Primer

WAVE regulatory complex

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Dynamic rearrangement of the actin cytoskeleton drives a myriad of processes in eukaryotic cells, such as cell migration and vesicle trafficking, and its dysregulation is deeply associated with various diseases, including cancer, immune deficiency, and neurological disorders. Members of the Wiskott-Aldrich syndrome protein (WASP) family, including WASP, N-WASP, WAVE, WASH, WHAMM, JMY, and the recently identified WHIMP, are ubiquitous regulators of actin dynamics. Although each WASP-family protein uses a different regulatory mechanism and participates in distinct cellular processes, they all act by integrating various upstream signals and transmitting them to their carboxy-terminal WCA (WH2-central-acidic, where WH2 stands for WASP homology 2) domain. This domain stimulates the actin nucleation activity of the Arp2/3 complex to promote the formation of new filaments from existing ones, creating branched actin networks that are crucial for dynamic deformations of membranes.

Among the WASP-family proteins, WAVE (WASP family Verprolin homolog — also known as SCAR for suppressor of cAMP receptor) is uniquely regulated through its constitutive incorporation into a large protein assembly of ~400 kDa, known as the WAVE regulatory complex (WRC). Since the Kirschner lab first discovered this complex in 2002 by biochemical purification, the WRC has attracted major attention from biologists and biochemists to understand its structure, regulation, and function. This is not only because the WRC is essential for membrane protrusion and cell migration and is widely implicated in human disease, but also because the size and complexity of the WRC make it a fascinating model for understanding membrane-to-actin signaling and allosteric regulation.

A series of tour de force studies in recent years combining cell biology, biochemistry, and structural biology have elucidated several fundamental mechanisms of the WRC and established it as a major signaling hub between the plasma membrane and actin in diverse processes. It is widely accepted that, in its basal state, the WRC is autoinhibited in the cytosol, but that a large variety of membrane ligands, including GTPases, phospholipids, membrane receptors, and kinases, can act cooperatively to activate and/or recruit the complex to the plasma membrane through direct interactions, and this in turn stimulates the Arp2/3 complex to polymerize actin. Many mechanistic questions, however, still remain unanswered, and new functions of the WRC are emerging. In this Primer, we summarize our current knowledge about the WRC and discuss major questions and challenges to be addressed for fully understanding its regulation and function.

Composition and assembly of the WRC

The WRC is assembled from five different protein subunits that are conserved in most eukaryotic species, including animals, plants, slime molds, and many protists and unicellular algae. For each subunit, vertebrates and multicellular plants usually have several orthologs in their genomes (Figure 1), including 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). For simplicity, in this article we use nomenclature based on the murine or human WRC, specifically the complex containing Sra1, Nap1, Abi2, HSPC300, and WAVE1, which is the major form of the WRC in the human brain and led to the first crystal structure of the complex.

It is remarkable that the composition and assembly of the WRC are highly conserved across species. First, orthologous subunits in a given organism can be exchanged to form different WRCs through a 'mix and match' type process, even though different orthologs may have a specific tissue distribution, interact with distinct regulatory molecules, or exhibit different kinetics in promoting Arp2/3-mediated actin polymerization. Second, the five subunits appear to have co-existed throughout evolution: an organism contains either all five components or none of them (as is the case in yeast). Third, knocking out or suppressing expression of a single subunit in cells often eliminates or reduces the expression of all other subunits, disrupting WRC-mediated

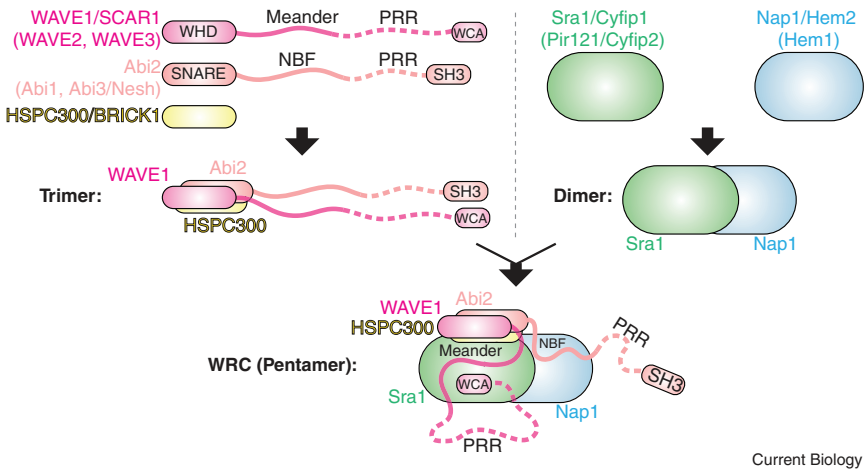

Figure 1. Composition and *in vitro* assembly of the WRC.
Schematic showing how the WRC is assembled from five different subunits through an *in vitro* biochemical reconstitution. Curved lines represent flexible sequences. Dotted lines represent unstructured sequences containing proline-rich regions (PRR) that were not included in crystal structures. NBF, Nap1-binding fragment; WHD, WAVE homology domain; WCA, WH2-central-acidic domain. Names of homologous proteins in vertebrates are shown in parentheses.

processes in a manner similar to that resulting from the removal of WAVE. Together, the above evidence emphasizes the importance of an intact, fully assembled WRC for its function. Although individual subunits have been suggested to participate in other protein complexes, such as Abi binding to N-WASP and Sra1 binding to the Fragile X mental retardation protein FMRP and the translation initiation factor eIF4E, these interactions either need further experimental validation or may have evolved as a function peripheral to their essential roles in the WRC.

How the WRC is assembled from newly synthesized subunits in cells and how the cell precisely balances the protein levels of the five subunits remain intriguing questions. *In vitro*, individually purified subunits are either sticky, aggregated, or unstable (which could cause various artifacts if a study only uses individual subunits as the experimental material — see next section). This indicates that, in cells, individual subunits or partially assembled subcomplexes may be unstable, requiring certain stabilization mechanisms before being assembled into the WRC. For example, HSPC300 was shown to form a homotrimer prior to entering the WRC. In addition, the dynein regulator Nudel was suggested to serve as a chaperone binding to and stabilizing subcomplexes including Sra1–Nap1–Abi1 and HSPC300.

Although little is known about WRC assembly in cells, on the basis of *in vitro* reconstitution and crystal structures the WRC can be readily dissected into two parts: a large elongated dimer of around 10 x 10 x 20 nm formed by Sra1 and Nap1, and a smaller trimer formed by WAVE1, Abi2, and HSPC300 (Figure 1). Almost the entire Sra1–Nap1 dimer is made up of α-helices, without clearly distinguishable subdomains. It is worth noting that, despite the low sequence similarity between Sra1 and Nap1, they share distant homology and define a protein family together with several remotely related proteins, including SWIP and Strumpellin (subunits of the related WASH regulatory complex), and the recently identified Rac1-binding protein CYRI. In contrast, the WAVE1–Abi2–HSPC300 trimer consists of a four-helix bundle that is immediately followed by two long tails extending from WAVE1 and Abi2, respectively. The four-helix bundle, aligned along the long axis of the Sra1–Nap1 dimer, is formed by the amino-terminal WAVE homology domain (WHD) of WAVE1, the amino-terminal SNARE-like helices of Abi2, and the entire helix of HSPC300 (Figure 1).

The long tail extending from WAVE1 consists of three parts: a sequence of around 90 amino acids known as the meander region, a long unstructured sequence of around 300 amino acids containing multiple proline-rich regions (PRR), and the WCA domain of around 75 amino acids at the carboxyl terminus. The long Abi2 tail also consists of three parts: a sequence of around 40 amino acids termed the Nap1-binding fragment (NBF), a long unstructured PRR of around 250 amino acids, and a carboxy-terminal Src homology 3 (SH3) domain (Figure 1). The meander region in WAVE1 literally 'meanders' across the surface of Sra1 as a loose collection of loops and helices, which together with Sra1 plays a key role in stabilizing WCA domain binding. In comparison, the seemingly unstructured NBF of Abi2 'crawls' along the surface of Nap1, forming an extensive interaction with the latter. The unstructured PRRs in both WAVE1 and Abi2 provide many potential binding sites for various SH3- or Ena/VASP homology 1 (EVH1)-domain-containing proteins, such as Eps8, p47phox, and Ena/VASP (Figure 2). Similarly, the SH3 domain of Abi2 can bind to proteins containing PRRs, such as Abl-family kinases. Finally, as the defining feature of WASP-family proteins, the WCA domain of WAVE1 serves as the output module of the entire complex. The W region is an actin-binding module found in various actin-binding proteins, and the C and A regions are critical for binding to and activating the Arp2/3 complex. The W and C elements form two short helices that are sequestered by multiple interactions with both Sra1 and the meander region of WAVE1, keeping the WRC inhibited. Activation of the WRC disrupts these interactions and releases the WCA domain, allowing this region to bind to and activate the Arp2/3 complex to promote actin polymerization.

Regulation of the WRC

Studies in the past 20 years have identified a large number of molecules that interact with the WRC, ranging from GTPases, inositol phospholipids, membrane receptors, and kinases, to various cytosolic proteins (with caveats discussed below), highlighting both the complexity and the central role of WRC regulation in linking diverse signaling pathways to actin (Figure 2). By contrast, we are only beginning to precisely understand their underlying mechanisms.

Central to WRC regulation is its activation. The Rho-family GTPase Rac1 is the ubiquitous activator of WRC — this connection was established for individual subunits even before the WRC was identified. The essential role of the Rac1–WRC–Arp2/3 axis in actin regulation is further underlined by the recent discovery of the Rac1-binding protein CYRI, which acts by specifically disrupting Rac1–WRC interactions using its A-site-analogous domain, also known as DUF1394 (Figure 2). Despite the importance of the Rac1–WRC interaction, several obstacles have stymied a mechanistic understanding of WRC activation for many years; these include the low affinity of Rac1 for the WRC, the cooperativity of Rac1 with other ligands, and the difficulties in purifying the WRC. Several conflicting models were not reconciled until the successful purification of the recombinant WRC by the Rosen lab, which subsequently led to a series of biochemical and structural studies establishing that the WRC is autoinhibited in the resting state and that Rac1 binding activates the WRC by allosterically releasing the WCA domain. Structural work identified two distinct Rac1-binding sites located at the opposite sides of the Sra1 subunit, named the A site and D site (for adjacent or distant to the WCA-binding site, respectively) (Figure 2). Neither site overlaps with the WCA-binding region, suggesting an allosteric activation mechanism. Biochemical studies showed that two Rac1 molecules can simultaneously bind to both sites, with the A site having around 40 times lower affinity for Rac1 than the D site and both sites being essential for WRC activation. In contrast, cell biological studies in both mammalian and *Dictyostelium* cells found that only the A site was crucial for WRC activation, whereas the D site served

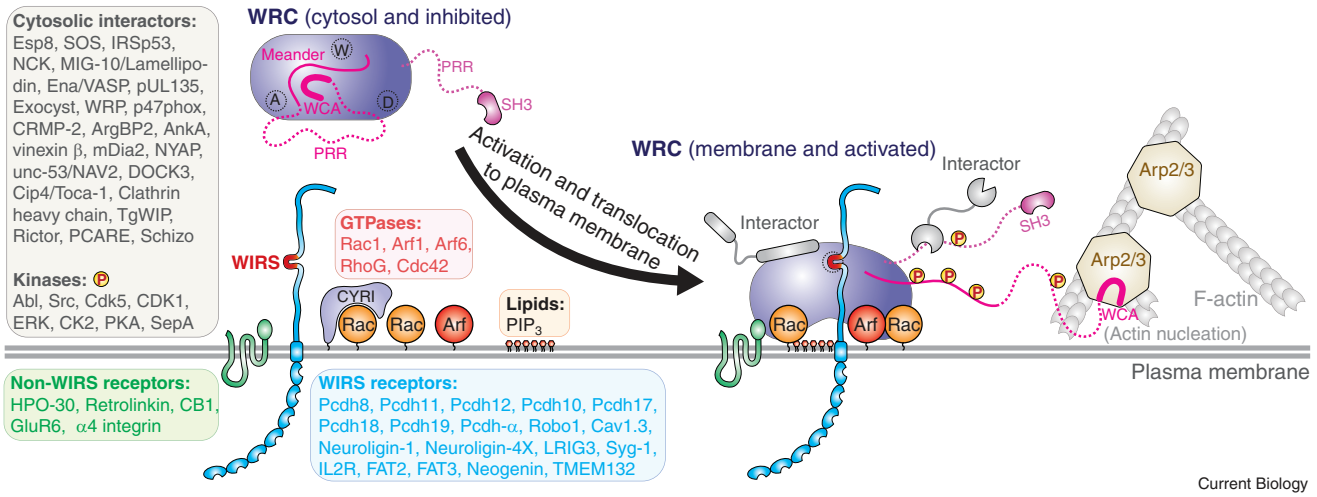

Figure 2. Activation and regulation of the WRC at the plasma membrane.
Schematic depicting concomitant activation of the WRC and its translocation to the plasma membrane by cooperative actions of major groups of membrane ligands, leading to the release of the WCA, which in turn binds to the Arp2/3 complex to stimulate actin nucleation and formation of branched actin networks. A, D, and W sites are indicated by dotted circles on the WRC. Each text box shows a ligand group and a list of representative ligands. Ligands that bind to individual subunits of the WRC but do not bind to the fully assembled WRC are not listed; these include N-WASP, FMRP, and eIF4E.

to promote or optimize WRC function. The discrepancy between biochemical and cellular studies suggests that WRC regulation is more complex in cells and highlights the need to develop new experiments and tools to further understand the contribution and mechanism of the two Rac1-binding sites both *in vitro* and *in vivo*.

Aside from Rac1, other GTPases have also been suggested to play a role in WRC activation, such as the Arf-family GTPases Arf1 and Arf6, or tune WRC activity, as in the case of the Rho-family GTPases RhoG and Cdc42 (Figure 2). While not sufficient for activating the WRC on their own, these GTPases might act cooperatively with Rac1 to optimize WRC activity, which may be important for linking Rac1–WRC–Arp2/3 signaling to processes mainly regulated by these GTPases, including vesicle trafficking and pathogen invasion. How these GTPases cooperate with Rac1 to drive WRC activation or output is mostly unknown. Furthermore, kinases also play an important role in activating the WRC (Figure 2). Both WAVE and Abi have many phosphorylation sites targeted by various kinases, such as Abl, Src, and cyclin-dependent kinase 5 (Cdk5). Phosphorylation in their unstructured PRRs may regulate interactions with auxiliary proteins, whereas phosphorylation in the meander region of WAVE can directly destabilize WCA binding and evoke WRC activation. Moreover, the WCA region is often found to be phosphorylated, which can fine-tune its kinetics in promoting Arp2/3-mediated actin polymerization.

Concomitant with activation, the WRC is translocated to and remains associated with the plasma membrane to sustain Arp2/3 complex activation. Rac1 (or its orthologs Rac2 and Rac3) plays a key role in this process. In addition, phospholipids, phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) and many transmembrane proteins may provide more precise, perhaps spatiotemporal, control of WRC localization at membranes (Figure 2). PIP_3 is known to enhance WRC activation by Rac1, likely by binding to the positively charged surface of the WRC. Various transmembrane receptors with diverse functions can directly recruit the WRC to membranes using a peptide motif named WIRS (WRC interacting receptor sequence), which binds to a surface pocket on the WRC (which we name the W or WIRS site) (Figure 2). The W site comprises amino acids from Sra1 and Abi2 that are 100% conserved in animals, suggesting that these WIRS receptors connect intact WRCs, instead of individual subunits, to processes specific to animals, such as immune responses and neural morphogenesis and functions (Figure 3). Notably, sequences flanking the WIRS motifs can also modulate WRC activity, either by directly interacting with the complex or by recruiting regulatory molecules such as Rac guanine nucleotide exchange factors (GEFs), which can synergistically promote WRC activation by increasing local Rac concentrations.

Finally, some membrane receptors that do not contain a WIRS motif as well as many cytosolic proteins may interact with WRC, which can potentially link the WRC and actin regulation to a much broader range of cellular activities (Figure 2). In addition to host proteins, pathogen effectors may also interact with the complex to hijack actin regulation and facilitate pathogen entry or immune evasion, such as pUL135 from human cytomegalovirus, AnkA from *Anaplasma phagocytophilum*, and TgWIP from *Toxoplasma gondii*. In most cases, however, the precise mechanism and function of the aforementioned interactions warrant careful further examinations, both *in vitro* and in cells. One major caveat to bear in mind is that many of these interactions have been identified and characterized using isolated WRC subunits, which — apart from Abi–SH3, Abi-PRR and WAVE-PRR — are unstable and tend to form aggregates and non-specific interactions. It will thus be critical to further examine these interactions in the context of fully assembled WRCs in the future.

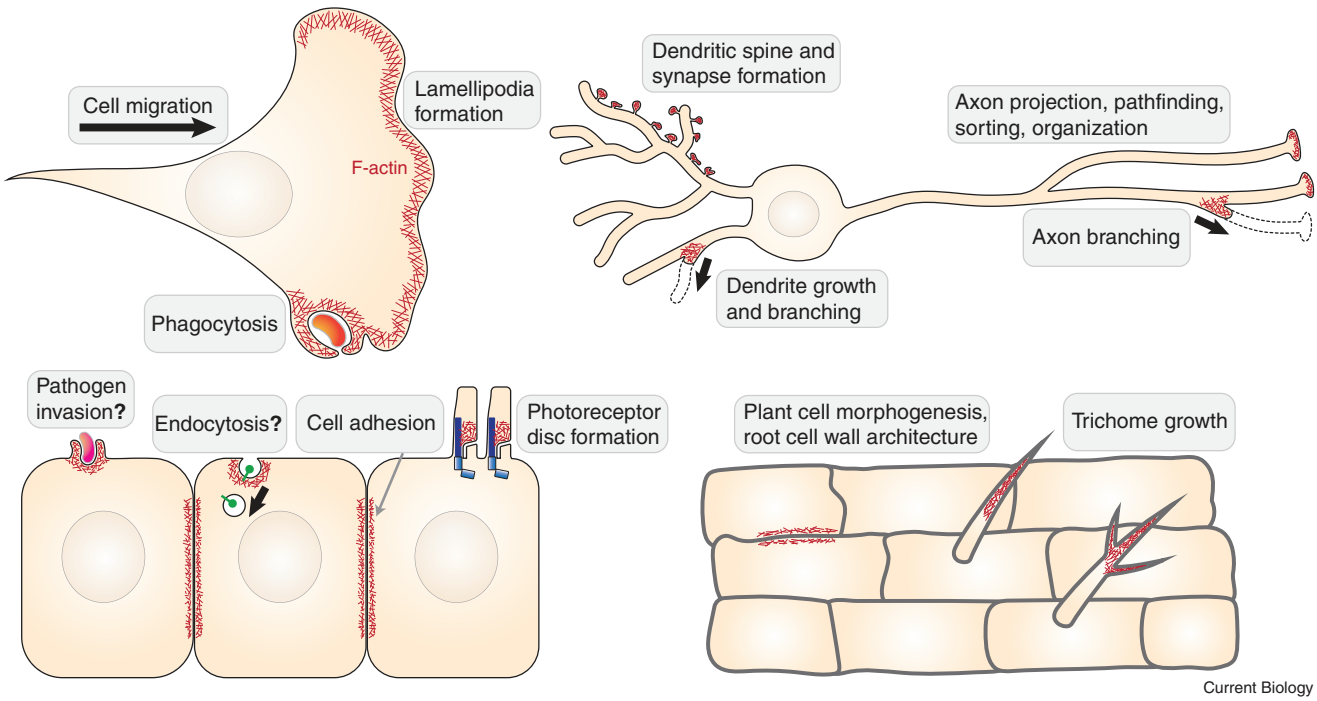

Figure 3. Biological functions of the WRC.
Schematic showing major cellular processes, indicated by grey text boxes, that are regulated by WRC-mediated actin network formation (represented by F-actin in red; WRC not shown). Processes that are still controversial or in which a general function for WRC remains to be confirmed are indicated by question marks.

Biological/organismic functions of the WRC

Consistent with the long list of WRC-interacting ligands and the importance of Rac–WRC–Arp2/3 signaling in actin dynamics, the WRC performs many important functions in biology. In most cases, the WRC is crucial for morphogenetic processes that rely on dynamic rearrangements of Arp2/3-mediated actin networks formed at the plasma membrane (Figure 3).

Best known among these processes in animal cells are lamellipodia protrusion and membrane ruffling, which can significantly enhance the efficiency of cell migration in various cell types and tissue contexts. The WRC unequivocally drives these processes in many different model systems, such as migration of melanoma or immune cells in mice, cell motility in zebrafish, fruit fly and nematode worms, and pseudopod-mediated crawling in *Dicytostelium discoideum*. In neurons, WRC function is essential for the formation of various cell protrusions rich in actin filaments, which can generate dendritic spines and neuromuscular synapse junctions, drive branch formation of dendrites and axons to establish complex neural circuits, and guide axon growth, organization, and precise projection to target tissues.

Other processes involving Rac-mediated actin remodeling, such as cell–cell adhesion, phagocytosis, and photoreceptor disc formation, also require WRC function (Figure 3). Furthermore, the complex has been implicated in the invasion of pathogens, including Gram-positive *Listeria* and Gram-negative *Rickettsia* and *Salmonella*, albeit to variable extents in different studies, warranting additional, more detailed investigations. Interestingly, the WRC has even been linked with specific types of endocytosis, which is usually considered to be a major function of N-WASP. In these instances, endocytosis required direct binding of the WRC to receptors through WIRS (interleukin-2 receptor) or non-WIRS (retrolinkin) interactions. Additional examples will be required in the future to examine whether the WRC plays a general role in endocytosis.

The importance of the WRC is reflected by the severe phenotypes resulting from the deletion of individual WRC subunits in animals. Deleting ubiquitously expressed subunits , such as Sra1, WAVE2, Nap1, and Abi1, in mammals is embryonically lethal, whereas deleting more tissue-specific orthologs, such as WAVE1 (nervous system) and Hem1 (hematopoietic cells), produces offspring with significant defects in neural or immune functions, respectively. In contrast, deletion of Cyfip2, which is enriched in the brain, is perinatally lethal, but reducing Cyfip2 levels, i.e. in heterozygous animals, or specifically deleting Cyfip2 in postnatal forebrain excitatory neurons causes neurobehavioral phenotypes, consistent with the effects of various mutations in this subunit in human patients (see next section).

The WRC also plays many important roles in plant cells, but very little is known about the molecular or biochemical mechanisms involved due to the functional redundancy of many orthologs of WRC subunits in plants and the lack of biochemical reconstitution of a plant WRC (Figure 3). Although plant cells lack dynamic membrane protrusions, they rely on proper remodeling of actin networks

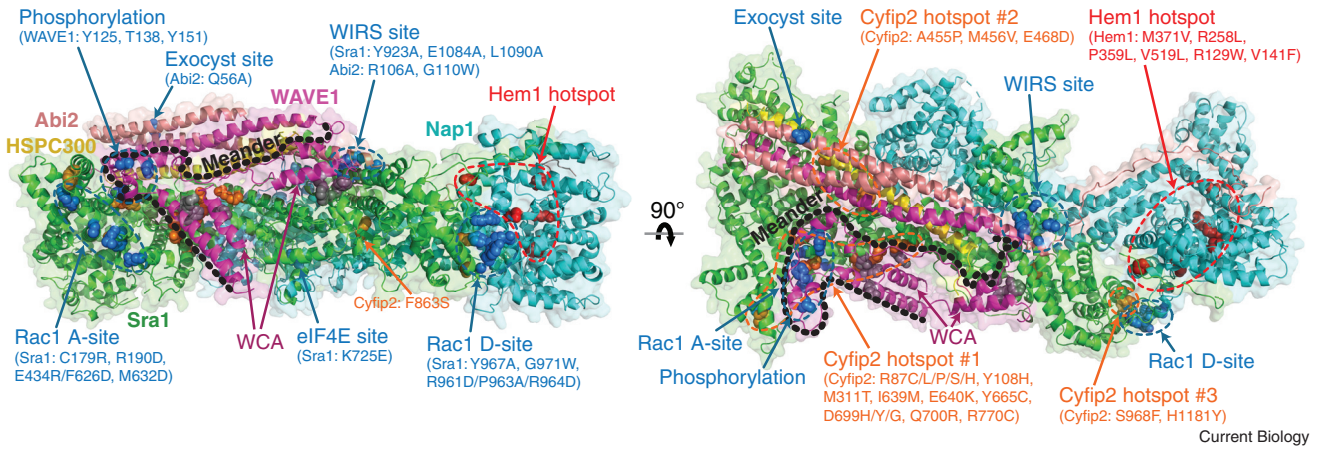

Figure 4. Regulatory sites and missense mutations in the WRC.
Cartoon representation of the WRC crystal structure in the inhibited state (PDB: 3P8C). For clarity, the meander sequence in WAVE1 is traced by black dotted lines. Amino acids important for regulation or that, when mutated, disrupt ligand binding or cause disease are shown as spheres. Regulatory sites, including phosphorylation sites and binding sites for Rac1, WIRS receptors, exocyst, and eIF4E are colored blue (the eIF4E site is buried and not compatible with WRC assembly). Shown in orange are amino acid changes in Cyfip2 (ref sequence: NP_001032410.1) that were identified to cause neurodevelopmental disorders, including West syndrome, early-onset epileptic encephalopathy, intellectual disability, seizures, drug addiction, and binge eating. Shown in red are amino acid changes in Hem1 (ref sequence: NP_005328.2) that led to immunological disorders, including immunodeficiency, lymphoproliferation, and autoimmunity. Mutations previously designed to disrupt WCA inhibition are colored in grey (but not labeled), including L697D, Y704D, L841A, F844A, W845A and F686E in Sra1, and W161E and K162D in WAVE1.

in the cytosol to regulate transport of polysaccharides to their cell wall destinations. It is well established that, by controlling actin polymerization required for plant wall biosynthesis, the WRC regulates various aspects of plant development and cell function, including cell division, cell morphology, trichome growth, root rigidity, drought response, cell–cell junction formation, symbiosis and host–pathogen interactions.

Implications in disease

Given the broad functions of the WRC in various cell types, especially in the immune and nervous systems, it is not surprising that perturbations of WRC subunits or its many ligands are associated with various genetic diseases, including neurological disorders, immune deficiencies, and cancer.

Since the WRC carries out its function as an integral complex and the expression level between subunits is tightly regulated, mutations affecting the expression of a single subunit can readily disturb the total levels of WRC, leading to disease. Copy number variations (CNVs) arising from chromosome microdeletions, nonsense mutations or gene duplications, regardless of whether they reduce or elevate WRC levels, are common causes of WRC-associated disorders. For example, the 15q11.2 region of the human genome, which contains *Cyfip1* (encoding Sra1), is a hotspot of chromosomal microdeletions or duplications. Anomalies in this region are heavily associated with neurological disorders, including autism spectrum disorder (ASD), schizophrenia, epilepsy, and intellectual disability (ID). Similarly, heterozygous deletions or premature truncations of Cyfip2, Nap1, WAVE1, or Hem1 are tightly associated with early-onset epileptic encephalopathy (EOEE), Alzheimer's disease, ASD, ID, seizures, immune deficiency, or autoimmunity. In addition, overexpression (sometimes deletion) of WRC components, including WAVE1/2/3, Sra1, Nap1, Hem1, Abi1/3, and HSPC300, are frequently found in various types of cancers (such as breast, ovary and lung) and are often associated with tumor invasiveness and poor prognosis.

Compared with mutations that cause changes in expression levels, missense mutations in patients are more rare, but provide invaluable opportunities for understanding the mechanisms of WRC regulation and for developing therapeutic strategies to target these mechanisms. The crystal structures shown in Figure 4 summarize an up-to-date collection of missense mutations associated with human diseases (denoted as orange and red in Figure 4). As a reference point, amino acids previously identified to be important for WRC regulation are also labeled, including residues important for ligand binding, phosphorylation sites, and mutations designed through structural, biochemical, and cell biological analyses (denoted as blue and grey in Figure 4). It is intriguing that most missense mutations are clustered at a few spatially concentrated hotspots in the WRC structure, even though their positions in the primary sequence may be distant. Structural analysis suggests that these mutations act either by destabilizing protein folding to cause WRC degradation or by disrupting WRC autoinhibition. Cyfip2 contains three hotspots, and these mutations are typically involved in neurodevelopmental disorders such as EOEE, ID, seizures, hypotonia, West syndrome, eating disorders, and altered drug addiction. It is worth noting that hotspot #1 is located close to the A site or the meander sequence, and hotspot #3 is underneath the D site, suggesting that these mutations act by disrupting autoinhibition and/or modulating Rac1 binding. Hem1 contains one hotspot, and these mutations were identified to cause novel syndromes that combine immunodeficiency and autoimmunity. Most mutations in Hem1 seem to

disrupt Hem1 structure, leading to WRC degradation, except for the M371V mutation, which has been suggested to disrupt Arf1–WRC signaling.

Outlook and future directions

Many important questions remain to be answered for a complete understanding of the function and regulation of the WRC, from both a biochemical/structural and cell biological perspective. A key remaining question is how the WRC interacts with or becomes activated by various ligands, including Rac1, Arf1, PIP_3 and many other molecules, both individually and cooperatively, and both *in vitro* and at the plasma membrane of cells. As an example, it is still unclear whether WRC activation can be separated from its recruitment to the membrane. Answering all of these questions will promote the development of inhibitors, activators, and chemical or optogenetic tools to control or track WRC functions in cells, which will be of both scientific and potential medical relevance. Such tools might also provide us with the ability to unravel additional, perhaps less canonical, functions to those summarized above. How different WRC variants containing distinct combinations of subunits are differentially regulated in cells is emerging as yet another exciting future topic. Additional questions that have remained almost entirely unanswered include the regulation of WRC assembly, recycling and degradation, as well as biochemical mechanisms of WRC regulation in plants. Last, but not least, it will be essential that a full understanding of WRC regulation and function establishes how its individual subunits also participate in other complexes, such as Sra1 with FMRP–eIF4E, and how cells balance all of these individual subunit activities in normal development and disease.

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Primer

Formins

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Actin is one of the most abundant proteins in eukaryotes. Discovered in muscle and described as far back as 1887, actin was first purified in 1942. It plays myriad roles in essentially every eukaryotic cell. Actin is central to development, muscle contraction, and cell motility, and it also functions in the nucleus, to name a spectrum of examples. The flexibility of actin function stems from two factors: firstly, it is dynamic, transitioning between monomer and filament, and, secondly, there are hundreds of actin-binding proteins that build and organize specific actin-based structures. Of prime importance are actin nucleators — proteins that stimulate *de novo* formation of actin filaments. There are three known classes of actin nucleators: the Arp2/3 complex, formins, and tandem WASP homology 2 (WH2) nucleators. Each class nucleates by a distinct mechanism that contributes to the organization of the larger structure being built. Evidence shows that the Arp2/3 complex produces branched actin filaments, remaining bound at the branch point, while formins create linear actin filaments, remaining bound at the growing end. Here, we focus on the formin family of actin nucleators.

Formins are crucial proteins for a range of cellular processes, as demonstrated by their links to various pathologies, including cardiomyopathies, cancers, intellectual disabilities and other neuronal disorders, nonsyndromic deafness, and renal disorders. It follows that understanding how formins help to build actin-based structures is essential to our knowledge of normal physiology as well as many pathologies. In this Primer, we highlight the biochemical activities underlying actin assembly by formins and, where possible, we weave in links between biochemistry and biological roles. Finally, we discuss outstanding questions about formins.

The importance of nucleators

Nucleators are essential for tightly regulated actin assembly. The initial step of actin filament