of double-KO T cells [20], as well as of WIP KO cells (in which WIP deficiency also results in the depletion of WASp) [19], are greater than those of WASp KO alone.

Indeed, in human T cells, WIP deficiency causes impaired proliferation, a defective response to IL-2, and abrogates chemotaxis completely toward the C-X-C motif of the chemokine immune protein-10. In cells taken from WAS patients, however, the proliferative response was only diminished partially [13].

Interestingly, whereas B cells from $WIP^{-/-}$ mice exhibit defects in the actin network, they demonstrate increased proliferation in response to BCR ligation. This suggests that an intact actin cytoskeleton stabilized by WIP may negatively regulate B cell activation [19].

In NK cells, WIP is part of a large protein complex, consisting of WIP, WASp, actin, and myosin IIA [24]. Following NK cell activation, WIP phosphorylation is essential for the formation of this complex, which is recruited to the NK cell, target cell contact site, where it is involved in actin polymerization. At the NKIS site, WIP was shown to colocalize with lytic granules [25]. It is, as yet, unclear whether the interaction of WIP with lytic granules is direct or whether it is mediated by surface granule proteins, such as actin, which are binding partners of WIP. These binding partners may thus serve as an interface between WIP and cytolytic granules. As WIP affects WASp levels in NK cells, and WASp is important for IS stability in T [78] and NK cells [112], it is possible that the decreased WASp levels caused by WIP KO may result in the instability of the IS, thus impairing cytotoxicity. Indeed, WIP knockdown in NK cells results in a nearly complete loss of cytotoxicity of the cells, whereas WASp knockdown results in partial loss of cytotoxicity [24], indicating that the function of WIP in lytic granule polarization goes beyond its role in the maintenance of WASp stability. It is possible that the entire multiprotein complex could be involved in the transport of the granules, whereas WIP may serve as the link between the actin cytoskeleton and lytic granule secretion. It was suggested that myosin IIA transports WIP and WASp while moving along the actin cytoskeleton to the IS, where WASp can induce Arp2/3-dependent actin polymerization, and WIP can stabilize the actin filaments [24].

TABLE 1. A Comparison of WASp and WIP Deficiencies in Humans and in Mice

	WASp deficiency		WIP deficiency	
	Human (WAS)	Mouse	Human	Mouse
Pathology	Thrombocytopenia, eczema, recurrent infections, increased incidence of autoimmune disorders, and a tendency toward hematopoietic malignancies [116]	Immunodeficiency and autoimmune diseases, such as colitis [117]	Recurrent infections, eczema, and thrombocytopenia[13]	Ulcerative colitis, pneumonitis, splenomegaly, and premature death [109]
Hematology	Severe thrombocytopenia and low-platelet volume[118]; platelets coated with IgG antibodies [119]	Moderate reduction in platelet number, less of a reduction than in humans [110]; no accumulation of platelet-associated IgAs [115]	Thrombocytopenia; platelet volume is normal [13].	Normal platelet number and volume were reported in $WIP^{-/-}$ mice; moderate thrombocytopenia described in another study [109, 115].
	Lymphopenia, caused mainly by decreased T cell number; B cell numbers are moderately depressed [2].	Substantial decrease in lymphoid cell numbers [107]	Low B and T cell numbers [13]	Platelets are coated with IgA antibodies; impaired GPVI signaling [115].
	Normal or elevated NK cell percentages [88]	NK cells expressing WASp are in higher percentages relative to $WASp^{-/-}$ cells as a result of enhanced proliferation and/or survival [120].	Increased number and percentage of $CD16^{+}CD56^{+}$ NK cells [13]	Granulocytosis, severe lymphopenia with reduced B and T cell numbers; increased hematopoietic tissue and red pulp and reduced white pulp [109]
Cellular function T cells	Reduced T cell proliferation in response to TCR stimulation [108, 111]	Reduced TCR-induced proliferation [107]	Defective T cell proliferation and chemotaxis [13]	T cells fail to proliferate [19].
	Reduced IL-2 production[111]	Reduced IL-2 production [107, 110, 122]	Impaired response of T cells to IL-2 [13]	IL-2 responsiveness and IL-2R signaling were impaired [20] in double-KO mice; another study detected a reduction in IL-2 production [19].
	Partial defects in T cell immune synapse formation with anomalous morphology [121]	Immune synapse initially forms [122], but its stability is decreased [78].		Defective conjugate formation with superantigen-presenting B cells and anti-CD3 bilayers [19]
	Defective actin rearrangement [63, 121]	Actin polymerization disrupted in some mouse models [107] but not in others [122, 123]		T cells fail to polarize and to increase their F-actin content following TCR ligation [19].

(continued on next page)

Table 1. ***(continued)***

	WASp deficiency		WIP deficiency	
	Human (WAS)	Mouse	Human	Mouse
	Abnormal morphology [108]			
B cells	Defective signaling of B cells [124]; F-actin content is markedly reduced [125].	Mild hyper-responsiveness; cells exhibit reduced BCR internalization [126].	No cellular function assays have been reported.	B cells proliferate more extensively and exhibit an overall enhanced response to various stimuli [19].
NK	Impaired cytotoxicity of NK cells [88]	NK cytotoxicity partially impaired [127]	Impaired NK cell cytolytic activity and reduced expression of NKG2D, NKp30, and NKp46 [13]	Significant reduction in cytotoxicity
				Lytic-granule polarization and conjugation of NK cells to the target cells are impaired [24, 25].
Phagocytes	Macrophages and DCs isolated from WAS patients completely lack podosomes [87].	DCs and macrophages fail to form functional podosomes; large focal adhesion structures are assembled instead [14].	No cellular function assays have been reported.	$WIP^{-/-}$ DCs fail to form podosomes, instead forming large focal adhesion structures [14].

NK cells from WAS patients have impaired cytotoxicity and migration [88, 113]. Interestingly, IL-2 treatment of WASp-deficient NK cells restores their function, i.e., increases their cytotoxicity and the F-actin content at the NKIS site. It was shown that this restoration is independent of WASp/N-WASp activity, but rather, it is a consequence of the activation of the WASp homolog WAVE2. Indeed, the activity of WAVE2 was shown to be responsible for the induction of NK cell function in the absence of WASp [114]. These data strongly indicate that WAVE2 and WASp are responsible for the activation of parallel and independent pathways that control F-actin reorganization and NK cell function. With the consideration that WAVE2 stability and function are not dependent on WIP and as these cells are functional and lack WASp, WASp-deficient NK cell treated with IL-2 can serve as a model system for studying the unique, WASp- independent functions of WIP in leukocyte biology.

Mast cells

In mast cells, degranulation and secretion are associated with the rearrangement of the actin cytoskeleton, suggesting a role for WIP in mast cell effector function. Indeed, WIP was shown to play essential roles in IL-6 secretion, following high-affinity FcεR ligation and mast cell degranulation [26]. WIP was also found to inhibit the degradation of Syk, a tyrosine kinase essential for FcεR signal propagation and mast cell degranulation. The mechanism by which WIP protects Syk may involve interference with calpain activity and the prevention of Syk targeting for proteosomal degradation. The kinetics of cellular shape changes induced by FcεR in BMMCs from $WIP^{-/-}$ mice are altered, and their baseline F-actin level is reduced compared with normal BMMCs [26].

Platelets

WAS patients present a wide range of phenotypes that often include thrombocytopenia associated with reduced platelet volume. Recently, it was shown that WIP KO in mice causes increased platelet clearance and results in thrombocytopenia [115]. These results contradict a prior study showing normal platelet counts in WIP KO mice [109]. WIP KO platelets exhibit defects in α-granule secretion, integrin $\alpha_{IIb}\beta_3$ activation, calcium mobilization, and actin assembly. Interestingly, WIP KO platelets accumulate platelet-associated Ig of the IgA class. These antibodies impair GPVI responses but do not affect platelet survival. The mechanism for the binding of IgA to the platelet surface is currently unclear.

Macrophages and DCs: WIP and podosomes

WASp-dependent and -independent functions of WIP in phagocytes, i.e., macrophages and DCs, have been related to their migration via the formation of podosomes. DCs and macrophages cultured from WAS patients completely lack podosomes, despite the presence of N-WASp, demonstrating that WASp serves as a key function in these cells [87]. WASp deficiency in mice also results in a failure of podosome formation

(see Table 1). As expected, WIP-null DCs possess an almost identical phenotype to that of WASp-null DCs and are generally devoid of podosomes, preferentially displaying long-living actin structures, termed "large focal contacts" [10] (**Table 1**). Additionally, it was shown that WIP is required for the localization of WASp to podosomes [10]. The interaction between WIP and WASp is important for monocyte chemotaxis, cell polarization [65], and podosome formation [15].

The migration of these cells requires the assembly of podosomes and their disassembly. The process of podosome disassembly and turnover is required to allow the progression of migrating cells. Calpain activity in the regulation of podosome composition is essential for the proper turnover of podosomes; calpain was shown to cleave WASp in migrating DCs [128]. WASp cleavage permits podosome disassembly and thus, plays a role in the regulation of podosome dynamics. As WIP is known to protect WASp from the degradation process in T cells [7, 9], it would be interesting to clarify further the role of WIP in the regulation of WASp degradation in the case of podosome turnover.

Recently, it was found that in DCs, the cortactin domain of WIP is necessary for podosome maturation and matrix degradation. This study indicates that whereas WIP is essential for podosome formation, the interaction of WIP with cortactin is important for the development and maturation of podosome structure and for the recruitment and secretion of MMPs [23].

A summary of various functions of WIP, organized according to their reliance on WASp, is presented in Fig. 3.

CONCLUDING REMARKS

WIP was first discovered as a binding partner and regulator of WASp, but further research has revealed its important functions that go beyond its role as a WASp chaperon. Intense research has uncovered direct roles of WIP in the regulation of actin polymerization, formation of actin-rich cytoplasmic protrusions, gene expression, and other cellular functions in mouse and human systems. The understanding of the regulation of WIP, its interaction with its associated proteins, and its complex cross-talk with immune cell activation and cytoskeleton systems can greatly enhance our understanding of the immune response. Furthermore, investigation of the roles of WIP in the immune system may yield valuable insights leading to the development of novel therapeutic approaches for immunopathologies, e.g., WAS and XLT.

AUTHORSHIP

M.B-S., S.F., O.M., and E.N. performed the literature search, compiled information, and prepared drafts of the manuscript. M.B-S. is the senior author; she corrected the drafts and provided feedback, as well as suggestions regarding presentation of the information.

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DISCLOSURES

The authors declare no conflict of interest.

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