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Phosphoregulation of the WAVE Regulatory Complex and Signal Integration

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Abstract

The WAVE2 Regulatory Complex (WRC) induces actin polymerization by activating the actin nucleator Arp2/3. Polymerizing actin pushes against the cell membrane and induces dramatic edge protrusions. In order to properly control such changes in cell morphology and function, cells have evolved multiple methods to tightly regulate WRC and Arp2/3 activity in space and time. Of these mechanisms, phosphorylation plays a fundamental role in transmitting extracellular and intracellular signals to the WRC and the actin cytoskeleton. This review discusses the phosphorylation-based regulatory inputs into the WRC. Signaling pathways that respond to growth factors, chemokines, hormones, and extracellular matrix converge upon the WAVE and ABI components of the WRC. The Abl, Src, ERK, and PKA kinases promote complex activation through a WRC conformation change that permits interaction with the Arp2/3 complex and through WRC translocation to the cell edge. The neuron-specific CDK5 and constitutively active CK2 kinases inhibit WRC activation. These regulatory signals are integrated in space and time as they coalesce upon the WRC. The combination of WRC phosphorylation events and WRC activity is controlled by stimulus, cell type, and cell cycle-specific pathway activation and via pathway cross-inhibition and cross-activation.

Keywords

Abl; Ableson-interacting protein (ABI); Cyclin-dependent kinase 5 (CDK5); Extracellular-regulated kinase (ERK); phosphorylation; WASp-family verprolin homologous protein (WAVE)

1. Introduction

1.1 Structure and Function of the WRC

The WAVE Regulatory Complex (WRC) activates the Arp2/3 actin nucleator to induce actin polymerization during cell motility and neurite extension. Actin monomers are abundant in the cytoplasm and join existing actin filaments to cause rapid polymerization. However, newly initiated actin dimers and trimers are unstable. The WRC-activated Arp2/3 complex acts as a catalyst to overcome this energy barrier and nucleates new actin filament branches at 70° angles from pre-existing filaments (for review, see [1, 2]). The resulting crosslinked network of actin induces protrusion events necessary for cell movement during

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development, wound healing, immune responses, and cancer cell metastasis, and for new axon and dendrite formation during neuronal development.

The WRC is a stable pentamer of isoforms of the following five proteins: WASp-family verprolin homologous protein (WAVE), Ableson interacting protein (ABI), NCK-associated proteins (NAP), Specifically Rac-associated 1 (SRA1), and Hematopoietic stem progenitor cell 300 (HSPC300) (Fig. 1A) [3-7]. In this review, WAVE, ABI, and NAP refer to conclusions drawn from multiple isoforms of each protein that are considered to be universal. WAVE, ABI, and HSPC300 form a trimer with actin-polymerizing activity that rests upon a NAP:SRA1 dimer [4, 5, 7-9]. The recent WRC crystal structure revealed that SRA1 and NAP exhibit very similar domain organization, suggesting they belong to the same protein family even though they have minimal sequence identity [8].

WAVE proteins have four conserved domains: a WAVE homology domain (WHD, also called SCAR homology domain for the *Dictyostelium* homolog SCAR) at the N-terminus, a basic region, a polyproline-rich domain (PRD), and a VCA region (Fig. 1B). The WHD mediates complex formation with ABI, NAP, SRA1 and HSPC300 [5, 8]. The polyproline-rich region binds Src-homology-3 (SH3) domains and WW domains in other regulatory proteins. ABI also has a PRD and an SH3 domain, suggesting WAVE and ABI may together to contribute to WRC recruitment or oligomerization by SH3 or WW-domain containing proteins (Fig. 1B, reviewed in [10]). The VCA region is a 3-part domain at the WAVE C-terminus called the VCA region is named for its Verprolin homology domain (V), hydrophobic Central or Cofilin homology domain (C), and Acidic domain (A). The function of the VCA region was elucidated in the related WASP proteins and is assumed to be similar for the WAVES. The C-A regions bind the Arp2 and Arp3 subunits of the Arp2/3 complex, which are homologous to actin [11-13]. VCA binding to Arp2/3 induces changes in the tertiary and quaternary structure of the Arp2/3 complex so that the Arp2 and Arp3 subunits are in a “closed” conformation [14-16]. The V domain binds to G-actin and brings it in close proximity to the Arp2 and/or Arp3 subunits, thereby creating a pseudotrimer nucleus that grows into a new filament on its own [13, 15, 17].

1.2 Activation of the WRC

The WRC is a stable and intermolecularly-inhibited complex. In many cell types, knockdown or knockout of any one component causes a reduction in the levels of the remaining components, indicating WAVE, ABI, NAP, SRA1, and HSPC300 are stabilized by their incorporation into the WRC [5, 6, 18-21]. Alone, the WAVE VCA region strongly stimulates actin nucleation [5, 9]. The recent WRC crystal structure revealed that the VCA region is normally inhibited through interaction with the meander region of WAVE (WAVE1 residues 82-184) and SRA1 [8]. Upstream signals are needed to activate the WRC. However, these signals do not disrupt the WRC, but rather induce a conformational change that releases the VCA region so that it can bind Arp2/3 and actin [5, 8, 9, 20, 22]. Improper purification of the WRC, such as heating, freeze-thaw cycles, prolonged storage on ice, or lack of glycerol as a cryo-protectant can also lead to complex denaturation and erroneous actin polymerization activity [9, 22-24]. Past conclusions from overexpression and *in vitro* studies need to be carefully reconsidered to determine if the WAVE was uncomplexed, since uncomplexed WAVE2 is artificially and constitutively active [5, 9, 19, 22, 24-27].

The WRC is a signal integrator. Signals from the Rac GTPase, phospholipids, and protein kinases that sense growth factor and substrate adhesion coalesce upon components of the WRC to induce WRC membrane localization and activation at the right time. Rac-GTP binds the WRC through SRA1 [8, 28]. The binding competes with sequestration of the WAVE VCA region, so Rac-GTP binding makes the VCA region accessible to the Arp2/3 complex and actin [8].

WRC binding to negatively-charged phospholipids contributes to WRC recruitment to the cell membrane and WRC activation of the Arp2/3 complex. Phosphatidylinositol(3,4,5)-triphosphate (PtdIns(3,4,5)P₃), and to a lesser degree phosphatidylinositol(4,5)-diphosphate (PtdIns(4,5)P₂), binds the basic region of WAVE2 [29]. Lipid binding is necessary for WAVE2 translocation to the leading edge and Rac-induced protrusion [29]. In addition, liposomes containing PtdIns(3,4,5)P₃ are required to fully activate the *in vitro* actin polymerization activity of Rac-WRC [22]. Other negatively-charged lipids can induce partial activity in a charge-dependent manner [22]. Activation of the WRC is highly cooperative and two VCA domains bind the Arp2/3 complex with greater affinity than a VCA monomer. Thus, phospholipid recruitment of the WRC to the cell membrane may increase activation of the Arp2/3 complex via WRC oligomerization [22, 30].

Phosphorylation is the transfer of a phosphate group to another molecule. It is a post-translational mechanism for transmitting extracellular signals, such as growth factor, cytokine, and adhesion status, to the cell machinery. Kinases catalyze the transfer of the γ -phosphoryl group (PO₃²⁻) from an ATP molecule to tyrosine, serine, or threonine residues in the WRC complex. Phosphatases remove the phosphates, although most of the phosphatases involved in WRC de-phosphorylation have yet to be identified. Kinase and phosphatase interaction with substrates is generally transient and the phosphorylations can be unstable during protein purification due to phosphatase release. Some kinases, such as the Extracellular Regulated Kinase (ERK)-mitogen-activated protein kinase (MAPK), also require docking sites within their substrate to both confer specificity and aid recruitment and positioning of the kinase. This review discusses the signaling pathways that are known to phosphorylate the WRC, how the phosphorylations regulate WRC localization and function to control the actin cytoskeleton, and putative kinases responsible for the verified but as yet uncharacterized phosphorylations.

1.3 Isoform-specific Activities

The phospho-residues identified in WAVE and ABI are not uniformly conserved between their respective isoforms, warranting a brief discussion of their different expression patterns and functions. There are three WAVE, four ABI, two NAP, and two SRA-1 genes in mammalian cells. The individual WAVE homologs do not appear to have specificity in terms of pairing with specific ABI, NAP, or SRA1 proteins beyond their expression patterns [7]. Some functional specificity likely exists though, as upregulated ABI2 cannot substitute for ABI1 loss to maintain the stability of the other WRC components in mouse embryonic fibroblasts [31].

WAVE2 is the more ubiquitous WAVE homolog in mammals [27, 32]. WAVE2, but not WAVE1, is essential for lamellipodia protrusion induced by Rac and growth factors [20, 29, 33-36]. WAVE1 and WAVE3 are also expressed in many cell types, but are enriched in the brain [32, 37, 38]. WAVE1 activity is required for dorsal ruffle formation in fibroblasts and for neurite outgrowth in neurons [24, 34]. Less is known about the specific functions of WAVE3, although, similar to WAVE2, WAVE3 is involved in lamellipodia protrusion and cell migration [35, 39, 40]. ABI1 is ubiquitously expressed and required for lamellipodia protrusions, dorsal ruffling, and cell migration [5, 19-21, 31, 41]. ABI2 is enriched in the eye and brain and like WAVE1, knockout mice exhibit abnormal neuron migration and dendritic spine morphology [24, 42, 43]. Little is known about the specific functions of ABI3.

2. Tyrosine Phosphorylation of the WRC

2.1. Abl

Abl (named for its identification as the cellular proto-oncogene in the Abelson murine leukemia virus) is a non-receptor tyrosine kinase. Abl phosphorylates tyrosine residues in the consensus sequence I/V/L-Y-X-X-P/F. Abl activity is controlled by cytokines and growth factors and also by cellular differences in its nuclear versus cytoplasmic localization (reviewed in [44]). In fibroblasts and epithelial cells, a small fraction of Abl is in the cytoplasm associated with the plasma membrane and actin cytoskeleton, but most Abl is nuclear-localized. In contrast, Abl is mostly cytoplasmic and cytoskeleton-associated in hematopoietic cells and neurons and most experiments addressing the role of Abl phosphorylation in WRC function have been carried out in these latter cell types. Oncogenic Abl, in which the Breakpoint Cluster Region (BCR) protein is fused to Abl, induces cytoskeletal remodeling, cell migration, and leukemia [45, 46]. It now appears that these effects are mediated, in part, through Abl phosphorylation and activation of the WRC.

ABI1 and ABI2 were initially identified as proteins that interact with the Abl [47, 48]. The Abl kinase has an SH3 domain and Proline-rich sequences at its C terminus that mediate SH3-PXXPP interactions with the ABI PRD and SH3 domain [47-49]. Abl localizes to the cell periphery with the WRC and phosphorylates both WAVE and ABI [50, 51]. The ABI SH3 domain and Proline-rich sequences are required for Abl interaction with the other WRC members and for Abl phosphorylation of WAVE2 [51-54]. A “kinase dead” Abl mutant, in which the activation loop lysine that coordinates interaction with ATP is mutated to arginine, exhibits reduced interaction with the WRC [53].

Abl phosphorylates WAVE2 on the conserved Tyr150 (Tyr151 in WAVE1 and WAVE3, Fig. 2) [51, 53]. Based on the WRC crystal structure, Abl phosphorylation of Tyr150 is predicted to disrupt interaction between the WAVE meander region and SRA1 so that the VCA region is released and available for interaction with the Arp2/3 complex [8]. A phospho-mimetic Y151E WAVE1 mutant exhibits high actin polymerization activity *in vitro* and *in vivo* [8]. An un-phosphorylatable Y150F mutant cannot rescue loss of membrane ruffling and microspike formation in *Wave2* knockout cells [53], indicating that Abl phosphorylation of WAVE is necessary for WRC-mediated activation of the Arp2/3 complex and actin polymerization. While mutation of Tyr150 blocks all Abl-induced Tyr phosphorylation on WAVE2, Abl additionally phosphorylates Tyr248, Tyr337, and Tyr486 on WAVE3 [39, 51, 53]. Only when all four tyrosines are mutated is Abl-mediated phosphorylation of overexpressed WAVE3 completely abrogated [39]. Only Tyr486 is conserved in WAVE1 and WAVE2, but its sequence E-Y-S-D-S does not fit the canonical Abl motif. None of these additional sites are conserved in invertebrates. Thus, further validation of Abl's ability to phosphorylate the additional WAVE3 sites and whether the phosphorylations have functional consequence is needed.

In *Drosophila* S2 cells, Abl phosphorylates and the tyrosine phosphatase PTP1B dephosphorylates ABI Tyr148, Tyr155, Tyr248, and Tyr285 [55]. The *Drosophila* ABI protein is the human ABI1 homolog and the four phosphorylation sites are conserved between ABI1 and ABI2 in vertebrates (Fig. 3). While Abl localizes to the cells edge and sites of active actin polymerization, PTP1B is purely cytoplasmic [55]. This differential localization may spatially control WRC phosphorylation to promote the complex's translocation to the cell edge. Indeed, Abl is required for WAVE2 localization to the immune synapse, a site of actin polymerization, and the ABI 4YF mutant exhibits reduced membrane localization and reduced lamella structures [45, 55]. Oncogenic Abl also induces membrane translocation of both ABI and WAVE [52]. Interestingly, the WAVE2 Y150F mutant abrogates tyrosine phosphorylation of both WAVE2 and co-overexpressed ABI1,

suggesting Abl must first phosphorylate WAVE before the ABI sites become accessible [51]. Together, Abl phosphorylation of both WAVE and ABI may control both the WRC's activity towards the Arp2/3 complex and localization to the cell edge.

Somewhat confoundingly, Abl phosphorylation of the *Drosophila* ABI was reported to promote ABI1 protein stability, while oncogenic Abl induced the ubiquitin-dependent degradation of ABI2 in hematopoietic cells [49, 52, 55, 56]. Tyrosine to phenylalanine mutation of all four Abl sites (ABI 4YF) decreased *Drosophila* ABI1 protein half-life [55]. Further, Abl co-overexpression increases ABI1 levels and this is abrogated by expression of PTP1B [55]. It remains to be determined if endogenous Abl also induces ABI2 degradation and if ABI2 is phosphorylated at unique sites to be differentially regulated by Abl. Regardless, Abl activity alone is unlikely to regulate ABI protein stability or its incorporation into the WRC, since Abl inactivation and activation through serum starvation, growth factor stimulation, and treatment with Abl kinase inhibitors does not alter ABI levels or the formation of WRC [5, 53]. An additional layer of regulation acting at the level of Abl, such as phosphorylation of Abl or interaction with adaptor proteins, may control Abl interaction with and stabilization of specific ABIs. For example, the p21-activated kinase (PAK) phosphorylates Abl Ser637 and Ser638 adjacent to the PXXP motif that binds the SH3 domain of ABI and this inhibits Abl interaction with ABI2 [57]. ArgBP2 negatively regulates Abl by mediating the formation of an Abl-Cbl complex that promotes Abl ubiquitination and degradation [58]. ArgBP2 overexpression has also been found to enhance Abl interaction with and phosphorylation of WAVE1 and ubiquitination of WAVE2, indicating ArgBP2-binding might impact the extent and effects of Abl phosphorylation of WAVE and ABI [50, 59]. While more work needs to be done to clarify how and under what circumstances Abl regulate ABI and WAVE protein levels, Abl is an accepted positive regulator of WRC activity.

2.2. Src

The Src non-receptor tyrosine kinase is the canonical and most well-studied member of the Src family kinases. Src is named after the Rous Sarcoma virus, from which it was originally cloned. Src phosphorylates tyrosines in Y-S and Y-D/E motifs [60]. Src also interacts with phospho-tyrosines and PXXXP domains through its Src homology 2 (SH2) and Src homology 3 (SH3) domains, respectively. Src interacts with, is activated by, and modulates the downstream signaling of growth factor receptors and integrins, receptors for the extracellular matrix (reviewed in [61-63]). Src is myristoylated, which causes it to localize to the plasma membrane and endomembranes. Upon activation, Src accumulates at sites of actin polymerization, such as lamellipodia induced by Rac activation [64].

Src phosphorylates many components of focal adhesions to promote actin remodeling and adhesion turnover [61-63]. An additional mechanism by which Src may promote actin polymerization may be through its phosphorylation of WAVE. Src phosphorylates WAVE1 Tyr125, which is conserved in WAVE2 and 3 and from animals to plants (Fig. 2 [8, 65]). Tyr125 phosphorylation is predicted to destabilize the meander region of WAVE and lead to release of the VCA region [8]. Indeed, Src co-transfection increases Arp3 interaction with overexpressed WAVE1 and a Tyr125F mutation blocks the interaction [65]. A phospho-mimetic Y124D WAVE2 mutant increases lamellipodia formation in *Wave2* knockout cells [8].

3. Serine/Threonine Phosphorylation of the WRC

3.1. Cyclin-dependent Kinases (CDKs)

Cyclin Dependent Kinases (CDKs) are serine/threonine kinases that function as heterodimers of a catalytic subunit, which is inactive on its own, and a cognate cyclin.

Cyclin expression and degradation fluctuates with the cell cycle, causing CDK activity to similarly fluctuate. CDK activity is also regulated by activating and inhibitory phosphorylations and by the binding of inhibitory molecules. The CDKs are proline-directed kinases and their minimal phospho-acceptor consensus motif includes a proline in the +1 position and a basic residue in the +3 position (S/T-P-X-K/R, reviewed in [66]).

CDK1 phosphorylates ABI1 at Ser216 both *in vitro* and *in vivo* during mitosis (Fig. 3 [54]). CDK1 is activated at the end of interphase by A-type cyclins and promotes mitotic entry [67]. Following nuclear envelope breakdown and cyclin A degradation, CDK1 is activated by B-type cyclins and promotes mitotic progression (reviewed in [68]). CDK1 phosphorylation of ABI1 inhibits tyrosine phosphorylation of ABI1 and WAVE2 [54]. Indeed, mitotic cells do not exhibit tyrosine phosphorylation of WAVE2 or ABI1 [54]. The affected tyrosine phosphorylation is assumed to be that of Abl, since the ABI1 S216D phospho-mimetic mutant reduces the presence of F-actin rich spots in hematopoietic cells while oncogenic Abl increases the percentage of cells with F-actin rich spots [54]. However, mitotic CDK1 phosphorylation of ABI Ser216 is unlikely to simply inhibit Abl interaction with and phosphorylation of ABI1. ABI1 phosphorylated at Ser216 actually exhibits increased interaction with Abl. Further, while ABI1 S216D exhibits no tyrosine phosphorylation, ABI1 S216A exhibits less tyrosine phosphorylation than the wildtype ABI1 [54]. It is possible that CDK1 phosphorylates additional residues on ABI1 and serine to alanine mutation of one phosphosite increases CDK1 phosphorylation of the other sites. The *in vitro* CDK1 – ABI1 kinase assay detected the CDK1-mediated phosphotransfer by Western with a pS216 antibody, rather than ³²P detection with a PhosphorImager, which precludes the detection of other phosphosites [54]. More mechanistic studies with double mutants of both the CDK1 and Abl phosphosites are needed to determine if CDK1 phosphorylation of ABI inhibits WRC function and motility during mitosis [54].

CDK5 phosphorylates WAVE1 on Ser310, Ser397, and Ser441 to high basal stoichiometry [24, 69]. CDK5 is activated upon association with the p35 or p39 proteins, whose expression is restricted to post-mitotic neurons (reviewed in [70]). The binding proteins have little sequence homology with cyclins, but function to activate their cognate CDK in a similar manner. CDK5 is required for neuronal spine formation and migration, processes dependent upon WAVE1 and actin-driven protrusions [24, 70]. Although Ser310, Ser397, and Ser441 do not have a basic residue in the +3 position, they are all phosphorylated to high stoichiometry in a CDK5-dependent manner. The extracellular signaling regulated kinase (ERK) can also phosphorylate proline-directed serine and threonine residues (see section 3.2 below), but WAVE1 Ser310, Ser397, and Ser441 phosphorylation is not affected by ERK pathway inhibitors [24]. The sites are located within the PRD, but are not conserved in WAVE2 or WAVE3. CDK5 phosphorylation of WAVE1 inhibits WRC and free WAVE1 *in vitro* actin polymerization activity (Fig. 2 [24]). A S310A mutant exhibits elevated *in vitro* activity and increased ability to complement spine defects in *wave1* knockout neurons, while the S310D mutant is inactive *in vitro* and *in vivo* [24]. PP2A and PP2B dephosphorylate the CDK5 sites on WAVE1 [71].

CDK5 has also been shown phosphorylate WAVE2 at Ser137 within the WHD in oligodendrocytes, glial cells of the central nervous system [69]. WAVE2 Ser137 is conserved as a threonine in WAVE1 and WAVE3. The phosphorylation is predicted to perturb the structure of the meander region and thereby contribute to WRC activation [8].

3.2. Extracellular signal-regulated kinase (ERK)

ERK is a mitogen-activated protein kinase (MAPK) that functions as the major effector of the Ras oncoprotein. ERK is a serine/threonine proline-directed kinase closely related to the

CDKs. ERK phosphorylates PXS/TP motifs, with an absolute requirement for the proline in the +1 position [72]. Ras is a small GTPase that when bound to GTP, can interact with the MAPKKK Raf, which phosphorylates and activates the MAPKK MEK, which then phosphorylates and activates the effector MAPK ERK. Ras and ERK are activated by growth factors, polypeptide hormones, neurotransmitters, chemokines, and phorbol esters (reviewed in [73, 74]). Raf, MEK and ERK collectively bind to scaffold proteins that direct the kinases to specific subcellular locales and insulate their signaling from phosphatases (reviewed in [75, 76]). Within minutes after growth factor stimulation and Ras activation, phospho-ERK can be detected at the cell edge [20, 77]. ERK activity has long been known to be required for cell motility, but only recently was it found to act through the WRC.

ERK phosphorylates WAVE2 on Ser343, Thr346, Ser351 within the PRD [20, 78, 79]. In some cell lines, ERK also phosphorylates WAVE2 Ser308 [20, 79]. ERK phosphorylation of these sites is supported by *in vivo* labeling, *in vitro* kinase assays, and phospho-antibody studies from multiple labs and in multiple cell types. Ser308 and Ser351 are evolutionarily conserved in vertebrates and Ser343 and Thr346 are conserved in mammals, but none of these sites are present in WAVE1 or WAVE3 (Fig. 2). WAVE1 and WAVE3 do not exhibit growth factor induced or MEK inhibitor-sensitive electrophoretic mobility shifts, further supporting their lack of phosphorylation by ERK [79]. The recent WRC crystal structure used WAVE1 and did not include the WAVE PRD because the disordered nature of the region inhibited crystallization [8]. The phosphorylations in the PRD likely affect interaction with SH3 domain-containing binding partners, as PDGF-stimulated WAVE exhibits reduced interaction with Grb2 adaptor protein [80]. In addition, growth factor stimulation increases interaction of WAVE2-containing WRC with the Arp2/3 complex and ERK inhibition abrogates this increase [20]. Serine to alanine mutation of the four ERK phosphorylation sites on WAVE2 induces an unregulated WAVE2 interaction with Arp2/3 and actin [20], suggesting some basal hypophosphorylation of the sites may be needed for proper cycling of Arp2/3 binding, activation, and release. In NIH 3T3 cells, which exhibit dorsal and peripheral membrane ruffles independent of WAVE2, a WAVE2 S343A, T346A, S351A triple mutant does not affect membrane ruffles but increases cell migration speed by reducing Golgi apparatus polarization, a function independent of Rac activity [78]. The mechanistic role of the WRC and ERK phosphorylation of WAVE2 in this phenotype remains to be clarified.

ERK also phosphorylates ABI on numerous residues within its PRD. Phosphorylation of ABI1 Ser183, Ser216, Ser225, Ser392, Thr394 and Ser410 was discovered by mass spectrometry of ABI purified from growth factor-treated cells and ABI *in vitro*-phosphorylated by ERK [20, 22]. Two additional Abi1 residues are predicted ERK-phosphosites (Thr265, Ser2672) [81]. These eight sites are conserved in vertebrates and in ABI2 (Fig. 3). Mutation of all the sites is necessary to completely remove ERK's *in vitro* and *in vivo* phosphorylation of ABI1 (Fig. 3 [20]). ERK-dependent phosphorylation of S225 was confirmed with a phospho-specific antibody [20]. As discussed above, ABI1 Ser216 is phosphorylated by CDK1 during mitosis [54]. In our hands, S183A/S216A and T265A/S216A double mutants are phosphorylated less than the corresponding S183A and T265A single mutants in an *in vitro* ERK kinase assay, indicating ERK can phosphorylate ABI1 Ser216 (unpublished data). However, we did not test the individual contribution of Ser216 phosphorylation to the ERK-dependent phosphorylation of ABI1 *in vivo* [20]. Mutation of the 8 ERK phosphorylation sites on ABI1 to alanines inhibited WRC interaction with Arp2/3 and actin, suggesting ERK phosphorylation of ABI participates in release of the VCA region and WRC activation [20]. Mutation of the eight sites to phospho-mimicking aspartic and glutamic acids increase WRC interaction with Arp2/3 and actin. Thus, if Ser216 is not a true ERK site *in vivo*, its phosphorylation is unlikely to inhibit WRC interaction with Arp2/3 when ABI is phosphorylated by ERK.

While more work with site-specific mutants is needed to sort out the contribution and order of the individual ERK phosphosites, ERK phosphorylation of WAVE2 and ABI together promotes WRC activity. Introduction of WRC complexes in which all the WAVE2 and ABI1 phosphosites are inactivated by mutation to alanines or mimicked by mutation to aspartic and glutamic acids indicate the phosphorylations are necessary for and promote actin-driven edge protrusion and cell migration *in vivo* [20].

3.3. CK2

Casein kinase 2 is a constitutively-active and ubiquitous serine/threonine kinase. CK2 has a preference for serine residues and is directed by acidic residues following the phosphoacceptor site. It recognizes the consensus sequence D/E/X-S/T-D/E/X-D/E/X-D/E/X, where X is any residue except a basic or proline residue and the D/E at the +3 position is absolutely required. Phosphoserine can replace an aspartic acid or glutamic acid at any position, meaning phosphorylation by other kinases can prime a substrate for phosphorylation by CK2 and CK2 often progressively phosphorylates multiple serine and threonine residues (reviewed in [82, 83]).

CK2 phosphorylates WAVE2 on Ser482, 484, 488, 489, and 497 within the acidic domain of the VCA region [84]. Ser482, 484, and 488 are conserved in all three WAVES and in vertebrates. Ser489 is unique to WAVE2 and Ser497 is unique to WAVE2 and WAVE3 (Fig. 2). In the amoeba *Dictyostelium discoideum*, the surrounding sequence is different, but the general composition of acidic residues following the phosphorylated serines is similar [85]. The five CK2 phosphorylations inhibit actin-driven pseudopod formation in *Dictyostelium* [85]. Phosphorylation of the first two sites, Ser482 and Ser484, appear to be the most critical for inhibiting VCA function. A S482A, S484A double mutant could equally rescue the protrusion defects of *wave* knockout *Dictyostelium* [85]. The WAVE 2A and 5A mutants actually exhibit hyperactivity compared to wildtype WAVE. The mutants induce abnormally large, dominant pseudopods that rarely bleb or split in *Dictyostelium* and cause frequent direction changes during fibroblast cell migration [84, 85]. The acidic domain of the VCA region is proposed to form an intramolecular interaction with the WAVE basic region that is strengthened upon CK2 phosphorylation [85]. Such an interaction would block WAVE interaction with the Arp2/3 complex, indicating that an as yet unidentified phosphatase must remove the CK2-mediated phosphorylations for WRC activation. However, the Arp2/3 activation and actin polymerization activity of free VCA region *in vitro* was elevated with the 2A and 5A mutations [79, 84]. This result suggests the CK2 phosphorylation of the VCA domain may inhibit VCA domain activity independent on the WAVE basic region, but is confounded by the potential for the GST tag used to purify the VCA region to induce multimer formation.

4. Protein Kinase A

Protein Kinase A is a Ser/Thr kinase in the AGC kinase family that preferentially phosphorylates R-R/K-X-S/T motifs (reviewed in [86]). PKA is activated by hormones, chemokines, and neurotransmitters that bind G protein-coupled receptors and activate adenylate cyclase. cAMP binds to the PKA regulatory subunit and releases its inhibition of the catalytic subunit [86-88]. PKA phosphorylates the regulatory subunits of protein phosphatase 2A (PP2A) to activate PP2A, which mediates dephosphorylation of the inhibitory CDK5 sites on WAVE1 [71]. An agonist for the neurotransmitter dopamine and forskolin, both of which activate cyclic AMP (cAMP) production, reduce WAVE1 Ser310, Ser397, and Ser441 phosphorylation and increase spine and protrusion formation [24]. PKA also physically interacts with WAVE1 and WAVE2 and could increase the local concentration of active PP2A near WAVE2-containing WRCs [89, 90].

5. Conclusion

The rapid and highly reversible nature of phosphorylation provides a mechanism for motile cells to rapidly respond to changes in their extracellular environment. The WRC integrates cues from the extracellular and intracellular environments via multiple signaling pathways. Growth factors, cytokines, hormones, neurotransmitters, and extracellular matrix signals activate Abl, Src, and ERK. These kinases directly phosphorylate WAVE and ABI1 to promote WRC localization to the cell edge, release of WAVE's VCA domain, and Arp2/3 activation. Activating signals must additionally induce phosphatases to remove CDK and CK2-mediated inhibitory phosphorylations.

The WRC regulatory pathways crosstalk with each other and can indirectly affect phosphorylation at other phosphosites. For example, CDK1 phosphorylation of ABI1 inhibits Abl-induced tyrosine phosphorylation of ABI1 [54] and PKA promotes dephosphorylation of the CDK5 sites on WAVE1 [24] [71]. Abl and Src can promote ERK activation upon adhesion or receptor tyrosine kinase activation [91, 92]. Thus, an activating signal can be amplified through cross-activation of additional pathways that converge upon the WRC. Determining which combination(s) of phosphorylations induces maximal WRC activity will be a significant step towards understanding how signaling pathways control WRC and Arp2/3 activity.

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- Abl, Src, ERK, CDKs, and CK2 phosphorylate WAVE and ABI at unique sites
- Extracellular stimuli activate Abl and ERK to phosphorylate and activate the WRC
- The constitutively-active CK2 phosphorylates WAVE to inhibit WRC activity
- In neurons, CDK5 phosphorylates WAVE and inhibits WRC activity
- PKA activates a phosphatase to remove the inhibitory CDK5 phosphorylations

Fig.1. Components and domains of the WRC

(A) The WRC is a stable heterocomplex of WAVE, ABI, NAP, SRA1, and HSPC300 isoforms. WAVE is also called SCAR, after its *Dictyostelium* homolog. NAP is also called Hematopoietic protein (Hem). SRA1 is also called p53-inducible mRNA121 (PIR121) or cytoplasmic FMRP interacting protein (CYFIP). HSPC300 is also called BRICK. WRC activation involves release of the VCA domain from intermolecular interaction with the meander region of WAVE and SRA1. (B) WAVE and ABI interaction domains. WAVE interacts with ABI and HSPC300 through its WHD. WAVE interacts with negatively-charged phospholipids, such as phosphatidylinositol(3,4,5)-triphosphate (PIP₃), through its basic region. Both WAVE and ABI interact with regulatory proteins through their

polyproline rich domains (PRDs) binding to Src Homology 3 (SH3) domains in the regulatory proteins. WAVE interacts with and activates the Arp2/3 complex through the WAVE VCA region.

A WHD Phosphorylations

			Src						Abl				
Hs wave1	123	E T	Y	D	V	C	...	148	K F	Y	T	N	P S Y ...
Mm wave1	123	E T	Y	D	V	C	...	148	K F	Y	T	N	P S Y ...
Xl wave1	123	E T	Y	E	I	C	...	148	K F	Y	T	N	P S Y ...
Dm scar	125	D T	Y	A	Q	C	...	148	K F	Y	T	N	P N Y ...
Dd scar	127	T V	Y	E	K	C	...	177	K F	Y	T	N	P D Y ...
Hs wave2	122	E T	Y	N	T	C	...	147	K F	Y	T	D	P S Y ...
Mm wave2	122	E T	Y	N	S	C	...	147	K F	Y	T	N	P S Y ...
Xl wave2	122	E T	Y	S	T	C	...	147	K F	Y	T	D	P S Y ...

B PRD Phosphorylations

		CDK5/ERK										ERK		ERK		ERK						CDK5				CDK5														
Hs wave1	308	P	Q	S	P	A	T	...	365	-	-	A	P	A	V	P	P	P	A	P	L	Q	...	395	Q	P	S	P	P	V	...	439	P	S	S	P	V	T	...	
Mm wave1	308	P	Q	S	P	A	A	...	365	-	-	A	P	A	V	P	P	P	A	P	L	Q	...	395	Q	P	S	P	P	V	...	439	P	S	S	P	V	A	...	
Xl wave1	310	P	Q	S	P	A	M	...	374	-	-	T	T	V	V	P	P	P	T	P	L	Q	...	404	Q	A	S	P	P	V	...	445	P	S	S	P	I	A	...	
Dm scar	327	G	Q	G	V	M	S	...	390	P	P	P	V	P	D	V	I	S	P	M	S	G	M	...	425	A	G	S	P	N	S	...	491	P	V	E	E	G	M	...
Dd scar	274	-	-	-	-	-	...	343	-	-	N	M	P	P	P	P	P	M	Q	Q	-	...	343	V	A	A	P	A	S	...	364	P	M	A	K	V	A	...		
Hs wave2	306	L	G	S	P	P	G	...	341	F	G	S	P	G	T	P	P	P	S	P	P	S	...	373	A	D	Y	P	T	L	...	404	P	P	P	F	T	G	...	
Mm wave2	306	L	S	S	P	P	G	...	341	F	G	S	P	G	T	P	P	P	S	P	P	S	...	373	A	D	Y	P	-	M	...	401	P	L	P	F	S	G	...	
Xl wave2	294	I	G	S	P	I	G	...	321	-	D	A	P	Y	L	P	-	P	P	S	P	P	...	355	L	D	Y	P	A	A	...	388	P	P	P	A	F	S	...	

C VCA Region Phosphorylations

			CK2 CK2				CK2CK2						CK2												
Hs wave1	542	E Y	S	D	S	E	D	D	S	-	E	F	D	E	V	D	W	L	E						
Mm wave1	542	E Y	S	D	S	E	D	D	S	-	E	F	D	E	V	D	W	L	E						
Xl wave1	541	E Y	S	D	S	E	D	D	S	-	E	F	D	E	V	E	W	L	E						
Dm scar	589	E L	S	E	S	E	D	S	D	S	E	D	D	S	E	G	W	M	E	P	N	E	T	S	A
Dd scar	427	G D	S	D	S	E	D	E	S	D	D	F	S	D	-	W	-	D							
Hs wave2	480	E Y	S	D	S	E	D	D	S	S	E	F	D	E	D	D	W	S	D						
Mm wave2	479	E Y	S	D	S	E	D	D	S	S	E	F	D	E	D	D	W	S	D						
Xl wave2	460	E Y	S	D	S	E	D	D	S	S	E	F	D	E	D	D	W	S	D						

Fig. 2. Phosphosites on WAVE

(A) Src and Abl tyrosine phosphorylate the WAVE WHD. (B) CDK5 and ERK serine/threonine phosphorylate the PRD of the WRC. These sites are unique to specific WAVE isoforms. CDK5 regulates WAVE1 and ERK regulates WAVE2. (C) CK2 phosphorylates the VCA region. Sequences were aligned using the Clustal W method. Phosphosites that promote WRC activity are in green and those that inhibit WRC activity are in red. Other residues necessary for directing the kinase to the phosphosite are highlighted in yellow.

			Abl													Abl													CDK1 ERK													ERK												
Hs	abi1	134	V	R	Y	I	R	K	P	I	D	Y	T	V	L	D	...	211	N	D	Y	M	T	S	P	A	R	-	-	L	G	S	Q	H	-	S	P	G	R	...														
Mm	abi1	134	V	R	Y	I	R	K	P	I	D	Y	T	V	L	D	...	211	N	D	Y	M	T	S	P	A	R	-	-	L	G	S	Q	H	-	S	P	G	R	...														
Xl	abi1	134	V	R	Y	I	R	K	P	V	D	Y	T	V	L	D	...	211	N	D	Y	M	T	S	P	A	R	-	-	L	G	S	Q	H	-	S	P	G	R	...														
Dm	abi1	146	I	K	Y	V	R	K	P	I	D	Y	S	M	L	D	...	246	S	H	Y	A	P	N	-	-	-	-	Y	P	I	G	H	P	K	R	M	S	...															
Hs	abi2	134	V	R	Y	I	R	K	P	I	D	Y	T	I	L	D	...	205	N	D	Y	V	P	S	P	T	R	N	M	A	P	S	Q	Q	-	S	P	V	R	...														
Mm	abi2	134	V	R	Y	I	R	K	P	I	D	Y	T	V	L	D	...	205	N	D	Y	V	P	S	P	T	R	N	M	A	P	S	Q	Q	Q	-	S	P	V	R	...													
Xl	abi2	134	V	R	Y	I	R	K	P	I	D	Y	T	V	L	D	...	205	N	D	Y	V	P	S	P	T	R	N	M	A	P	P	Q	Q	-	S	P	V	R	...														

			Abl													ERK ERK													ERK ERK													ERK												
Hs	abi1	246	-	-	-	S	G	S	R	...	262	A	V	P	T	P	S	P	P	T	...	390	A	D	S	P	T	P	P	P	...	408	D	D	S	P	P	P	...															
Mm	abi1	246	-	-	-	S	G	S	R	...	262	A	V	P	T	P	S	P	P	T	...	364	A	D	S	P	T	P	P	P	...	381	D	D	S	P	P	P	...															
Xl	abi1	246	-	-	-	S	G	S	R	...	262	A	V	P	T	P	S	P	P	T	...	385	A	D	S	P	T	P	P	P	...	403	D	D	S	P	P	P	...															
Dm	abi1	283	A	G	Y	S	A	L	P	...	303	N	L	P	S	A	G	M	M	Q	...	369	S	Q	S	P	P	L	P	P	...	387	F	G	R	P	P	P	...															
Hs	abi2	247	S	S	R	S	S	S	R	...	266	A	V	P	T	P	S	P	P	S	...	361	S	D	T	P	-	-	-	P	...	376	D	E	S	P	P	P	...															
Mm	abi2	247	S	S	R	S	S	S	R	...	266	A	V	P	T	P	S	P	P	S	...	333	-	D	T	P	-	-	-	P	...	347	D	E	S	P	P	P	...															
Xl	abi2	247	S	S	R	S	S	S	R	...	267	A	V	P	T	P	S	P	P	S	...	345	T	E	T	P	-	-	-	P	...	439	D	E	S	P	P	P	...															

Fig. 3. Phosphosites on ABI

Abl, ERK, and CDK1 phosphorylate the ABI proteins in the region and within the PRD. Sequences were aligned using the Clustal W method. Phosphosites that promote WRC activity are in green. Other residues necessary for directing the kinase to the phosphosite are highlighted in yellow. The Ser216 site (blue) has been found to be both an ERK and CDK1 site and it remains to be resolved whether the phosphorylation activates or inhibits the WRC.