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WASP Family Proteins: Molecular Mechanisms and Implications in Human Disease

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Abstract

Proteins of the Wiskott-Aldrich syndrome protein (WASP) family play a central role in regulating actin cytoskeletal dynamics in a wide range of cellular processes. Genetic mutations or misregulation of these proteins are tightly associated with many diseases. The WASP-family proteins act by transmitting various upstream signals to their conserved WH2-Central-Acidic (WCA) peptide sequence at the C-terminus, which in turn binds to the Arp2/3 complex to stimulate the formation of branched actin networks at membranes. Despite this common feature, the regulatory mechanisms and cellular functions of distinct WASP-family proteins are very different. Here, we summarize and clarify our current understanding of WASP-family proteins and how disruption of their functions is related to human disease.

Introduction

Dynamic rearrangements of the actin cytoskeleton are essential to all eukaryotic organisms. Polymerization of actin provides locomotive forces to drive fundamental processes that involve membrane deformation, such as cell migration, neuron growth, and vesicle trafficking (Blanchoin et al., 2014; Luo, 2002; Pollard and Cooper, 2009). The rate limiting step of actin polymerization is the formation of a nucleus formed by several actin monomers, which creates a barbed end that can spontaneously grow a new actin filament. Efficient actin polymerization in the cell requires a regulatory factor to facilitate the nucleation process (Pollard, 2016). One central factor that promotes actin nucleation is the Arp2/3 complex (actin-related-protein 2/3 complex), which acts by binding to the side of an existing actin filament to generate a new, branched filament (Blanchoin et al., 2000; Machesky et al., 1994). Basally inactive, the Arp2/3 complex needs to be activated by proteins collectively known as nucleation promoting factors, or NPFs, through direct protein-protein interaction (Goley and Welch, 2006; Higgs and Pollard, 2001; Rotty et al., 2013). Proteins of the Wiskott-Aldrich syndrome protein (WASP) family are major NPFs, playing an essential role in directing a large diversity of upstream signals to Arp2/3-mediated actin polymerization in many important processes throughout the cell (Figure 1).

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WASP-family proteins in mammals include nine different members, falling into five different groups based on their sequence similarity: WASP and N-WASP (neuronal-WASP); WAVE1, WAVE2, and WAVE3 (Wiskott Aldrich syndrome protein and verprolin homologue, also known as SCAR for suppressor of cAMP receptor); WASH (Wiskott-Aldrich syndrome protein and SCAR homologue); WHAMM (WASP homolog-associated protein with actin, membranes and microtubules) and JMY (junction-mediating and -regulatory protein); and the recently discovered WHIMP (WAVE homology in membrane protrusions) (Alekhina et al., 2017; Campellone et al., 2008b; Derry et al., 1994; Kabrawala et al., 2020; Kollmar et al., 2012; Linardopoulou et al., 2007; Miki et al., 1998a, 1996; Shikama et al., 1999; Suetsugu et al., 1999; Zuchero et al., 2012). The feature that defines all WASP family proteins is their conserved C-terminal WCA (WASP homology 2 (WH2), central, acidic) sequence, which is able to bind to and activate the Arp2/3 complex (Figure 1, left) (Higgs et al., 1999; Machesky and Insall, 1998). The WH2 motif—sometimes multiple WH2 motifs in a row—binds to actin monomers and is necessary for recruiting actin to the Arp2/3 complex. The WH2 motif is also found in a variety of other actin regulators, such as Spire, Cobl, WIP, and VopL/VopF (effector proteins from bacterial pathogen *Vibrio*) (Husson et al., 2011; Paunola et al., 2002; Pernier et al., 2013; Quinlan et al., 2005; Vaduva et al., 1999). The CA sequence in the WCA is responsible for binding Arp2/3. Two individual CA sequences can bind to two distinct locations on Arp2/3, which induces conformational changes necessary for initiating actin polymerization (Marchand et al., 2001; Padrick et al., 2011, 2008; Padrick and Rosen, 2010; Panchal et al., 2003; Shaaban et al., 2020; Zimmet et al., 2020). Except this common WCA sequence, WASP-family proteins contain distinct N-terminal domains and linker sequences, which dictate their differences in activity regulation, membrane localization, ligand interaction, and cellular functions (Figure 1). In this review, we summarize what is known of the structure and regulation of each WASP-family protein and the different ways in which they are associated with disease. We also provide analysis showing WHAMM and JMY are homologous proteins and may share a similar mechanism for regulation.

WASP and N-WASP

WASP and N-WASP are the founding members of the WASP protein family and are the best understood compared to other WASP-family proteins (Imai et al., 2003; Snapper and Rosen, 1999; Thrasher and Burns, 2010). Here, we discuss them together as the two proteins share similar domain architectures, regulatory mechanisms, and cellular functions. One major distinction between the two proteins is that WASP is specifically expressed in hematopoietic cells and N-WASP is ubiquitously expressed in human tissues (Miki et al., 1996; Uhlén et al., 2015). Therefore, mutations in WASP usually have a profound impact on the immune system, while N-WASP is involved in broader physiological processes.

WASP was first discovered in 1994 as the gene product responsible for Wiskott-Aldrich syndrome (WAS), an X-linked immune disorder affecting roughly 1 in 300000 males and characterized by the “trifecta” of eczema, thrombocytopenia, immune deficiencies, and chronic, recurring infections (Derry et al., 1994; Massaad et al., 2013; Notarangelo et al., 2008; Orange et al., 2002). N-WASP was soon discovered in 1996 as a Grb2-binding protein from brain lysate, which shared ~50% sequence homology with WASP (Miki et

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al., 1996). Even before the discovery of WASP, the connection between WAS and the actin cytoskeleton was noticed from anomalies associated with the actin cytoskeleton in the white blood cells of WAS patients, including reduced microvilli structure on neutrophils, defects in the actin cytoskeleton of T-cells and platelets, and reduced actin polymerization when treated with antigen (Facchetti et al., 1998; Gallego et al., 1997; Kenney et al., 1986; Molina et al., 1992). WASP was soon found to bind the Rho-family GTPase Cdc42 and use its C-terminal 59 amino acids (now known as the WCA sequence) to promote actin polymerization through the Arp2/3 complex in cells (Marchand et al., 2001; Miki and Takenawa, 1998; Panchal et al., 2003; Symons et al., 1996). Since then, a plethora of biochemical, structural, and cellular work over the past ~25 years has revealed how the activity of WASP and N-WASP is finely regulated by several layers of control.

WASP and N-WASP contain three domains, an N-terminal WASP homology 1 (WH1) domain, a central GTPase binding domain (GBD), and the C-terminal WCA domain (with N-WASP containing an additional W element, herein denoted as WWCA). Connecting these domains are long, unstructured sequences with important regulatory roles, including the proline-rich region (PRR) between GBD and WCA, and the polybasic sequence next to the GBD. WASP/N-WASP are autoinhibited in the basal state, and Cdc42 plays a central role in relieving the inhibition and activating WASP/N-WASP towards the Arp2/3 complex (Abdul-Manan et al., 1999; Higgs and Pollard, 2000; Kim et al., 2000; Rohatgi R. et al., 1999). In the autoinhibited state, the C-helix in WCA is associated with the GBD, which harbors the Cdc42/Rac Interacting Binding (CRIB) motif. This conformation keeps WCA sequestered from accessing Arp2/3 (Figure 2A, left). Cdc42 binding to the CRIB motif leads to a dramatic structural rearrangement in GBD, which both eliminates the inhibited conformation and occludes the surface that is required for sequestering WCA. Thus, Cdc42 uses a direct competition mechanism to release WCA from GBD, which in turn can bind Arp2/3 to trigger actin polymerization (Figure 2A, middle) (Prehoda et al., 2000; Rohatgi et al., 2000).

Many other mechanisms often act cooperatively with Cdc42 to promote WASP/N-WASP activation. These include phosphorylation, acidic phospholipid binding, and adaptor protein binding (Figure 2). Phosphorylation of WASP by various families of kinases, including Src (Hck, Fyn, and Lck), Tek (Itk and Btk, CK2, PAK4, FAK, Ack), Abl (Abl1 and Arg), and DYRK (Dyrk1a), is clustered in the WH1, GBD, PPR, and WCA regions (Banin et al., 1996; Bunnell et al., 1996; Cory et al., 2003, 2002; Finan et al., 1996; Guinamard et al., 1998; Hornbeck et al., 2015; Labno et al., 2003; Miller et al., 2010; Park et al., 2012; Suetsugu et al., 2002; Wu et al., 2004; Yokoyama et al., 2005; Zhao et al., 2017). Phosphorylation in the WH1 domain (e.g., Y102 in WASP) can disrupt WIP-WASP interaction to destabilize WASP and reduce WASP protein level and activity. By comparison, phosphorylation in the GBD (e.g., Y291 in WASP) and the WCA (e.g., S483, S501 in WASP) typically enhances WASP activity, either by relieving the GBD-WCA auto-inhibition or by increasing the affinity of WCA to Arp2/3 (Badour et al., 2004; Cory et al., 2002; Guinamard et al., 1998; Hornbeck et al., 2015; Thrasher & Burns, 2010). It is worth noting that phosphorylated Y291 in WASP can in turn provide a docking site for SH2 domain-containing proteins such as Src-family kinases, which can further stabilize the activated conformation by sterically blocking WCA binding (Torres and Rosen, 2003). Inositol phospholipids (e.g., PIP_2) interact with the

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polybasic sequences near the GBD domain, which can facilitate WASP/N-WASP activation likely through two distinct mechanisms simultaneously: 1) increasing membrane localization and local protein concentration of WASP/N-WASP and 2) influencing GBD-WCA stability and shifting it from the autoinhibited to activated conformation (Higgs and Pollard, 2000; Papayannopoulos et al., 2005).

Various multiple-domain scaffolding proteins, including WASP Interaction Protein (WIP), Toca-1/2, NCK, IQGAP1, Grb2, CIP4, Intersectin, Tuba, FCHSD2, Nebulin, and Amphiphysins I and II provide another important layer of control over the localization and organization of WASP/N-WASP at cell membranes (Figure 2) (Almeida-Souza et al., 2018; Bu et al., 2009; Carlier et al., 2000; Falcone et al., 2014; Ho et al., 2004; Hussain et al., 2001; Le Clainche et al., 2007; Ramesh et al., 1997; Rohatgi et al., 2001; Salazar et al., 2003; Takano et al., 2010; Yamada et al., 2009). Among them, WIP, and possibly its homologues CR16 and WICH, plays a unique role. It forms a stable complex with the WH1 domain of WASP/N-WASP in the cell (Antón et al., 2007; Ho et al., 2001; Kato et al., 2002). This interaction does not directly contribute to WASP/N-WASP activation but plays an essential role in protecting WASP/N-WASP from degradation. WIP can also link WASP/N-WASP to other adaptor proteins, such as CrkL and Zap70, which are key adaptors for recruiting WASP to the immunological synapse (Figure 2) (Sasahara et al., 2002; Volkman et al., 2004; Zettl and Way, 2002).

Many adaptor proteins, such as Nck, Grb2, WISH, Intersectin I, CrkL, and Tks5, contain multiple SH3 domains, and they can regulate WASP/N-WASP activity by simultaneously binding many SH3-binding motifs located in the PRR of WASP/N-WASP (Figure 2A, right) (Fukuoka et al., 2001; Hussain et al., 2001; Oda et al., 2001; Oikawa et al., 2008; Ramesh et al., 1997; Rivero-Lezcano et al., 1995; She et al., 1997). The multivalent interactions between SH3-containing proteins and the PRR of WASP/N-WASP promote protein oligomerization, which can concomitantly trigger liquid-liquid phase separation (LLPS). Through LLPS, relevant signaling molecules are quickly confined and organized in condensed protein droplets, which can produce a sharp increase in stimulating Arp2/3-mediated actin polymerization (Banjade and Rosen, 2014; Li et al., 2012). In addition to their ability to promote LLPS, these SH3-containing adaptors often harbor other protein-protein interaction domains, which can connect WASP/N-WASP to upstream signaling molecules through additional multivalent interactions. One of the most commonly seen domains is the SH2 domain, which binds to phosphorylated tyrosine residues in the intracellular domains (ICD) of membrane proteins, such as nephrin (an adhesin molecule in kidney podocytes) and LAT (a membrane protein important to T-cell receptor, or TCR, signaling). These ICDs usually contain multiple tyrosine-phosphorylation sites, which can be quickly phosphorylated/dephosphorylated in response to kinase signaling, allowing for docking multiple SH2-containing adaptors in a highly tunable manner. These additional multivalent interactions, plus the clustering of membrane proteins, are important not only for further enhancing the nonlinear LLPS behavior of WASP/N-WASP on the surface of lipid bilayers, but also for linking specific signaling events on the membrane to WASP/N-WASP-mediated actin polymerization. Together, the above mechanisms provide highly precise and tunable spatiotemporal control of actin polymerization at the plasma membrane to support a particular process, such as TCR signaling and kidney podocyte foot process

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formation. Such adaptor protein-mediated signaling has now emerged as a common theme for the regulation of WASP and N-WASP activity in the cell (Figure 2A, right) (Banani et al., 2017; Banjade et al., 2015; Case et al., 2019b, 2019a; Ditlev et al., 2019; Kim et al., 2019).

WASP expression is limited to hematopoietic cells, and WASP-mediated actin polymerization plays a key role in T-cell receptor signaling in the formation of the immunological synapse between T cells or natural killer (NK) cells and antigen presenting cells (APC) (Blundell et al., 2010; Burns et al., 2004; Calvez et al., 2011; Dupré et al., 2002). This explains the profound impact of WASP mutations on the immune system (Massaad et al., 2013). Disruption of WASP activity impairs immunological synapse formation, which will reduce infection clearance and antibody production (De Meester et al., 2010; Gismondi et al., 2004; Orange et al., 2002). WASP is also important for cell motility, intercellular trafficking, phagocytosis, chemotaxis of macrophages, and the ability of NK cells to screen and clear infected or malignant cells, which can contribute to lymphoreticular tumors and leukemia malignancies (Burns et al., 2004; Derry et al., 1994; Menotti et al., 2019; Murga-Zamalloa et al., 2017; Snapper et al., 2005). In addition, WASP (with N-WASP and WAVE as well) plays a role in autoimmunity, where it dampens B-cell signaling by triggering actin polymerization to prevent B-cell receptor (BCR) clustering and by stimulating the removal of activated BCR from the membrane through endocytosis (Liu et al., 2013; Massaad et al., 2013; Recher et al., 2012; Volpi et al., 2016; Westerberg et al., 2012). Besides its function in the cytosol, WASP also plays an important role in promoting Arp2/3-mediated actin polymerization in the nucleus, which is required for driving double-strand breaks into discrete sub-nuclear clusters to facilitate homology-directed DNA repair (Schrank et al., 2018).

Mutations in WASP are the cause of Wiskott-Aldrich syndrome and related syndromes, including X-linked thrombocytopenia (XLT) and X-linked neutropenia (XLN). Since the discovery of WAS, sequences from over 400 different patients have been studied, leading to a wealth of data on mutations in WASP that can cause disease (reviewed in Jin et al., 2004 and compiled online at http://pidj.rcai.riken.jp/waspbase/). The mutations include nonsense, frameshift, and splicing site mutations, as well as over 50 individual missense mutations across the entire sequence. While nonsense and frameshift mutations diminish the production of functional full-length protein, missense mutations can have effects ranging from protein level to regulation (Kolluri et al., 1995; MacCarthy-Morrogh et al., 1998). In general, mutations that result in the loss of WASP protein cause more severe symptoms than those that leave WASP protein levels reduced or unaffected (Imai et al., 2003).

The missense mutations found in human patients are primarily localized to the WH1 domain (with some scattered in the GBD and WCA region, as indicated by “hotspots” in Figure 2B), which is believed to impair the interaction between WASP and WIP. Consequently, this leads to the degradation of WASP and disrupts its localization at the immunological synapse (Antón et al., 2007; Jin et al., 2004; Volkman et al., 2004). By comparison, missense mutations in the GBD or the WCA domain may affect the inhibition or activation of WASP and its ability to promote Arp2/3 activation. While most mutations impair WASP activity, several mutations have an opposite, stimulatory effect. For example, a mutation in

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the GBD, L270P, which was found in patients suffering from X-linked Severe Congenital Neutropenia (XLN), was shown to disrupt the autoinhibition of the GBD-WCA interaction to produce a constitutively active WASP (Devriendt et al., 2001). The different, albeit similarly pathogenic, effects of disruptive vs. stimulatory mutations speak for the importance for the cell to have a precise control of WASP activity level. In addition to diseases caused by genetic mutations in WASP, mutations in WASP regulators like WIP and Cdc42 are also often associated with diseases closely related to WAS. For example, nonsense mutations in WIP cause Wiskott-Aldrich syndrome 2 (WAS2), likely due to the loss of WIP expression and the subsequent destabilization of WASP (Schwinger et al., 2018). Many missense mutations in Cdc42 lead to Takenouchi-Kosaki syndrome (TKS), which is a complex congenital developmental disorder affecting multiple organ systems, including the psychomotor, cardiac, and hematologic/lymphatic system, as well as recurrent infections (Martinelli et al., 2018).

The only effective therapy for WAS to date is hematopoietic stem cell transplantation from non-affected donors (Burroughs et al., 2020; Massaad et al., 2013). Wiskostatin, a small-molecule inhibitor of WASP that binds to the GBD to stabilize the autoinhibited conformation, has a potential for treating conditions caused by hyperactive WASP, but there is still a long way to go before its clinical application (Guerriero and Weisz, 2007; Peterson et al., 2004).

By comparison, N-WASP is ubiquitously expressed and plays a wide variety of roles in physiology, even though no genetic mutations have been reported to directly link N-WASP to a particular disease. On the cellular level, N-WASP has an essential role in endocytosis, where its activity is required for assembling actin filaments at clathrin-coated pits, which is important for pinching off the pits and propelling the internalized vesicle from the plasma membrane into the cytosol (Benesch et al., 2005; Hussain et al., 2001; Innocenti et al., 2005; Qualmann et al., 1999; Salazar et al., 2003; Shin et al., 2008; Taunton et al., 2000; Yamada et al., 2009; Zhang et al., 2009). Moreover, N-WASP is important to a variety of cellular structures, including the formation of filopodia (finger-like protrusions at the leading edge of migrating cells or growing neurons), podosomes, invadopodia, tight junctions, and the maintenance of Golgi morphology – including anterograde trafficking from the trans-Golgi network (TGN) to the endoplasmic reticulum (ER) and dispersal of the Golgi network after DNA damage (Figure 1) (Bhattacharya et al., 2016; Ivanov et al., 2005; Kovacs et al., 2011; Linder et al., 1999; Luna et al., 2002; Mizutani et al., 2002; Taunton et al., 2000; Tsuboi et al., 2006; Wen et al., 2020; Yamaguchi et al., 2005). Similar to WASP, N-WASP also plays an important role in the nucleus, where it can interact with the PSF-NonO complex and promote nuclear actin polymerization to regulate RNA polymerase II transcription activity (Wu et al., 2006).

On the tissue level, N-WASP is required for the proper differentiation and/or development of many different cell types, including hematopoietic cells, fibroblasts, muscle cells, and neurons. In hematopoietic cells, N-WASP function is partially redundant with WASP in the control of immunological synapse development, B-cell development and signaling, antigen uptake, and chemotaxis (Jain and Thanabalu, 2015; Liu et al., 2013; Westerberg et al., 2012). Fibroblasts lacking N-WASP expression fail to form the hallmark actin fibers and show

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reduced contractility, adhesion, and spreading (Cai et al., 2012; Misra et al., 2007). N-WASP is also required for the function and development of muscle cells, in which N-WASP is recruited to Z disks of myofibrils to induce actin polymerization through the PRR-SH3 interaction with the 900-kDa actin-binding protein Nebulin (Takano et al., 2010). N-WASP is also important to the proper positioning of nuclei in muscle cells through the PRR-SH3 interaction with Amphiphysin-II (Falcone et al., 2014). Mutations in Amphiphysin-II that have been linked to the muscle disorders Autosomal Recessive Centronuclear Myopathy (ARCNM) and myotonic dystrophy are found in the SH3 domain and likely act by disrupting its interaction with N-WASP, which affected both the localization and protein levels of N-WASP in muscle cells (Falcone et al., 2014). In neurons, N-WASP participates in various aspects of neuron development, including neurite outgrowth, formation of dendritic spines and synapses, and myelination (Irie and Yamaguchi, 2002; Katanov et al., 2020; Pinyol et al., 2007; Shekarabi et al., 2005; Suetsugu et al., 2004, 2002; Wegner et al., 2008; You and Lin-Chao, 2010). Elevated levels of N-WASP have been found in the brains of patients with Alzheimer’s and intractable epilepsy, both diseases characterized by aberrant sprouting of neurites (Kitamura et al., 2003; Xiao et al., 2008).

As a major driver of actin polymerization, N-WASP levels are often mis-regulated in cancer cells (reviewed in Biber et al., 2020). Changed expression levels of N-WASP are correlated with reduced survival in several cancer types, including pancreatic ductal adenocarcinoma (PDAC), hepatocellular carcinoma (HCC), renal cell carcinoma, nasopharyngeal carcinoma, and lung, breast, cervical, and gastric cancers (Frugtniet et al., 2017; Guo et al., 2014; Hou et al., 2017; Jin et al., 2013; Martin et al., 2012, 2008; Sanchez et al., 2010; Wang et al., 2010; Yanagawa et al., 2001; Yang et al., 2020; Yu et al., 2012). The roles of N-WASP in cancer progression vary depending on the cancer type, but there are several common factors. One of the most commonly accepted factors is the role of N-WASP in the formation and maintenance of invadopodia, filopodia-like structures that extend into the extracellular matrix (ECM) to promote ECM degradation by matrix metalloproteinases (MMPs), which in turn promotes cancer cell migration, invasion, and metastasis (DesMarais et al., 2009; Gligorijevic et al., 2012; Lorenz et al., 2004; Martin et al., 2008; Mizutani et al., 2002; Oser et al., 2009; Sanchez et al., 2010; Sarmiento et al., 2008; Shortrede et al., 2016; Yamaguchi et al., 2005). In parallel, N-WASP also promotes the delivery of MMPs to and maintenance of MMP levels at the tip of invadopodia (Yu et al., 2012). While reduced N-WASP levels could be ameliorated by gene therapy or retrovirus-mediated expression of N-WASP in cancer cells, overactive protein could be targeted using the inhibitor wiskostatin and its derivatives, as well as nanobodies raised against the N-WASP WCA (Hebbrecht et al., 2017; Peterson et al., 2004).

It is remarkable that many pathogens have co-opted the N-WASP-mediated signaling to facilitate their infection, either by driving their attachment to or entry into target cells or by facilitating their intercellular movement (Snapper et al., 2001). The intracellular bacterial pathogen *Shigella flexneri*, which is the main cause of dysentery, utilizes its effector protein IcsA (or VirG) to recruit N-WASP to the bacterial surface, where IcsA binds to the N-WASP GBD to competitively release WCA and drive actin-based motility throughout the host cytoplasm (Egile et al., 1999; Mauricio et al., 2017; Suzuki et al., 1998). Similarly, *Chlamydia trachomatis*, a common sexually transmitted infection, uses

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its effector protein TmeA to bind the N-WASP GBD, which is important for recruiting N-WASP to the site of invasion (Faris et al., 2020; Keb et al., 2021). The vaccinia virus uses its membrane protein A36R to recruit Nck, WIP, and N-WASP to drive actin-based motility (Frischknecht et al., 1999; Moreau et al., 2000). Other intracellular pathogens, such as the bacteria *Ehrlichia chaffeensis* (causative agent of the life-threatening tick-borne disease monocytic ehrlichiosis) and the protozoan parasite *Trypansoma cruzi* (causative agent of Chagas disease, which affects 6–7 million people worldwide) recruit N-WASP (sometimes WAVE as well) to the sites of invasion, but the molecular mechanisms remain unknown (Bonfim-Melo et al., 2018; Kumar et al., 2015). The parasite *Listeria monocytogenes*, a leading food borne pathogen causing meningitis and death in pregnant women, infants, and the elderly, is known to use ActA to coopt the host cell actin machinery to propel itself within host cytosol. In order to spread between cells, the protein InlC is secreted and binds to the SH3 domain of the scaffolding protein Tuba. This prevents Tuba from interacting with N-WASP, leading to weakened tight junctions and allowing for easier passage between cells for the bacteria (Cossart and Bierne, 2001; Rajabian et al., 2009). Furthermore, the extracellular pathogen, enterohaemorrhagic *Escherichia coli* (EHEC), a major cause of hemorrhagic colitis and pediatric kidney failure in developed countries, uses its effector protein $EspF_U$ injected into the host epithelial cells to activate N-WASP, which locally stimulates the formation of actin pedestals, an actin-rich membrane protrusion underneath the EHEC attachment site (Campellone et al., 2004; Hartland and Leong, 2013; Kalman et al., 1999; Lommel et al., 2004, 2001). $EspF_U$ promotes N-WASP activation through at least two mechanisms in parallel: 1) it binds to GBD of WASP with high affinity, which directly competes off WCA to drive WASP activation, and 2) it uses multiple repeats of this GBD binding sequence to achieve high efficiency, likely through clustering WASP at membranes (Campellone et al., 2008a; Cheng et al., 2008; Sallee et al., 2008).

In summary, WASP and N-WASP play important roles in many fundamental processes. Even though studies in the past two decades have revealed many important mechanisms underlying the regulation and function of WASP and N-WASP on the molecular, cellular, and organismal levels, new mechanisms and functions are still emerging, and many questions remain to be answered.

WAVE and WAVE regulatory complex (WRC)

The second group of WASP-family proteins, WAVE, contains three orthologs in vertebrates: WAVE1, WAVE2, and WAVE3. The three proteins differ mainly in their middle, unstructured regions encompassing the PRR sequence, while their N-terminal and C-terminal structured regions share ~80% sequence homology (Figure 1, left) (Suetsugu et al., 1999). One other major difference between the three proteins is their expression pattern, with WAVE1 and WAVE3 enriched in the brain and WAVE2 widely expressed in all tissues and particularly in peripheral blood leukocytes (Suetsugu et al., 1999; Uhlén et al., 2015).

WAVE1 was discovered as a WASP-related protein by multiple laboratories at nearly the same time in 1998. One approach identified it as a suppressor of cAMP receptor signaling in *Dictyostelium*, which led to its first name SCAR (suppressor of cAMP receptor) and the initial indication of its human homologs through a sequence database search (Bear et al.,

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1998). In the meantime, two other approaches, one searching for Arp2/3-interaction partners through a yeast-two-hybrid assay and the other searching for novel WH2-containing proteins in the human sequence database, identified the human homolog SCAR1 and introduced the name WAVE (WASP-family Verprolin-homologous protein) (Machesky and Insall, 1998; Miki et al., 1998b). Shortly after that, WAVE2 and WAVE3 were cloned and shown to have similar properties in driving Arp2/3-mediated actin polymerization (Suetsugu et al., 1999). Since then, the name WAVE has been widely used in vertebrates, while SCAR is used more often in invertebrates and plants. For clarity, we herein use WAVE to refer to all WAVE orthologs, unless an isoform is specifically mentioned.

Similar to WASP/N-WASP acting downstream of the Rho-GTPase Cdc42, WAVE acts downstream of another Rho-GTPase, Rac1 (Miki et al., 1998b). Distinct from WASP/N-WASP, however, WAVE did not contain a recognizable GTPase binding domain, nor could it directly bind Rac1 (Miki et al., 1998b). Furthermore, the recombinantly purified WAVE had high basal activity towards the Arp2/3 complex, suggesting it is not autoinhibited as a standalone protein as WASP/N-WASP are (Innocenti et al., 2004). Instead, the biochemical purification of WAVE from various animal tissues or cultured cells and subsequent recombinant reconstitution studies established that WAVE was constitutively incorporated in a large complex of ~400 kDa that consisted of five different protein subunits: Sra1 (Cyfip1, or its ortholog Pir121/Cyfip2), Nap1 (Hem2, or its ortholog Hem1), Abi2 (or its orthologs Abi1 and Abi3/Nesh), HSPC300 (BRICK1), and WAVE1 (SCAR1, or its orthologs WAVE2 and WAVE3) (Derivery et al., 2009a; Eden et al., 2002; Gautreau et al., 2004; Innocenti et al., 2004; Ismail et al., 2009; Lebensohn and Kirschner, 2009). This complex was thereafter named the WAVE Regulatory Complex (WRC or WAVE complex) (Figure 3). The identification of the WRC explained the previously observed links between Rac1 and WRC components and their importance in the formation of lamellipodia, thin sheet-like membrane ruffling often found at the leading edge of migrating cells (Kobayashi et al., 1998; Nobes and Hall, 1995; Ridley et al., 1992; Rogers et al., 2003; Scita et al., 1999). These studies also reconciled the original debates about WRC activity regulation, establishing that the WRC is basally inhibited in the cytosol and is recruited to and activated at the plasma membrane by various ligands to promote Arp2/3-mediated actin polymerization (Figure 3A) (Kurisu and Takenawa, 2009; Rottner et al., 2021).

Biochemical and structural studies have now elucidated the mechanism underlying the assembly and autoinhibition of the WRC and have started to explain how the WRC interacts with various ligands to promote activation and membrane recruitment (Rottner et al., 2021). The WRC can be viewed as an assembly of a large, elongated dimer formed by Sra1 and Nap1, and a smaller trimer formed by WAVE1, Abi2, and HSPC300. Sra1 and Nap1 are predominately alpha helical, whereas the N-termini of WAVE1 (i.e., part of its WHD domain), Abi2, and HSPC300 form a four-helix bundle aligning along the long axis of the Sra1-Nap1 dimer (Figure 3). Following the helical bundle, WAVE1 and Abi2 extend long tails that have important regulatory roles. The long tail of WAVE1 consists of three parts: a "meander" region of ~90 amino acids (a.a.), which "meanders" across the surface of Sra1 and is critical for the inhibition and activation of the WRC; a long, unstructured sequence of ~300 a.a. that contains multiple PRRs; and the C-terminal WCA region of ~75 a.a. The long tail of Abi2 also consists of three parts: a sequence of ~ 40 a.a. named the Nap1 binding

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fragment (NBF), which "crawls" on the surface of Nap1 and is critical for WRC assembly; a long, unstructured sequence of ~250 a.a. that contains multiple PRRs; and a C-terminal SH3 domain. The WRC keeps WAVE1 inhibited *in trans* by sequestering the W and C elements of WCA to a conserved surface formed by both Sra1 and the meander region of WAVE1 (B. Chen et al., 2014a; Chen et al., 2010a). This is distinct from WASP/N-WASP, in which the WCA is sequestered *in cis* by binding to the GBD in the same polypeptide chain. It is believed other WRC assemblies, such as WRCs containing Cyfip2, Hem1, or WAVE2, are similarly assembled and autoinhibited, although their regulatory mechanisms may have some difference (X. J. Chen et al., 2014; Cook et al., 2020; Derivery et al., 2009a; Gautreau et al., 2004; Polesskaya et al., 2021).

A large number of interacting ligands of the WRC have been identified, and the list is still rapidly growing (Rottner et al., 2021). Like WASP/N-WASP, WRC directly interacts with small G proteins (Rho-family and Arf-family GTPases), inositol phospholipids (e.g., PIP_3), various kinases (Abl, Src, Cdk5, Cdk1, Erk, CK2, Pka, SepA) and many cytosolic or adaptor proteins, such as IRSp53, Nck, Lamellipodin, Ena/VASP, NHSL1, and WRP (Ardern et al., 2006; X. J. Chen et al., 2014; Dai and Pendergast, 1995; Danson et al., 2007; Kim et al., 2006; Kitamura et al., 1996; Kobayashi et al., 1998; V. Koronakis et al., 2011; Law et al., 2021, 2013; Leng et al., 2005; Mendoza, 2013; Miki et al., 2000; Miyamoto et al., 2008; Nakanishi et al., 2007; Oikawa et al., 2004; Pocha and Cory, 2009; Shi et al., 2021; S. P. Singh et al., 2020; Soderling et al., 2002; Stuart et al., 2006; Ura et al., 2012; Westphal et al., 2000; Xu and Quinn, 2012; Yamashita et al., 2011). Unlike WASP/N-WASP, which are indirectly linked to membrane proteins through various cytosolic adaptors, the WRC directly interacts with many membrane proteins. Together, these interactions suggest the WRC acts as an important signaling hub, through which various membrane signals can be directly funneled down to the regulation of the actin cytoskeleton (Rottner et al., 2021).

Despite the long list of WRC ligands, most of their interaction mechanisms remain unsolved largely due to 1) technical difficulties caused by the large size and complexity of the WRC and 2) the cooperative nature and weak affinity of many ligands. Another complication is that only the fully assembled WRC exists as a stable, functional unit in the cell and as well-behaving material in biochemical studies. Recombinantly produced isolated subunits or subcomplexes of the WRC are prone to protein aggregation and could even cause "artificial" interactions through surfaces that would not be available in the assembled complex. Moreover, although the five subunits have co-existed through evolution and show a strong interdependence in cellular expression, it is likely some subunits may have evolved extra functions outside the WRC (Blagg et al., 2003; Echarri et al., 2004; Kunda et al., 2003; Rogers et al., 2003; Steffen et al., 2004; Stephan et al., 2011; Veltman and Insall, 2010). Examples include Sra1 binding to the Fragile X mental retardation protein FMRP and the translation initiation factor eIF4E, and Abi binding to N-WASP (Innocenti et al., 2005; Napoli et al., 2008; Schenck et al., 2001). Therefore, it is important to rigorously validate WRC-related interactions that were identified using isolated subunits. These interactions should be carefully evaluated based on fully assembled WRC both *in vitro* and in cells.

Among the WRC ligands, the Rho-GTPase Rac1 ubiquitously plays a central role in activating the WRC. Recent biochemical reconstitution and structural work has led to the

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identification of two separate Rac1 binding sites on the WRC, which are located on opposite ends of the elongated Sra1 subunit (Figure 3). The site adjacent to the WCA binding site was named the A site, and the site distant to WCA named the D site (Chen et al., 2017, 2010a). The two sites have ~40-fold difference in the affinity for Rac1, but both sites played a key role in activating the WRC *in vitro* (Chen et al., 2017). The weak binding site (i.e., A site) seemed to be more important for WRC-mediated lamellipodia formation in cells (Schaks et al., 2018). Without a high-resolution structure of the WRC bound to both sites, the exact mechanism underlying WRC activation is still unknown. Nevertheless, since both Rac1-binding sites are distinct from the WCA binding site, WRC activation must use an allosteric mechanism to destabilize the sequestered WCA, in contrast to the direct competition mechanism in WASP/N-WASP activation by Cdc42 (Figure 3A). Why the WRC contains two Rac1 binding sites is unknown, but there are two possible explanations: 1) the two sites are cooperative—Rac1 binding to D site could enhance A site binding, and 2) when Rac1 concentration is low, D site binding can "prime" the WRC on the membrane without causing activation, and only when Rac1 concentration is high can it bind to the A site and trigger activation (Chen et al., 2017). It is remarkable that a recently identified protein, Fam49/CYRI (Cyfip-related Rac1 interacting), uses a structure homologous to the A site to sequester Rac1 from activating the WRC and locally suppress actin polymerization and membrane protrusions (Fort et al., 2018).

While Rac1 is the canonical activator of the WRC, other molecules can contribute to WRC activation and membrane localization, and they often act cooperatively with Rac1 (Figure 3B). These include the Arf-family GTPases, Arf1 and Arf6, other Rho-family GTPases (Cdc42 and RhoG), different kinases, acidic phospholipids (PIP_3), and various cytosolic and membrane proteins (Vassilis Koronakis et al., 2011; Rottner et al., 2021; Schaks et al., 2021; V. Singh et al., 2020). Arf1 and Arf6 were shown to act synergistically with Rac1 to promote WRC activation both *in vitro* and in various cellular processes (Anitei et al., 2010; Vassilis Koronakis et al., 2011). These interactions may provide additional control of the WRC and link the WRC to particular processes, such as intracellular trafficking and pathogen invasion. Exactly how Arf GTPases interact with the WRC or how Arf cooperates with Rac1 is unknown. Phosphorylation on the WRC by various kinases (e.g., Cdk5, Erk, Abl, Ck2, and Src) mainly occurs in the meander and WCA regions of WAVE, and the unstructured PRR sequences of both WAVE and Abi (Ardern et al., 2006; Danson et al., 2007; Kim et al., 2006; Mendoza, 2013; Nakanishi et al., 2007; Pocha and Cory, 2009; Sossey-Alaoui et al., 2007a). Particularly, phosphorylation in the meander sequence, including Y125 (by Src), T138 (by Cdk5), and Y151 (by Abl), has been shown to destabilize meander binding to Sra1, which in turn causes WCA release and WRC activation (Chen et al., 2010a). Phosphorylation in the WCA of WAVE and PRR regions of WAVE and Abi may influence WRC activity in the cell by tuning the interactions with PRR-binding proteins (e.g., SH3 domain-containing proteins) and Arp2/3. Similar to the importance of inositol phospholipids PIP_2 to WASP/N-WASP activation, PIP_3 are important to WRC activation likely through two distinct mechanisms: 1) increasing membrane localization and local protein concentration of the WRC by binding to the positively charged face of the complex, and 2) directly contributing to WCA release by interacting with the positively charged helix 6 in the meander sequence.

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The long PRR sequences in WAVE and Abi are the most variable regions among different WAVE and Abi orthologs (Figure 1, left). They provide a rich environment for recruiting different adaptor proteins, such as SH3-domain or EVH1-domain containing proteins (e.g., Ena/VASP). Similarly, the SH3 domain at the C-terminus of Abi can recruit certain PRR-containing proteins, such as the Abl kinase. While these interactions may not directly contribute to WRC activation, they can play an important role in connecting various upstream signals to the WRC (Miki et al., 2000; Soderling et al., 2002; Stuart et al., 2006; Takenawa and Suetsugu, 2007). In principle, binding to multivalent adaptor proteins could induce LLPS, as is seen for WASP/N-WASP, but LLPS has not yet been reported for the WRC. It has been shown that the WRC forms high-order, wave-like assemblies on the plasma membrane, but the underlying mechanisms driving this organization are unknown (Pipathsouk et al., 2021; Weiner et al., 2007). It has been proposed that the N-terminal helix 1 of Sra1 could bind to a neighboring complex, which could potentially polymerize the WRC, but no experimental evidence has been found to confirm or deny this (Chen et al., 2010b).

In addition to interactions mediated by PRR sequences or the SH3 domain of WAVE and Abi, many other ligands either use an unidentified interaction mechanism or were found to interact with structured, non-PRR regions of the WRC. Here, we describe several ligands that link the WRC to various unique processes. The exocyst complex was shown to directly interact with the Cyfip and Abi subunits, which provides a mechanism to coordinate polarized exocytosis mediated by exocyst with cell migration mediated by the WRC (Biondini et al., 2016; Zago et al., 2018). PCARE (photoreceptor cilium actin regulator) was found to recruit the WAVE3-containing WRC to the cilia of photoreceptor cells, where the WRC played a key role in promoting the actin polymerization required for the outer segment disk formation (Corral-Serrano et al., 2020). Moreover, the neuronal protein Pancortin/Noelin was found to recruit WAVE1 to the surface of mitochondria after ischemic stroke, where the WAVE1-Pancortin complex sequestered Bcl-xL to facilitate cytochrome C release and apoptosis (Cheng et al., 2007). In leukemia cells, where Bcl2 is overexpressed to protect cells from apoptosis, WAVE1 was found to interact with Bcl2 and promote its localization to the mitochondria to enhance its anti-apoptotic activity (Kang et al., 2010). The general importance of the WRC to mitochondria is also seen in another study, in which WAVE1 was important to mitochondria positioning in neural dendrites (Sung et al., 2008).

Besides the above ligands, the WRC directly interacts with a large variety of transmembrane or membrane associated proteins. The majority of these membrane proteins (>100 in the human genome by prediction) can bind the WRC using a short peptide motif named the WRC interacting receptor sequence (WIRS) in their intracellular domain (ICD), which is defined as Φ-x-T/S-F-x-x (Φ for bulky hydrophobic residues and x for any residues) (B. Chen et al., 2014a). The WIRS proteins include various cell adhesion molecules (e.g., protocadherins, Robo1, neuroligins, and Syg-1), ion channels, and GPCRs (B. Chen et al., 2014a). This WIRS interaction is strictly conserved throughout animals, from human to sponge, and is not found in other species such as plants, suggesting the importance of WIRS-WRC interactions to processes unique to animals, like in the nervous system. Indeed, many studies have shown different neuronal receptors use the WIRS-WRC interaction to

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recruit the WRC to their sites of action and provide actin polymerization to support various neuronal activities, such as axon pathfinding, branching, and synapse formation (Chaudhari et al., 2021; Chia et al., 2014; Fan et al., 2018; Lee et al., 2016; Xing et al., 2018), as well as tissue morphogenesis (Lee et al., 2016; Malin et al., 2022; Squarr et al., 2016). It is remarkable that the WIRS binding pocket (indicated by the "W" site in Figure 3) is formed by both Sra and Abi subunits, highlighting the importance of fully assembled WRC to its function. Together, the diverse array of WIRS receptors provides the cell a versatile means to recruit the WRC to the membrane in response to many different upstream signals and in different cell types. In addition to serving as a membrane localization signal, WIRS proteins can also use the sequence flanking the WIRS motif to modulate—either inhibit or further promote—WRC activity (B. Chen et al., 2014a). Besides WIRS-containing proteins, membrane proteins that interact with the WRC without using a WIRS motif are emerging, such as HPO-30, Retrolinkin, and CB1, but the exactly interaction mechanisms remain to be solved, and whether there are more non-WIRS receptors for the WRC remain to be explored (Monday et al., 2020; Xu et al., 2016; Zou et al., 2018). It is intriguing that many ICDs, either directly interacting with the WRC or not, may use other protein-protein interaction motifs to recruit additional adaptor proteins, including various GEFs and GAPs for Rac1 and Arf1 (Kong et al., 2015; Lucas and Hardin, 2017; Lundström et al., 2004; Paskus et al., 2019; Stavoe and Colón-Ramos, 2012; Villanueva et al., 2021; Woolfrey et al., 2009; Yang and Bashaw, 2006). These interactions could bridge related signaling molecules to the WRC to provide more spatiotemporal control of WRC activation (Zou et al., 2018). Finally, the importance of membrane localization to WRC activity is further supported by a recent observation that the force generated by actin filament elongation could dissociate the WRC from the lamellipodia tip to decrease WRC dwell time and activity, which may provide a negative feedback loop to fine tune membrane protrusions (Mehidi et al., 2021).

The exact composition of the WRC subunits can provide an additional layer of control of WRC activity. Although in principle the WRC can be similarly assembled by various orthologs of each subunit through a "mix-and-match" process, different WRCs may have distinct interaction partners, respond to the same ligand differently, or have different activity output. For example, WAVE2-containing WRC, but not WAVE1-containing WRC, requires the adaptor protein IRSp53 for optimal activation (Miki et al., 2000; Suetsugu et al., 2006). WAVE1- and WAVE2-containing WRCs were also shown to play overlapping, but distinct roles in promoting membrane protrusions, cell migration, and actin network architecture (Suetsugu et al., 2003; Sweeney et al., 2015; Tang et al., 2020). Moreover, Cyfip2-containing WRC was shown to be less responsive to Rac1 activation than Cyfip1-containing WRC (Polesskaya et al., 2021). Similarly, Hem1-containing WRC could not be activated by Rac1 *in vitro* in identical conditions that could activate Nap1-containing WRC (Cook et al., 2020; Polesskaya et al., 2021). The biochemical and structural mechanisms underlying the difference between different WRC isoforms remain to be explored.

The WRC plays an essential role in many fundamental processes, including lamellipodia formation, cell migration, adhesion, and fusion (Gromnitza et al., 2018; Kim et al., 2015; Kunda et al., 2003; Nowak et al., 2009; O'Leary et al., 2017; Suetsugu et al., 2003; Yamazaki et al., 2007). The broad diversity of its ligands links the WRC to various physiological systems, particularly the nervous system and the immune system (Rottner

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et al., 2021; Yamazaki et al., 2003). Because of the interdependence of all five subunits in maintaining WRC integrity and function, genetic mutations disrupting any subunit can potentially disrupt WRC function and lead to disease.

The WRC, especially the WAVE1- and WAVE3-containing WRCs, are enriched in the brain and play an essential role in neural morphogenesis, axon growth, dendrite branching, synapse formation, and synaptic transmission and plasticity (Nozumi et al., 2003; Soderling et al., 2007, 2003; Sung et al., 2008). This explains the broad implication of the WRC in many types of neurological conditions (Rottner et al., 2021). Over a dozen *de novo* mutations in WAVE1 have been identified as the cause of a newly defined neurological syndrome named Neurodevelopmental Disorder with Absent Language and Variable Seizures (NEDALVS), which exhibits features of intellectual disability (ID), autism, and epilepsy. The mutations include different nonsense or frameshift mutations clustered in the WCA region, which would lead to a truncated WCA and loss of WAVE1 activity towards the Arp2/3 complex (Ito et al., 2018; Shimojima Yamamoto et al., 2021; Srivastava et al., 2021). Copy number variant (CNV) mutations caused by microdeletion of WAVE1-containing regions from chromosome 6q21 were also observed, which would cause haploinsufficiency of WAVE1 and reduce overall WRC activity in the brain (Srivastava et al., 2021). In addition, four cases of missense mutations (W161C/R or K172E) were identified in NEDALVS patients (Srivastava et al., 2021; Zhao et al., 2021). Located in helix 6 in the meander region, both W161 and K172 make contacts with the C helix to keep WCA sequestered. Therefore, W161C/R or K171E would lead to a constitutively active WRC, instead of loss of function as seen in other NEDALVS patients. That the two opposite effects lead to the same syndrome emphasizes the importance of precisely controlling WRC activity *in vivo*.

In addition to WAVE1, Cyfip1 and Cyfip2 are frequently mutated in human patients. Many mutations in Cyfip2, including CNVs caused by chromosome 5q33 microdeletion, nonsense or frameshift mutations, and over 25 different missense mutations have been identified as the cause of a neurodevelopmental disorder named Developmental and Epileptic Encephalopathy-65 (DEE-65), which involves ID, Early-Onset Epileptic Encephalopathy (EOEE), seizures, muscular hypotonia, West syndrome, eating disorders (in mouse), and altered drug addiction (in mouse) (Begemann et al., 2021; Kirkpatrick et al., 2017; Kumar et al., 2013; Zweier et al., 2019). The missense mutations are spatially clustered around three different “hotspots” in the WRC structure (Figure 3B) (Rottner et al., 2021). Hotspot 1 contains the meander and WCA binding surface and the A site. These mutations would disrupt WCA inhibition to increase WRC activity (Nakashima et al., 2018). For example, one of the frequently mutated residues, R87, plays a key structural role in stabilizing WAVE1 Y151 binding. Phosphorylation of Y151 by Abl kinase or mutating R87 or Y151 caused constitutive activation of the WRC (Chen et al., 2010a; Schaks et al., 2020; Stuart et al., 2006; Zweier et al., 2019). Hotspot 2 is buried in the WRC, where mutations would disrupt protein folding and stability to reduce WRC activity. Hotspot 3 is located immediately underneath the D site, where mutations have been shown to both destabilize WRC in neurons and enhance Rac1 binding and WRC activation *in vitro*. Again, similar to WASP/N-WASP and WAVE1, both loss-of-function mutations (which cause gene loss or disrupt protein folding) or gain-of-function mutations (which constitutively activate WRC)

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in Cyfip2 lead to similar syndromes, reiterating the importance of finely controlling WRC activity in the cell. In contrast to Cyfip2, no missense mutations in Cyfip1/Sra1 have been associated with disease, but the 15q11.2 region of the chromosome, which contains Cyfip1, is a hotspot of chromosomal microdeletions and duplications. CNVs of 15q11.2 are heavily associated with various neurological disorders, including ID, autism, seizures, schizophrenia, and epilepsy (Yoon et al., 2014). These mutations would act by altering the protein and activity level of the Cyfip1-containing WRC in the brain.

Accumulating evidence suggests the WRC also plays a role in neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD), perhaps through its role in regulating migration and phagocytosis of microglia, the immune cells in the brain. WAVE was found to co-aggregate with the pathogenic hyper-phosphorylated Tau and CRMP2 proteins in neurofibrillary tangles and abnormal neurites of the AD brain (Takata et al., 2009). A rare variant of Abi3, S209F, was identified to be a risk factor for late-onset AD (Conway et al., 2018; Olive et al., 2020; Sims et al., 2017). The importance of Abi3 in AD pathogenesis was further confirmed by a recent mouse model, in which knocking out Abi3 disrupted microglia migration and phagocytosis, increased amyloid β (Aβ) accumulation, decreased microglia clustering around the Aβ plaques, and impaired long-term potentiation (Karahan et al., 2021). In addition, WAVE1 expression forms a negative feedback loop with Aβ production: the amyloid precursor protein (APP) binds to the promoter of WAVE1 to suppress WAVE1 expression, whereas reducing WAVE1 expression significantly reduced Aβ levels and restored memory deficits in a mouse AD model (Ceglia et al., 2015). Both elevated and decreased expression level of WAVE1 in AD patient brains was reported, suggesting the mechanism is complicated and may also depend on disease stage (Ceglia et al., 2015; Kitamura et al., 2003). In addition, WAVE2 was found to have a genetic interaction with LRRK2 (Leucine-rich repeat kinase-2), one of the commonly mutated proteins in Parkinson's disease (PD). This interaction increased the lifetime of the WAVE2 protein and increased the phagocytic activity of microglial cells to increase neuron death, mimicking the cell death commonly seen in Parkinson's (Kim et al., 2018).

Hem1-containing WRC, which usually contains WAVE2, is specifically expressed in hematopoietic cells and plays an essential role in immune system processes, including immune cell chemotaxis, phagocytosis, T-cell activation, immunological synapse formation, integrin-mediated adhesion, and B cell development and homeostasis (Castro et al., 2020; Cook et al., 2020; Nolz et al., 2006; Park et al., 2010; Salzer et al., 2020; Stahnke et al., 2021; Weiner et al., 2006; Zipfel et al., 2006). Recently, several missense mutations and an exon-deleting mutation in Hem1 have been identified to be the cause of a new immunological syndrome named Immunodeficiency-72 with an Autoinflammation (IMD72), which involves immunodeficiency and recurring infections mixed with atopy, lymphoproliferation, and cytokine overproduction (Castro et al., 2020; Cook et al., 2020; Salzer et al., 2020). All mutations are spatially clustered at a hotspot in the WRC proximal to the Rac1-binding D site, which was named Hem1 hotspot (Figure 3B) (Rottner et al., 2021). Most of the mutations are buried in the WRC structure and were shown to disrupt protein folding and stability, while the only mutation on a surface residue, M371V, seemed to affect Arf1-mediated but not Rac1-mediated WRC activation. Exactly how M371V disrupt Arf1-WRC interaction remains to be addressed. Loss of Hem1 also seemed to

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disrupt mTORC2 activation, leading to impaired Akt signaling, cytokine secretion, and T cell proliferation. The linkage of the Hem1-/WAVE2-containing WRC to mTOR signaling was further supported by a recent conditional WAVE2 knockout mouse model (Liu et al., 2021).

As a major driver of cell motility, the WRC is heavily involved in many types of cancers, including HCC, leukemia, and breast, gastric, prostate, ovarian, bladder, pancreatic, lung, colorectal, and cervical cancers (Biber et al., 2020; Kurisu and Takenawa, 2010; Miki et al., 1998b; Nozumi et al., 2003; Sossey-Alaoui et al., 2005, 2007b; Suetsugu et al., 1999, 2003; Yan et al., 2003). Overall, the mechanism of how WRC misregulation is linked to cancer is largely unclear, but in general overexpression (and sometimes deletion) of WRC components, including WAVE, Abi, HSPC300, Nap, and Cyfip is frequently observed in cancer cells and often associated with an increase in the motility and invasiveness of cancer cells, which is often accompanied with poor prognosis (Carmona et al., 2016; Huang et al., 2018; Jia et al., 2014; Taniuchi et al., 2018). This is likely due to transcriptional rewiring in cancer cells that elevates WRC expression and activity to help cancer cells alter the actin cytoskeleton and promote cell migration. WAVE was also found to affect the epithelial to mesenchymal transmission (EMT), which is critical for cancer metastasis and prognosis (Park and Kim, 2017; Taniuchi et al., 2018; Taylor et al., 2013). In addition to regulating cell migration, WAVE also regulates the expression level of matrix metalloproteinases MMP1 and MMP9, which localize to invadopodia and degrade ECM components (Sossey-Alaoui et al., 2005). The generally elevated expression of the WRC suggests inhibitors targeting WRC activity or inducing WRC degradation could be a potential therapeutic for difficult to treat cancers, such as prostate and triple-negative breast cancers (Cowell et al., 2017; Limaye et al., 2022; Loveless and Teng, 2021; Teng et al., 2016).

Various pathogens hijack WRC signaling to facilitate their infection. For example, *Salmonella* relies on WAVE2-containing WRC for effective internalization into epithelial cells (Shi et al., 2005). *Salmonella* does so through two signaling pathways, both merging on the WRC. First, the bacteria secrete its own Rac1-GEF, SopE/SopE2, into the host cell, which promotes Rac1 activation to recruit the WRC to the invading site. Second, the bacteria simultaneously recruit the host Arf-GEF, ARNO, to the invading site, which promotes Arf1 activation. Both Rac1 and Arf1 act cooperatively on the WRC to promote actin polymerization at the invading site and trigger bacterial entry through micropinocytosis (Humphreys et al., 2013, 2012). Interestingly, the cooperativity between Rac1 and Arf1 in promoting WRC activation is also hijacked, albeit in the opposite way, by two extracellular bacterial pathogens, enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli*. Both EPEC and EHEC can secrete an effector protein EspG into host cells, which sequesters both Arf6 and Arf1 to reduce WRC activation, allowing the pathogen to evade WRC-dependent phagocytosis (Humphreys et al., 2016). Other intracellular bacterial pathogens also rely on Rac1-WRC signaling to promote their internalization, including *Chlamydia trachomatis*, *Anaplasma phagocytophilum*, the tick-born bacterial pathogen *Rickettsia*, and various Gram-negative bacteria (such as *Neisseria gonorrhoeae*) that rely on EACAM3-mediated phagocytosis (Carabeo et al., 2007; Lin et al., 2007; Pils et al., 2012; Reed et al., 2012). Viruses can also act through the WRC to promote their infection. During HIV internalization, WAVE2-containing WRC and its associated ligands,

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including Rac1, Arp2/3, and Abl kinase, are required for the viral envelope-mediated membrane fusion, entry, and infection (Harmon et al., 2010; Spear et al., 2014). During human cytomegalovirus (HCMV) infection, the viral protein pUL135 directly binds to Abi and recruits the WRC to the plasma membrane, which, instead of promoting actin polymerization, reduces the efficiency of immune synapse (IS) formation to help infected cells escape immune surveillance (Rak et al., 2018; Stanton et al., 2014). In addition, the protozoan parasite, *Toxoplasma gondii*, one of the most prevalent parasites on earth and the cause of toxoplasmosis, was found to rely on a novel parasite protein, *Tg*WIP (*T. gondii* WRC interaction protein), to help the parasites disseminate from the primary infection site to distant organs (Sangaré et al., 2019). TgWIP contains a WIRS motif, which is believed to be important for binding the WRC and redirecting WRC-mediated actin polymerization in infected cells.

In addition to the WRC itself, mutations in various WRC ligands are also frequently associated with diseases that resemble the symptoms of WRC-associated disorders. For example, missense mutations in Rac1/2/3 cause autosomal dominant mental retardation-48 (MRD48), immunodeficiency-73A/B/C (IMD73A), and neurodevelopmental disorder with structural brain anomalies and dysmorphic facies (NEDBAF), respectively (Alkhairy et al., 2015; Ambruso et al., 2000; Costain et al., 2019; Hsu et al., 2019; Reijnders et al., 2017; White et al., 2018; Williams et al., 2000). Misregulation of Rac1 activity, including that caused by several hotspot missense mutations (such as P29S), was identified in human melanoma and lung, liver, and breast cancers (Bauer et al., 2007; Dokmanovic et al., 2009; Hodis et al., 2012; Kawazu et al., 2013; Krauthammer et al., 2012; Liu et al., 2008; Schnelzer et al., 2000; Stallings-Mann et al., 2012). Among them, P29S was shown to increase the Rac1 binding affinity to various downstream effectors, including the WRC (Chen et al., 2017; Hodis et al., 2012; Krauthammer et al., 2012). In addition, mutations in various WIRS-containing receptors, such as protocadherin19 and DCC, cause neurological/ developmental disorders, such as epilepsy and mental retardation in females (EFMR) and congenital mirror movement, respectively (B. Chen et al., 2014b; Depienne et al., 2011; Depienne and Leguern, 2012).

In summary, the WRC acts as a central signaling hub that links a large array of ligands at membranes to the actin cytoskeleton in many different normal and disease related processes. Exactly how the WRC interacts with different ligands and how the interactions modulate WRC localization and activity is still largely unknown and requires rigorous biochemical, structural, and cell biological studies to elucidate the underlying mechanisms.

WASH and WASH Regulatory Complex (SHRC)

WASH was identified as the third WASP-family protein about 10 years after the discovery of WASP/N-WASP and WAVE. In 2007, when examining the subtelomeric region of human chromosomes, once considered a “genetic junkyard” filled with duplications and variations, Linardopoulou et al. found the most telomerically duplicated human genes, MGC52000, coded a protein homologous to WASP/N-WASP and WAVE, and named it WASH (Linardopoulou et al., 2007). These genes had been reported in other non-primate animals as a single-copy gene without a known function (Gianfrancesco et al., 2001; Hansen

et al., 2005). Interestingly, the *WASH* gene was found to be extensively duplicated in primates. Even the six human individuals examined in their study were different from each other, having 15–20 copy numbers and 16 different chromosomal locations (Linardopoulou et al., 2007). The duplicated genes contained pseudogenes, different truncation variants, and full-length, intact ORFs. The full-length ORFs contained significant numbers of amino acid substitutions, with 95.8% identity among them. It was speculated that the subtelomeric variations in the human population might give slightly different functions, which might contribute to phenotypic differences between human individuals (Linardopoulou et al., 2007).

Soon after the discovery of WASH, a series of cellular and biochemical studies found that, similar to WAVE, WASH also existed in a large protein complex of ~500 kDa, thereafter named the WASH Regulatory Complex (SHRC, pronounced "shark") (Derivery et al., 2009b; Gomez and Billadeau, 2009; Jia et al., 2010). Consisting of five core subunits, SWIP (KIAA1033, Strumpellin- and WASH-interacting protein), Strumpellin (KIAA0196), Fam21 (Fam21A/B and Fam21C), CCDC53, and WASH, the SHRC was found to resemble the WRC in many ways. First, both isolated WAVE and WASH proteins are constitutively active towards the Arp2/3 complex, while their corresponding complex keeps WAVE or WASH basally inhibited (Jia et al., 2010). Second, although the two complexes share less than 15% sequence identity (aside from the WCA domain of WASH and WAVE), a more advanced profile search by HHPred revealed that four subunits in the SHRC shared distant, but significant homology with a corresponding subunit in the WRC. SWIP is homologous to Sra1, Strumpellin to Nap1, the N-terminal helix of CCDC53 to HSPC300, and the N-terminal helix of WASH to the N-terminal helix of WAVE (Hildebrand et al., 2009; Jia et al., 2010). Although HHPred could not identify homology between the N-terminal helices of Fam21 and Abi2, they might share structural homology that was below the detection threshold. Third, the distant homology between SHRC and WRC subunits suggests the two complexes may have a similar structural organization (Figure 4). It is possible that the two large subunits, SWIP and Strumpellin, form a large, elongated dimer platform similar to the Sra1-Nap1 dimer, along which aligns a helix bundle formed by the N-terminal helices of Fam21, CCDC53, and WASH (similar to the Abi2-HSPC300-WAVE helix bundle). The overall structural resemblance was supported by both structure-function analysis in cells and negative stain EM analysis of purified SHRC, but exactly how the SHRC is assembled and how it keeps WASH basally inhibited remain unknown (Jia et al., 2010).

Similar to Abi and WAVE, Fam21 and WASH extend long, unstructured sequences immediately following their N-terminal helices (Figure 4). These extended sequences play an important role in SHRC assembly and regulation. CCDC53 also contains an extended sequence at the C-terminus, but unlike its N-terminal helix, this extended sequence did not seem to be critical for the assembly or function of the SHRC (Gomez and Billadeau, 2009; Jia et al., 2010). The N-terminus of WASH contains two loosely defined conserved regions named WHD1 (WASH homology domain 1, a.a. 1–167) and WHD2 (a.a. 168–304), which was collectively named WAHD (WASH homology domain) (Gomez and Billadeau, 2009; Linardopoulou et al., 2007). The N-terminal part of WHD1 (a.a. 29–79) contains the helix homologous to the N-terminal helix of WAVE, which is presumably important for forming a helix bundle with the N-terminal helices of Fam21 and CCDC53. Deleting this

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helical sequence, or the N-terminal helix of Fam21 or CCDC53, abolished SHRC formation (Gomez and Billadeau, 2009; Jia et al., 2010). The remaining part of WAHD, including the C-terminal part of WHD1 and the entire WHD2, does not contain well defined secondary structures and possibly resembles the meander region of WAVE, which could interact with the SWIP-Strumpellin dimer and control SHRC inhibition (Figure 4). Indeed, WHD2 was not essential for assembling the SHRC, but deleting WHD2 abolished SHRC function in retromer-mediated cargo sorting, suggesting WHD2 plays an important regulatory role (Gomez and Billadeau, 2009). Following WAHD is a small stretch of PRR and then the C-terminal WCA sequence. It is unknown what proteins directly interact with the PRR sequence.

The C-terminal tail of Fam21 is over 1,000 a.a. long and harbors many protein-protein interaction sequences. First, structure-function analysis suggested the sequence of ~120 a.a. immediately following the N-terminal helix of Fam21 plays an essential role in maintaining SHRC assembly, likely through a mechanism similar to the NBF region of Abi2 binding to Nap1 in the WRC (Jia et al., 2010). Following this sequence, the long C-terminal tail Fam21 contains 21 copies of a unique L-F-$[D/E]_{3-10}$-L-F motif (LFa, Figure 4), each motif being able to directly interact, with different affinities, with VPS35, a subunit in the cargo-selective complex named retromer (Harbour et al., 2012, 2010; Helfer et al., 2013; Jia et al., 2012). This multivalent interaction tightly links SHRC to retromer-mediated endosomal sorting and likely provides a mechanism for the cell to finely tune SHRC membrane recruitment based on the retromer density on endosome membranes. The C-terminal tail of Fam21 also contains a conserved capping protein binding motif, which binds to CapZ in the cell and inhibits its capping activity (Derivery et al., 2009b; Gomez and Billadeau, 2009; Jia et al., 2010). This binding activity was recently shown to competitively remove CapZ from the dynactin complex. As a result, the "de-capped" dynactin could provide an actin mini-filament that could elongate and prime SHRC-Arp2/3-mediated actin polymerization (Fokin et al., 2021; Fokin and Gautreau, 2021). The Fam21 tail also interacts with many other molecules, but the exact interaction mechanism is not known. For example, the N-terminal part of the Fam21 tail (a.a. 356–600) directly interacts with the C-termini of CCDC22 and CCDC93 of the COMMD/CCDC22/CCDC93 (CCC) complex, a key regulator of endosomal recycling. This interaction links SHRC to the CCC-retriever-mediated, retromer-independent cargo sorting (Figure 4A, right) (Harbour et al., 2012; McNally et al., 2017; Phillips-Krawczak et al., 2015). TBC1d23, a protein essential for endosome-to-Golgi trafficking, was shown to bind to the Fam21 tail, linking endosomal trafficking to the Trans-Golgi network (Wenjie et al., 2019). In addition, the C-terminus of Fam21 tail (a.a. 937–1341) interacted with various acidic phospholipids *in vitro*, in particular $PI(3,5)P_2$ (which is enriched in early endosomes) and PI4P (which is enriched in the Golgi), which might serve as a retromer-independent mechanism for recruiting the SHRC to endosome membranes (Buckley et al., 2016; Gomez and Billadeau, 2009; McNally et al., 2017). The Fam21 tail also binds to FKBP15 and recruits it to endosome membranes, and binds to RME-8 to coordinate with the membrane-tubulating function of the sorting nexins (Freeman et al., 2014; Harbour et al., 2012). The N-terminus of Fam21 (a.a. 1–356) was shown to bind ANKRD50, but we speculate the binding region is located in the unstructured sequence (a.a. 120–356) (Kvainickas et al., 2017). Moreover, the Fam21 tail contains both a nuclear

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localization signal sequence (NLS) and a nuclear export signal (NES), and binds multiple components of the nuclear factor κB (NF-κB) pathway (such as the p50 and p65 (RelA) NF-κB subunits), suggesting the isolated Fam21 has a distinct role in the nucleus (Deng et al., 2015). The Fam21 C-terminal also tail contains many phosphorylation sites, which may fine tune protein-protein or protein-lipids interactions (Hornbeck et al., 2015).

While the aforementioned ligands that bind to the C-terminal tail of Fam21 play important roles in linking SHRC to endosomal membranes and other regulators of endosomal trafficking, they don't directly bind to the core, structured part of the SHRC and, therefore, may not directly contribute to SHRC activation. Analogous to WASP/N-WASP and the WRC, several other mechanisms could directly contribute to SHRC activation through the core structured region and likely act in a cooperatively manner. These include post-translational modifications (phosphorylation and ubiquitination), binding to GTPases, and perhaps binding to novel ligands. WASH contains several phosphorylation sites in the WAHD region, which may promote SHRC activation analogous to the phosphorylation of WAVE meander region (Hornbeck et al., 2015). For example, Y141 in the WHD1 was shown to be phosphorylated by Lck in NK cells, and Y262 (Y261 in mouse WASH) in the WHD2 was phosphorylated by Btk in both *Drosophila* and mouse models (Huang et al., 2016; Tsarouhas et al., 2019). Y141F inhibited trafficking of lytic granules to the immune synapse, leading to impaired cytotoxicity of NK cells, while Y262 phosphorylation caused SHRC activation. It remains to be tested whether phosphorylation alone is sufficient to activate the SHRC. In addition to phosphorylation, ubiquitination at K220 in the WHD2 region by the MAGE-L2-TRIM27 E3 ubiquitin ligase was shown to be important for SHRC-mediated actin polymerization on endosomes and sufficient to activate the SHRC *in vitro* (Hao et al., 2013). Ubiquitination-mediated activation might not be unique to SHRC, as ubiquitination in the WAVE meander region (e.g., K161 in WAVE2 and K162 WAVE3) was observed in multiple high-throughput proteomic studies (Hornbeck et al., 2015).

Small GTPases have a central role in activating WASP/N-WASP (Cdc42) and the WRC (Rac1 and Arf). Does SHRC activation similarly require a GTPase? The answer is currently unknown, but a few clues suggest it is likely "yes". First, small GTPases, especially the large number of Arf (~30 in human) and Rab GTPases (~60 in human), are found all over the endomembrane system (Donaldson and Jackson, 2011; Stenmark, 2009). They play important roles in virtually every step of intracellular trafficking (including endosomal trafficking) where they regulate biogenesis, sorting, tethering, fusion, tubulation, and fission of various organelles (Molendijk et al., 2004). It is possible one or a group of them activates the SHRC, but the interaction may be weak or transient and cooperative with other membrane ligands, similar to the interaction between Rac1 or Arf1 and the WRC, which can elude conventional identification methods. Second, a study in *Drosophila* showed the GTPase Rho directly interacted with WASH in a nucleotide-dependent manner (Liu et al., 2009; Jeffrey M Verboon et al., 2015). Other studies using purified human proteins, however, could not detect the interaction between SHRC and RhoA, but detected nucleotide-independent interaction between SHRC and Rac1 (Jia et al., 2010). Neither Rac1, RhoA, nor Cdc42 was able to activate SHRC *in vitro*, suggesting some other ligands or conditions may be involved (Jia et al., 2010). Third, by using an *in situ* APEX2-mediated proximal labeling method, a recent study found Rab21 and Rab7 interacted with the SHRC and revealed the

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importance of Rab21 in endosomal cargo sorting (Del Olmo et al., 2019). In addition, Rab9 was also linked to SHRC-mediated retrograde trafficking (Dong et al., 2013). It is, however, unknown if these interactions are direct and how they contribute to SHRC activation. *In vitro* reconstitution methods will be important for validating and characterizing these potential interactions.

Other ligands may also interact with the core structure of SHRC. It is possible that, similar to the WRC, SHRC may contain a positively charged surface to directly bind to inositol phospholipids, independent of the lipid-binding activity of Fam21 C-terminal tail (Figure 4) (Derivery et al., 2009b; Gomez and Billadeau, 2009). Strumpellin-SWIP were shown to interact with the caveolar protein CAV1, which was important for maintaining CAV1 homeostasis required for integrin-mediated cell adhesion (Lee et al., 2020). The WAHD region of WASH is implicated in many interactions, which may not only link SHRC to other ligands, but also potentially contribute to SHRC activation. The WHD2 region of WASH directly interacted with tubulin, which may link microtubules to the actin cytoskeleton in endosome trafficking (Gomez and Billadeau, 2009; Liu et al., 2009). The overall WAHD region of WASH was reported to interact with two subunits of the exocyst complex, Sec3 and Exo84, to regulate exocytosis of transmembrane type 1 matrix metalloproteinase (MT1-MMP) at invadopodia (Monteiro et al., 2013). The middle region of WAHD was found to directly interact with Beclin1, which provided a mechanism to suppress autophagy (Xia et al., 2013). In addition, SHRC interacted with Hermansky-Pudlak syndrome complex BLOC-1 and its cargo phosphatidylinositol-4-kinase type IIα, likely through a direction interaction between the WAHD region of WASH and the BLOS2 subunit of BLOC-1 complex (Monfregola et al., 2010; Ryder et al., 2013). WASH may also directly interact with VPS35, which could further facilitate retromer binding (Harbour et al., 2012). Again, most of the above interaction mechanisms and their effects on SHRC activity remain largely unknown.

Unlike WASP/N-WASP and the WRC, which mainly regulate actin polymerization at the cell membrane, the SHRC mainly regulates actin assembly at early and recycling endosome membranes (Figure 1, right) (Duleh and Welch, 2010; Fokin and Gautreau, 2021; Seaman et al., 2013; Wang et al., 2018). The activity of the SHRC in promoting actin assembly plays a key role in maintaining tubular structures of the recycling endosomes and facilitating protein sorting (Derivery et al., 2009b; Fokin et al., 2021; Gomez et al., 2012; Gomez and Billadeau, 2009). SHRC-dependent actin networks are often found as discrete patches at endosomal membranes (Derivery et al., 2012, 2009b; Gomez and Billadeau, 2009). These actin-rich domains promote tubulation of membrane structures where specific cargo proteins are clustered, and they further facilitate dynamin-mediated membrane scission to release the vesicles (Puthenveedu et al., 2010). Loss of SHRC activity collapses endo-lysosomal membranes and impedes receptor recycling, leading to excessive degradation by the lysosome, which affect homeostasis of numerous important membrane proteins, such as CI-MPR, TfnR, EGFR, β2AR, TCR, CD28, LFA-1, GLUT1, LDLR, SR-BI, ATP7A, CAV1, α5β1-integrin, and ACE2 (the entry receptor for SARS-CoV-2 infection) (Bartuzi et al., 2016; Courtland et al., 2021; Gomez et al., 2012; Gomez and Billadeau, 2009; Lee et al., 2020; Phillips-Krawczak et al., 2015; Piotrowski et al., 2013; Puthenveedu et al., 2010; Temkin et al., 2011; Wijers et al., 2019; Zech et al., 2011; Zhu et al., 2021).

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In addition, new roles of the SHRC in regulating autophagy are emerging, where SHRC is required for trafficking of autophagy proteins, suppressing autophagy, and driving V-ATPase removal from lysosomes and lysosome neutralization, which is required for efficient phagocytic and autophagic clearance (Carnell et al., 2011; King et al., 2013; Nagel et al., 2017; Park et al., 2013; Xia et al., 2014, 2013; Zavodszky et al., 2014).

Given the fundamental role of the SHRC in endolysosomal biology, it is not surprising that disruption of SHRC function has a profound impact on homeostasis and signal transduction in various physiological processes, including cellular signaling, cholesterol clearance, and immune function (Simonetti and Cullen, 2019). Complete loss of the SHRC in animals severely interrupts development and leads to early embryonic death (Gomez et al., 2012; Linardopoulou et al., 2007). Mutations or aberrant activities of the SHRC and its closely related partners, including retromer, retriever, and the CCC complex, are the cause of various complicated developmental/neurological disorders, including hereditary spastic paraplegia 8 (HSP8), Ritscher-Schinzel syndrome, autosomal recessive intellectual developmental disorder-43 (MRT43), and Parkinson's disease 17 (PARK17) (Gangfuß et al., 2022; Ginanneschi et al., 2020; Gjerulfsen et al., 2021; Rahman and Morrison, 2019). Without a high-resolution structure of the SHRC, it is currently unknown exactly how various missense mutations cause disease.

At least 4 types of homozygous and heterozygous missense mutations in SWIP, including P1019R, Q442*/D1048G, K1079R/H503R, and Y1014C, have been identified as the cause of autosomal recessive intellectual developmental disorder-43 (MRT43), which shows severely impaired developmental and intellectual development, poor learning and motor skills, short stature, dysmorphic features, and recurrent infections (Assoum et al., 2020; Gangfuß et al., 2022; Ropers et al., 2011). Among them, the P1019R mutation may interfere with protein folding, as the patient cells showed significantly reduced expression of SWIP, Strumpellin, and WASH, which resulted in loss of SHRC function (Ropers et al., 2011). Similar phenotypes were shown in a recent mouse model, in which $\mathrm{SWIP}^{\mathrm{P1019R}}$ was found to reduce SHRC level and significantly disrupt both endosomal and lysosomal pathways (Courtland et al., 2021). By contrast, Y1014C did not seem to affect SHRC expression and therefore may be located in a region important for SHRC activity regulation or ligand binding (Gangfuß et al., 2022).

Many mutations in Strumpellin have been associated with hereditary spastic paraplegia 8 (HSP8) and Ritscher-Schinzel Syndrome (Ginanneschi et al., 2020; Gjerulfsen et al., 2021). HSP8 is an autosomal dominant neurologic disorder characterized by late onset lower limb spasticity and hyperreflexia. At least 12 different mutations in Strumpellin have been identified in HSP8 patients, including various missense mutations, an internal exon, and a frameshift mutation (de Bot et al., 2013; Ginanneschi et al., 2020; Valdmanis et al., 2007). Cellular and biochemical studies of N471D, L619F, and V626F did not detect clear defects in protein expression, ligand binding, or endosomal trafficking (Freeman et al., 2013; Jia et al., 2010). A recent mouse model of $\mathrm{Strumpellin}^{\mathrm{N471D}}$ showed this mutation recapitulated HSP8 phenotypes in human patients and mildly altered the brain proteome, albeit without affecting protein levels of SHRC or related ligands, suggesting this mutation (or other mutations) might disrupt ligand binding (Clemen et al., 2021).

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In addition to mutations that lead to HSP8, a splice site mutation (c.3335+2T-A) in Strumpellin is a cause of Ritscher-Schinzel Syndrome, also known as cranio-cerebello-cardiac syndrome or 3C syndrome, which is characterized by craniofacial abnormalities, congenital heart defects, and cerebellar malformations. This mutation causes frameshift and premature termination of Strumpellin and significantly reduces protein expression level (Elliott et al., 2013).

It is worth noting that mutations in closely related SHRC ligands, including CCDC22 (a subunit of the CCC complex) and VPS35L (a subunit of retriever), were the cause of two subtypes of Ritscher-Schinzel Syndrome (Gjerulfsen et al., 2021; Kato et al., 2020; Kolanczyk et al., 2015). Additionally, the D620N mutation in VPS35 (a subunit of retromer) is the cause of Parkinson's disease 17 (PARK17) (Rahman and Morrison, 2019). The mutation was found to impair VPS35 binding to Fam21 and subsequently disrupt endosome-to-TGN transport of CI-MPR and the turnover of mitochondrial DLP1 complex causing mitochondrial fragmentation and dysfunction in neurons (McGough et al., 2014; Wang et al., 2016). The above suggests disrupting SHRC-CCC-retriever vs. SHRC-retromer recycling pathways results in separate disease conditions, supporting the notion that the two pathways play distinct roles in the cell (Figure 4A) (McNally et al., 2017; Phillips-Krawczak et al., 2015).

SHRC is also involved in cancer, mainly through its role in regulating the recycling of cell adhesion molecules (e.g., α5β1-integrin) and transmembrane matrix metalloproteinase (e.g., MT1-MMP), which are required for migration and invasion of cancer cells (Biber et al., 2020; MacDonald et al., 2018; Monteiro et al., 2013; Porkka et al., 2004; Zech et al., 2011). Finally, the intracellular bacterial pathogen, *Burkholderia cenocepacia*, the cause of severe pulmonary infections in cystic fibrosis and chronic granulomatous disease patients, was found to hijack SHRC-mediated endolysosomal recycling to escape phagosome maturation and facilitate survival and infection (Walpole et al., 2020). It is possible this mechanism is also used by other intracellular pathogens. Recently, a genome-wide CRISPR screen identified SHRC, the CCC complex, and retromer as important host factors that regulate the entry of SARS-CoV-2 virus (Zhu et al., 2021). Loss of any of these components led to a significant reduction of surface ACE2, the entry receptor critical for SARS-CoV-2 infection (Zhu et al., 2021).

In summary, the SHRC is a central regulator of the actin cytoskeleton on endosome membranes to support endolysosomal membrane trafficking. Disturbance of the function of SHRC and its closely related ligands has profound impact on many different signaling pathways associated with diseases. Compared to WASP/N-WASP and the WRC, most mechanistic questions of how the SHRC interacts with different ligands and how its membrane recruitment and activity are regulated still need to be answered.

WHAMM and JMY

We discuss WHAMM and JMY together here as they share similar domain structures and partially overlapping cellular functions (Figure 1 & 5, and see below) (Campellone et al., 2008b; Rottner et al., 2010). One major difference between WHAMM/JMY and

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aforementioned WASP/N-WASP, WAVEs, and WASH is that WHAMM and JMY are basally active in stimulating Arp2/3-mediated actin polymerization. Their WCA sequences are not autoinhibited, neither *in cis* in a single polypeptide chain as for WASP/N-WASP, nor *in trans* in a large protein complex as for WAVE and WASH (Figure 5) (Campellone et al., 2008b; Kabrawala et al., 2020; Zuchero et al., 2009). Nevertheless, their actin-nucleation activity is regulated in the cell by ligand binding and by control of their cellular localization, although very little is currently known about the underlying biochemical or structural mechanisms.

WHAMM was discovered in 2008 from a search in the human proteome for WCA-containing proteins (Campellone et al., 2008b). By contrast, JMY was initially discovered in 1999 as a cofactor for p53, where in response to DNA damage it accumulated in the nucleus and activated p53-mediated transcription in a complex with p300 and STRAP (Demonacos et al., 2001; Shikama et al., 1999). JMY was not recognized as a WASP-family protein until a genome database search in 2009 for WH2-containing proteins (Zuchero et al., 2009). WHAMM and JMY share ~27–35% amino acid identity throughout the entire sequence (Campellone et al., 2008b). WHAMM contains two consecutive WH2 motifs (making a WWCA domain at the C-terminus), while JMY contains three WH2 motifs (making a WWWCA domain at its C-terminus) (Figure 1, left). The domain assignment of their N-terminal sequences, however, has been ambiguous (Campellone et al., 2008b; Dai et al., 2019; Zuchero et al., 2009). In one commonly used assignment, WHAMM contains a WHAMM membrane-binding domain (WMD, a.a. 1–260) and a central coiled coil domain (CC, a.a. 260–570). Similarly, JMY also contains an NT domain (a.a. 1–392) and a CC domain (a.a. 393–793) (Campellone et al., 2008b; Dai et al., 2019).

At the writing of this review, protein structure prediction by AlphaFold 2 had just achieved tremendous success (Jumper et al., 2021). We thereby analyzed the structure models generated by AlphaFold 2 in an attempt to have a better definition of the structural organization of WHAMM and JMY. To our surprise, we found striking structural similarity between the N-terminal regions of JMY and WHAMM. The structural models of both proteins revealed two clearly defined domains, which were different from the domain organization previously derived from sequence analysis (Figure 5C). The relative orientation between the two domains differed between JMY and WHAMM models, but the individual domains aligned very well between the two models, giving a root-mean-square deviation (r.m.s.d.) of less than 1 Å (Figure 5C). This suggests 1) the two regions are likely independent domains, and 2) the N-terminal regions of JMY and WHAMM have very similar tertiary structures despite their relatively low sequence identity.

The first domain, formed by a.a. 26–174 in WHAMM and a.a. 26–69 and a.a. 224–316 in JMY, contains a core globular structure consisting of a three-helix bundle packed against a three anti-parallel β-strands (Figure 5D). Protruding from the core are two insertions, one between β2 and β3 and the other between α1 and α2, giving a L-shaped molecule (Figure 5D). We herein name this first domain as the W̲HAMM and J̲MY h̲omology d̲omain (WJHD) (Figure 1). Note in the insertion between β2 and β3 (insert 1 in Figure 5D), JMY contains a long, unstructured loop of ~150 a.a., which we name J-loop (for J̲MY-specific loop). This loop is not present in WHAMM and may carry functions unique to

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JMY. Remarkably, a.a. 1–314 in JMY was shown to have a cryptic actin-nucleation activity without a known mechanism (Hu and Mullins, 2019), while such an activity has not been reported for the corresponding region in WHAMM. It will be interesting to see if this activity comes from the structured WJHD domain or the unstructured J-loop.

Searching for structures similar to the WJHD in the PDB database using PDBeFold identified many different PH (phox-homology) domains, which aligned to the core structure, but not including the two inserts protruding from the core (see two examples in Figure 5D) (Krissinel and Henrick, 2004). The structural similarity between the WJHD and the PX domain is consistent with the functions of WHAMM and JMY, as PX domains usually bind inositol phospholipids and are found in various proteins involved in membrane binding, vesicle trafficking, cell signaling, and lipid metabolism (Chandra and Collins, 2019). As discussed below, WHAMM and JMY are key actin regulators in various membrane-associated processes in the endomembrane system. In addition, the previously defined WMD region of WHAMM, which covers the entire WJHD, was shown to bind different inositol phospholipids, especially PI(4,5)P_2. It is possible the WJHD of both WHAMM and JMY acts as a PX domain to bind inositol phospholipids and facilitate their membrane localization. In addition to binding lipids, the WJHD may also mediate protein-protein interactions, which is sometimes seen for PX domains. The two inserts protruding from the core structure were not found in canonical PX domains, which may alter the function or add new functions to the WJHD, including novel protein-protein interactions or activity regulation.

It is worth noting that the 26-a.a. unstructured sequence N-terminal of the WJHD contains a conserved LC3-interacting region (LIR) (Figure 1 & 5). LC3 is a protein specifically found on autophagosome membranes. The LIR in JMY was previously identified based on the consensus sequence found in various LC3-binding proteins, which was W/F/Y-x-x-L/I/V flanked by acidic residues and an S or T (Coutts and La Thangue, 2015). Mutating the conserved W and V in the LIR (ETLESD**W**VA**V**RP) abolished the autophagosome localization of JMY. It is not known if WHAMM also binds to LC3, but WHAMM contains a similar LIR (DSLEG**W**VP**V**RE) in the same region and also plays a role in autophagosome development and function (Campellone et al., 2008b; Dai et al., 2019; Kast et al., 2015). It is possible the juxtaposed LIR sequence and the WJHD act synergistically to facilitate the membrane recruitment of WHAMM and JMY to autophagosome.

The second domain, formed by a.a. 175–557 in WHAMM and a.a. 317–690 in JMY, contains coiled-coils stacked by 6 helices, for which we follow the previous name as the coiled coil domain (CC). Searching for similar structures did not give meaningful results due to the low-complexity nature of helical bundles. It is possible the predicted CC structure is either novel folding or inaccurate. In spite of this, these predicted structural models are exciting in providing new insights into the structure-function relationship in WHAMM and JMY. Experimental validation of these structural predictions will be important in future studies. Below we discuss features specific to each protein.

Purified WHAMM is a monomer and is constitutively active towards the Arp2/3 complex (Campellone et al., 2008b; Kast et al., 2015). The previously defined WMD directly

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binds to different inositol phospholipids, especially $PI(4,5)P_2$, which is responsible for recruiting WHAMM to various intracellular compartments, including the cis-Golgi, the ER-Golgi intermediate compartment, autophagosomes, and autolysosomes (Campellone et al., 2008b; Dai et al., 2019; Kast et al., 2015). The CC domain directly binds to microtubules (Campellone et al., 2008b; Liu et al., 2017; Shen et al., 2012). The WWCA domain of WHAMM is competent in promoting Arp2/3-mediated actin polymerization. This activity is not absolutely required for ER to Golgi transport, but is important for the elongation and stabilization of WHAMM-associated tubular membranes, ER to autophagosome biogenesis and trafficking, and autophagic lysosome reformation (Campellone et al., 2008b; Dai et al., 2019; Kast et al., 2015).

Only a few WHAMM-binding ligands have been identified, including inositol phospholipids, microtubules, and GTPases (Figure 5A). It is interesting that although purified WHAMM is constitutively active in promoting Arp2/3-mediated actin polymerization, several ligands inhibit this activity *in vitro*, suggesting WHAMM activity in the cell is dynamically regulated depending on the cellular context. For example, microtubule binding inhibited WHAMM activity in a dose-dependent manner, suggesting that binding microtubules and promoting actin polymerization are mutually exclusive for WHAMM function (Shen et al., 2012). Both activities, however, were required for proper function of WHAMM in facilitating membrane tubulation and anterograde transport (Campellone et al., 2008b). It is therefore possible that two spatiotemporally regulated populations of WHAMM exist to coordinate the activities at the interfaces between various endomembranes and the two cytoskeletal systems (Shen et al., 2012). Different GTPases, including Rab1, RhoD, and Rif, were found to bind the WMD region of WHAMM in a nucleotide dependent manner (Gad et al., 2012; Russo et al., 2016). Among them, the prenylated Rab1 (but not the unprenylated form) directly interacted with the WMD. This interaction inhibited WHAMM-mediated actin polymerization *in vitro* in a dose- and nucleotide-dependent manner (Russo et al., 2016). In addition, the BLOS1 subunit of the BLOC complex might interact with WHAMM in the initiation of autolysosomal tubulation (Wu et al., 2021). Without detailed biochemistry or high-resolution structures, it remains unknown how ligand binding to N-terminal domains inhibits the activity of WWCA, since the two parts are separated by a long unstructured sequence (Liu et al., 2017). It is also unknown if any posttranslational modifications are important for WHAMM regulation (Hornbeck et al., 2015).

WHAMM is only found in vertebrates and is broadly expressed, particularly in the brain (Campellone et al., 2008b). WHAMM plays an important role in regulating various endomembrane systems, including Golgi positioning and morphology, ER to Golgi transport, ER to autophagosome biogenesis and trafficking, and autophagic lysosome formation (Campellone et al., 2008b; Dai et al., 2019; Kast et al., 2015). In addition, WHAMM and its activity in promoting Arp2/3-mediated actin polymerization is required for apoptosis, cell adhesion, and migration (Gad et al., 2012; King et al., 2021). Furthermore, WHAMM plays an essential role in spindle actin polymerization, spindle formation and migration, and asymmetric cytokinesis in mouse oocytes (Huang et al., 2013; Jo et al., 2021).

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WHAMM mutations have been associated with several types of diseases. A missense mutation, R725W, in WHAMM was identified as a pathogenic factor in patients with inflammatory bowel disease (Ben-Yosef et al., 2021). In another case, homozygous frameshift mutations in *WDR73* together with closely linked *WHAMM* were identified as the cause of Galloway-Mowat syndrome (GMS), a nephrocerebellar syndrome characterized by microcephaly and nephrosis (Jinks et al., 2015; Mathiowetz et al., 2017). WHAMM was also suggested as a candidate gene associated with severe chronic periodontitis in genome-wide association studies, but the results remained inconclusive (Rhodin et al., 2014; Shang et al., 2015).

JMY shares many similarities with WHAMM, but is also unique among the WASP-family proteins in that it can polymerize actin in an Arp2/3-dependent and -independent manner (Firat-Karalar et al., 2011; Zuchero et al., 2009). In the presence of Arp2/3, JMY uses its WCA sequence to produce branched actin filaments similar to other WASP-family proteins, while in the absence of Arp2/3, JMY uses its tandem WH2 sequences to produce unbranched actin filaments using a mechanism similar to actin nucleation factors that also contain multiple WH2 sequences, such as Spire (Dominguez, 2016; Firat-Karalar et al., 2011; Zuchero et al., 2009). Also unique to JMY is that its N-terminal WJHD does not contain any identifiable actin-binding motifs, but is sufficient to promote actin polymerization *in vitro* independent of WWWCA or Arp2/3 (Figure 5B) (Hu and Mullins, 2019). This region also bound the autophagy regulator LC3, with the interaction enhancing its actin nucleation activity (Hu and Mullins, 2019).

Similar to WHAMM, purified JMY was constitutively active in promoting actin polymerization and did not seem to exist in a large regulatory complex (Zuchero et al., 2009). Inside the cell, JMY activity seemed to be suppressed (Figure 5B) (Firat-Karalar et al., 2011). The mechanisms suppressing its actin polymerization activity remain unclear, but cellular localization likely plays an important role. Unlike its homolog WHAMM, which is primarily located at endomembranes, JMY was found primarily in the nucleus, cytosol, and the leading edge of motile cells, depending on the cell type and conditions (Firat-Karalar et al., 2011; Zuchero et al., 2009). JMY is dynamically shuttled between the cytosol and the nucleus to balance its activity in promoting actin polymerization and in augmenting gene transcription of proteins involved in cell motility, such as RhoD and cadherins (Coutts et al., 2009; King et al., 2021). It is remarkable that the localization of JMY to the leading edge was correlated with cell motility, and its role in promoting cell migration required its Arp2/3-activating activity (Firat-Karalar et al., 2011; Zuchero et al., 2009). Shuttling JMY between the cytosol and the nucleus depended on its second nuclear localization sequence (NLS), which spanned the C-terminal part of the first WH2 and the linker that leads to the second WH2 (Figure 5B). Mutating these two WH2 domains to abolish their actin binding capacity or depleting monomeric actin from the cytosol dramatically shifted JMY to the nucleus, suggesting actin binding to WH2 competed against its nuclear import (Zuchero et al., 2012). This presents a novel mechanism of using monomeric actin to balance the activity of JMY in promoting actin polymerization in the cytosol and transcription in the nucleus.

JMY is widely expressed in various tissues, particularly in the brain, heart, and testes (Firat-Karalar et al., 2011; Shikama et al., 1999). Its cellular functions often overlap with

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its homolog WHAMM, including apoptosis, anterograde vesicle trafficking, autophagosome formation, and spindle actin polymerization and asymmetric cytokinesis in oocytes (Figure 1, right) (Coutts and La Thangue, 2015; Hu and Mullins, 2019; King et al., 2021; Liu et al., 2012; Schlüter et al., 2014; Sun et al., 2011). JMY also plays an important role in cell migration and neurite outgrowth, oligodendrocyte differentiation and maturation, and spermatogenesis (Azevedo et al., 2018; Coutts et al., 2009; Firat-Karalar et al., 2011; Liu et al., 2020; Zuchero et al., 2009).

Recent studies have started to reveal how JMY is localized to different membranes and how its activity is regulated (Coutts and La Thangue, 2015; Hu and Mullins, 2019). The N-terminal WJHD of JMY has two functions: binding to LC3 using the LIR sequence and promoting actin polymerization using an unknown mechanism independent of the C-terminal WWWCA region (Figure 5B). Binding to LC3 provided a mechanism to recruit JMY to LC3-containing autophagosomes. In the meantime, LC3 binding enhanced the actin-nucleating activity of the N-terminal sequence to promote autophagosome formation and maturation (Figure 5B) (Coutts and La Thangue, 2015; Hu and Mullins, 2019). Interestingly, STRAP, the nuclear partner of JMY, also exists in the cytosol to bind JMY and regulate autophagy. The interaction between STRAP and JMY potently inhibited the overall actin nucleation activity of JMY and counteracted the effect of LC3 (Hu and Mullins, 2019). In the nucleus, STRAP, JMY, and p300 form a complex to prevent JMY degradation by MDM2 and facilitate p53 activation (Demonacos et al., 2001). The interaction between STRAP and JMY was shown to involve N-terminal, central, and C-terminal sequences of JMY (Demonacos et al., 2001). It is plausible that the cytoplasmic STRAP inhibits the actin-nucleation activity of JMY by sequestering both its the N-terminal WJHD and C-terminal WWWCA (Figure 5B). It remains an open question exactly how LC3 and STRAP interact with JMY to regulate its activity and how the WJHD or the J-loop of JMY promotes actin polymerization.

It is worth noting that, although the dual function of JMY in the cytosol and the nucleus is a remarkable feature of JMY, other WASP-family proteins, actin, and Arp2/3 have also been shown to have important roles in the nucleus, either dependent on or independent of their activity in Arp2/3-mediated actin polymerization. Their nuclear functions are a separate topic of great interest and are not the focus of this review (Caridi et al., 2018; Kluge et al., 2018; Miyamoto et al., 2013; Schrank et al., 2018; Shikama et al., 1999; Taylor et al., 2010; Jeffrey M. Verboon et al., 2015; Weston et al., 2012; Wu et al., 2006).

The connection of JMY with human diseases is not yet established, although its role in augmenting the activity of the tumor suppressor p53 in response to DNA damage and its role in regulating cell migration suggests JMY could play a role in tumor invasiveness. In addition, JMY was found to be a candidate gene for susceptibility of ankylosing spondylitis in genome-wide analysis (Chai et al., 2013).

In conclusion, WHAMM and JMY are homologous proteins and form a distinct group of the WASP-family protein. They are not autoinhibited in the basal state as is seen for WASP/N-WASP, WAVEs, and WASH. They link the Arp2/3 complex to a diverse array of important functions in the cell that cover various endomembrane compartments, intracellular

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trafficking pathways, and the plasma membrane. Their activity is dynamically regulated in the cell, although currently very little is known on the cellular, biochemical, and structural levels about how the regulation is achieved.

WHIMP

The newest member of the WASP-family proteins, WHIMP, was identified in 2020 from a genomic sequence search for WH2-domain containing proteins (Kabrawala et al., 2020). WHIMP seems to be the least conserved of the WASP-family proteins, because even though it exists in many examined mammals it was not identified in the human genome. Furthermore, sequence conservation between different animals was also relatively low (Kabrawala et al., 2020). This suggests WHIMP may have appeared recently in evolution and may have special functions in animals. The N-terminal domain of WHIMP is distantly related to part of the WHD region of WAVE, which we herein name as WHD-like domain (WHDL) (Figure 1, left). WHIMP does not contain a PRR, which is found in all other WASP-family proteins. The activity of its C-terminal WCA sequence is relatively weak towards Arp2/3-mediated actin polymerization both *in vitro* and in cells (Kabrawala et al., 2020). WHIMP is ubiquitously expressed in mouse tissues with low expression in the brain. It is enriched in membrane ruffles and the leading edge of migrating cells, sharing the same localization with Arp2/3, N-WASP, and WAVE. WHIMP expression induced formation of large, highly dynamic membrane ruffles and non-selective micropinocytosis, promoted cell migration, and increased Src-mediated phosphorylation at membranes (Kabrawala et al., 2020). The increased membrane ruffling was dependent on the WHIMP WCA, Arp2/3, and Rac1-WRC signaling. Increased Src phosphorylation is believed to provide positive feedback to facilitate Rac1-WRC and/or Cdc42-N-WASP signaling to enhance actin polymerization and membrane protrusions (Kabrawala et al., 2020). It remains uncertain whether WHIMP also exists in humans and how its activity is regulated in the cell.

Summary and Prospects

Since the initial discovery of WASP, two decades of work has now expanded this protein family to 9 members in mammals and has greatly advanced our understanding of their cellular functions and regulation mechanisms (Alekhina et al., 2017; Padrick and Rosen, 2010; Takenawa and Suetsugu, 2007; Veltman and Insall, 2010). Even though all WASP-family proteins are able to use their C-terminal WCA domain to stimulate actin polymerization through the Arp2/3 complex, it is fascinating how they use various N-terminal sequences to regulate their cellular localization and biochemical activity and how these regulatory sequences allow them to channel a vast diversity of upstream signals to the remodeling of the actin cytoskeleton to serve different membrane-associated processes throughout the cell. The fundamental roles of WASP family proteins in various processes explain their broad involvement in many diseases. The fact that genetic mutations disrupting or enhancing their activity often lead to similar disease conditions emphasizes the need for precise control of their activity in cells.

Despite many major achievements in this field, important questions remain open, and new, exciting functions and regulatory mechanisms are rapidly emerging. One major knowledge

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gap is the understanding, or in many cases clarification, of the regulatory mechanisms of these proteins. Filling this gap will require a combination of advanced tools in gene editing, cell imaging, proteomics, *in vitro* reconstitution, quantitative biochemistry, and structural biology. The other major knowledge gap is the discovery of new functions and regulatory mechanisms, both *in vitro* and in different physiological processes *in vivo*. Advanced gene editing, such as conditional knock-out and knock-in in both cultured cells and live animals, is becoming a powerful tool to approach these physiological questions. Meanwhile, advances in large-scale clinical genomics and high-throughput, quantitative proteomics and interactomics will provide an unprecedented wealth of information for uncovering the structure-function mechanisms of disease-causing mutations and identifying new regulatory molecules. At the same time, structural bioinformatics and targeted drug design will in parallel promote the identification of novel regulatory molecules useful both in research and in medical interventions.

Due to space limit, this review is only focused on WASP-family proteins in mammals, particularly in humans, and only focused on their canonical functions in regulating the Arp2/3-mediated actin polymerization. Many new functions and mechanisms in other organisms, such as plants, or those independent of Arp2/3 or actin are also rapidly emerging (Ali et al., 2020; Chin et al., 2021; Facette et al., 2015; Gavrin et al., 2020; Miyamoto et al., 2013; Taylor et al., 2010; Weston et al., 2012). These exciting fields await rigorous cellular, biochemical, and structural studies to address many mechanistic questions.

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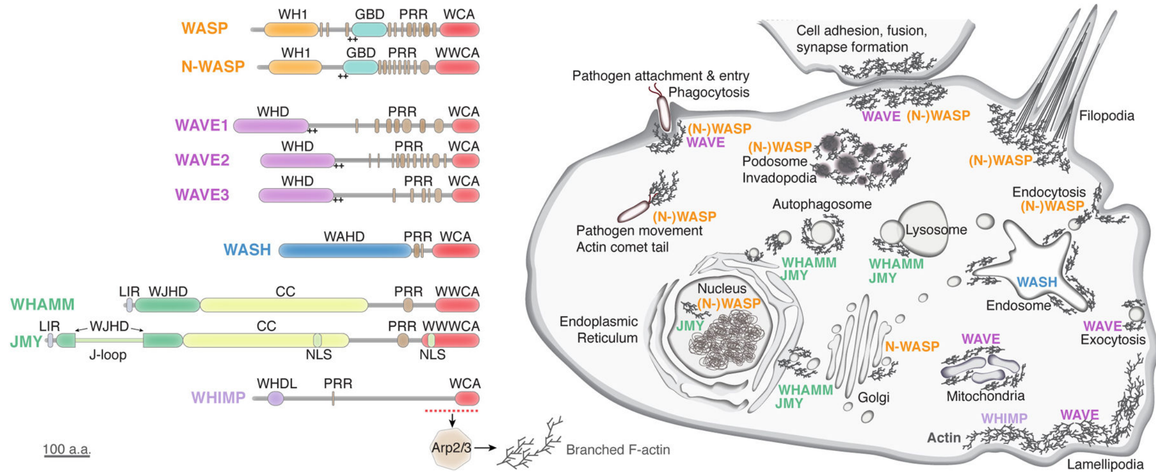

Figure 1. WASP-family protein domain structure and cellular function.

Schematic showing domain organization of different WASP-family proteins found in mammals (left) and their main functions and localizations in the cell (right). Domain structures are drawn to scale. Based on the structural homology from AlphaFold 2 prediction shown in Figure 5, we re-define WHAMM and JMY N-terminal domain as WJHD (WHAMM and JMY homology domain). WH1: WASP homology 1; GBD: GTPase binding domain; PRR: proline-rich region; WCA: WH2-central-acidic domain; WHD: WAVE homology domain; WAHD: WASH homology domain; LIR: LC3-interacting region; WJHD: WHAMM and JMY homology domain; J-loop: JMY-specific loop; CC: coiled coil domain; NLS: nuclear localization signal; WHDL: WAVE homology domain-like. “++” indicates positively charged sequence.

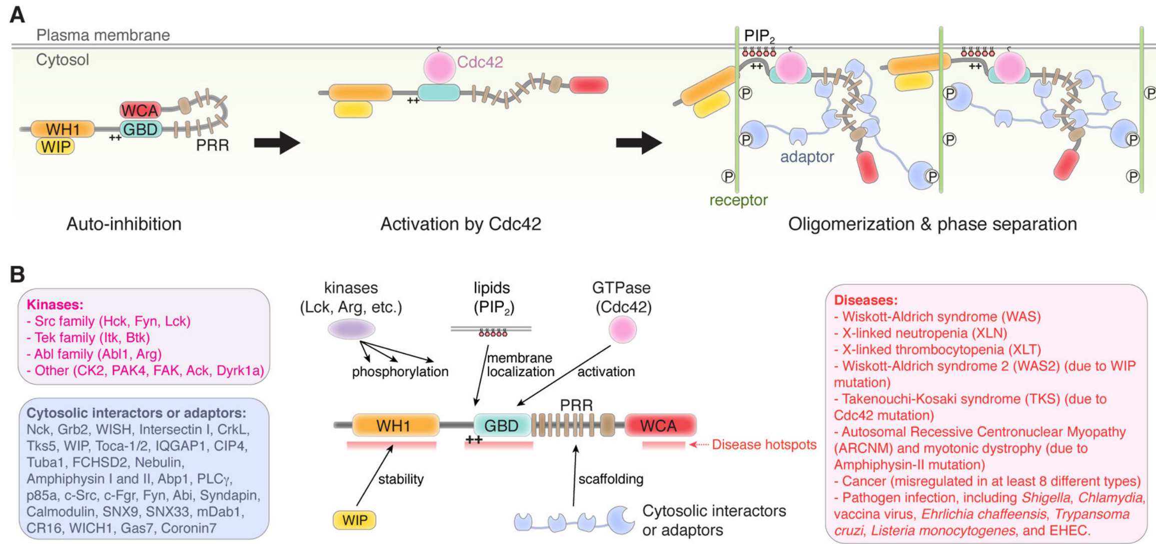

Figure 2. WASP and N-WASP.

(**A**) Major mechanisms underlying WASP and N-WASP auto-inhibition, activation, membrane localization, and oligomerization. (**B**) Schematic showing how different regulatory ligands interact with WASP and N-WASP. Text boxes show representative ligands in indicated category and diseases caused by or associated with WASP and N-WASP. Hotspots in WASP where most missense mutations in patients are clustered are indicated.

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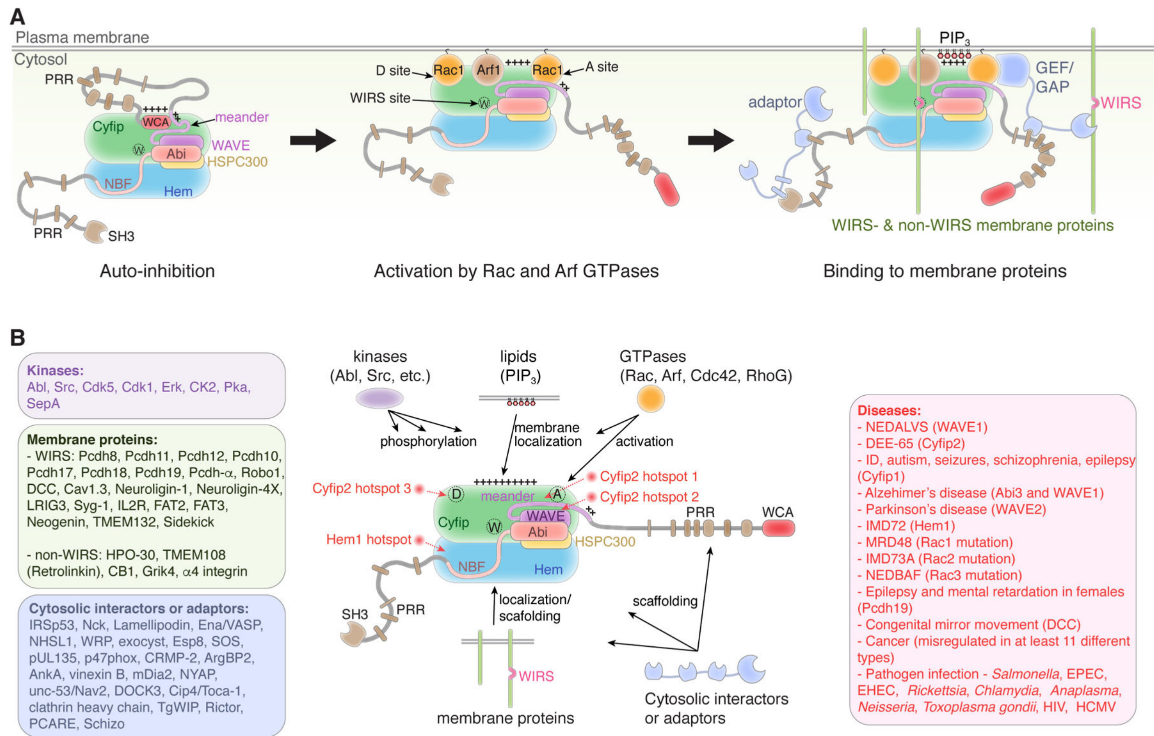

Figure 3. WAVE regulatory complex (WRC).

(**A**) Schematic showing mechanisms by which the WRC keeps WAVE auto-inhibited in the basal state, becomes activated by GTPase binding, and translocated to the membrane by directly interacting with membrane proteins and acidic phospholipids. "++++" indicates the positively charged side of the WRC. WAVE for WAVE1/2/3, Abi for Abi1/2/3, Hem for Hem1/Nap2, Cyfip for Sra1/Cyfip2. NBF: Nap1 binding fragment. (**B**) Schematic showing how different regulatory ligands interact with the WRC. Text boxes show representative ligands in indicated category and diseases caused by or associated with WRC subunits. Hotspots in the WRC where most missense mutations in patients are clustered are indicated. Ligands that bind to individual subunits of the WRC, but do not bind to the fully assembled WRC are not listed, such as N-WASP, FMRP, and eIF4E.

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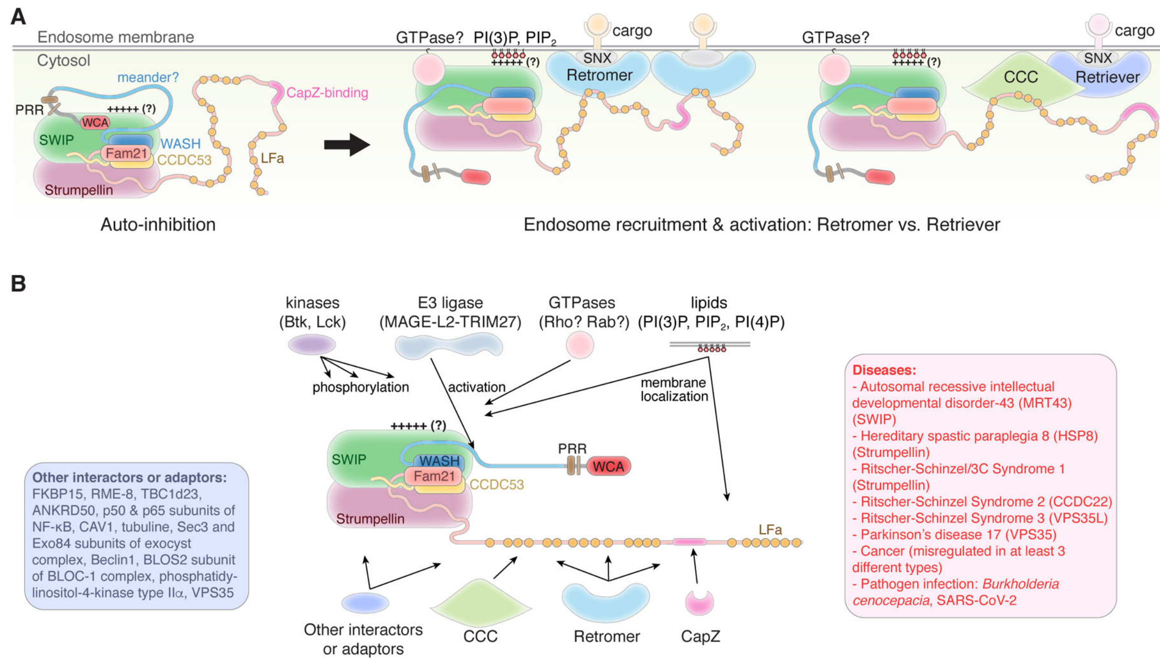

Figure 4. WASH regulatory complex (SHRC).

(**A**) Schematic showing mechanisms by which the SHRC may keep WASH auto-inhibited in the basal state and be activated and recruited to the endosomal membrane to regulate retromer- and CCC-retriever-mediated cargo sorting. The relative position of each subunit in the SHRC is based on its homology to the WRC. "++++?" indicates a positively charged surface possibly existing in the SHRC based on its resemblance to the WRC. "GTPase?" indicates the uncertainty of whether and what GTPase directly binds to the SHRC to induce activation. LFa: LF-(D/E)$_{3-10}$-LF sequence, which directly binds to VPS35 in retromer. (**B**) Schematic showing how different regulatory ligands interact with the SHRC. Text boxes show representative ligands in indicated category and diseases caused by or associated with SHRC subunits.

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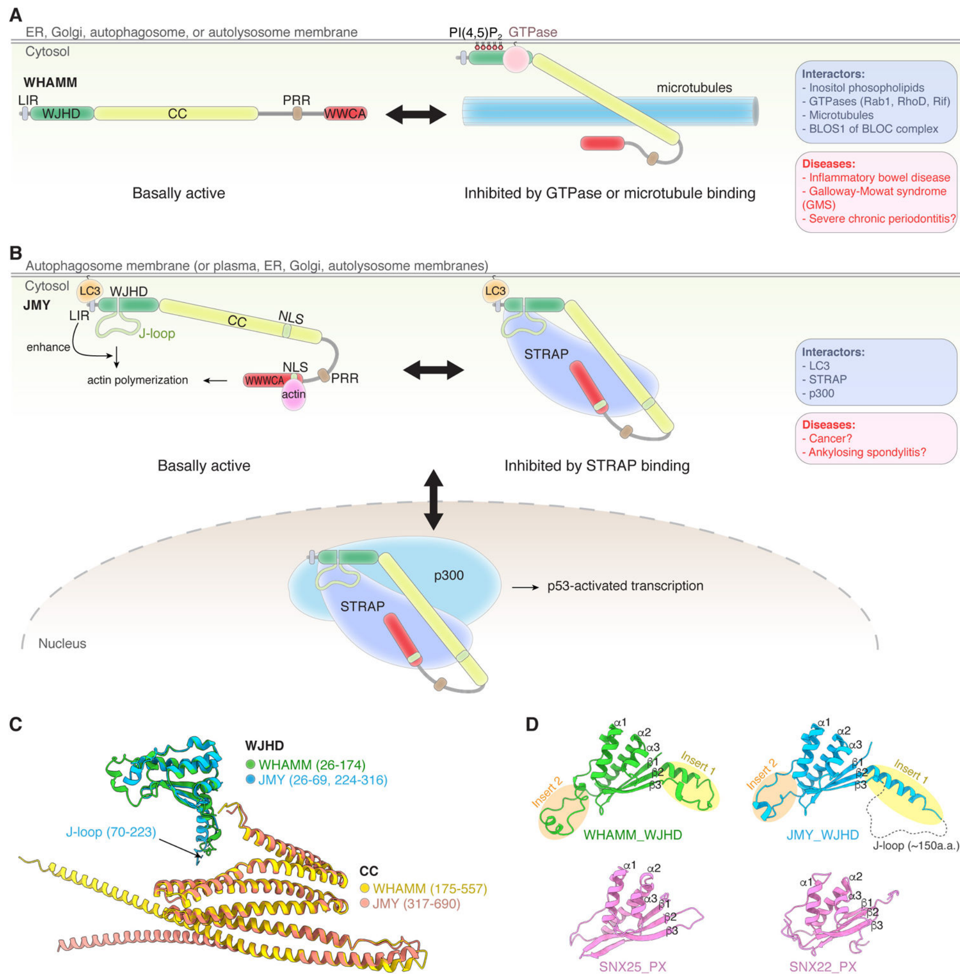

Figure 5. WHAMM and JMY.

(**A-B**) Mechanisms underlying WHAMM and JMY membrane localization and inhibition, respectively. Text boxes show known interacting ligands and associated diseases. (**C**) Overlay of the N-terminal structures of WHAMM and JMY predicted by AlphaFold 2. As the relative orientation between the two domains is different for WHAMM and JMY, shown in the cartoon is the two domains of JMY separately aligned to WHAMM. For clarity, unstructured sequences are removed from the presentation, including the J-loop insertion in the WJHD of JMY. (**D**) Comparison of WJHD with two representative PX domains, one from SNX25 (sorting nexin 25, PDB: 5WOE) and the other from SNX22 (PDB: 2ETT). The core PX folding is indicated by similar secondary structural elements. Also indicated are the structural inserts protruding from the core, which are unique to the WJHD and not found in PX domains. The J-loop unique to JMY is indicated by a dotted line.