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Surfing the Big WAVE: Insights into the Role of WAVE3 as a Driving Force in Cancer Progression and Metastasis

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Abstract

WAVE3 belongs to the WASP/WAVE family of actin cytoskeleton remodeling proteins. These proteins are known to be involved in several biological functions ranging from controlling cell shape and movement, to being closely associated with pathological conditions such as cancer progression and metastasis. Last decade has seen an explosion in the literature reporting significant scientific advances on the molecular mechanisms whereby the WASP/WAVE proteins are regulated both in normal physiological as well as pathological conditions.

The purpose of this review is to present the major findings pertaining to how WAVE3 has become a critical player in the regulation of signaling pathways involved in cancer progression and metastasis. The review will conclude with suggesting options for the potential use of WAVE3 as a therapeutic target to prevent the progression of cancer to the lethal stage that is the metastatic disease.

Keywords

WAVE3; cancer cell invasion; Invasion-Metastasis Cascade; microRNAs; TNBC; Invadopodia

1. Background

Actin cytoskeleton plays a key role in a number of cellular functions that include cell shape changes, cytokinesis, cell motility, cell proliferation and membrane traffic [1, 2]. Precise control of the assembly, dynamics and organization of the actin filaments that constitute the core of the actin cytoskeleton, is critical for these functions. Defects in proper actin regulation, however, can contribute to human disease including cancer metastasis, and Wiskott-Aldrich syndrome to name a few [3-7]. Several genes were identified to be involved in the regulation of actin-cytoskeleton organization including the genes encoding for the WASP/WAVE family of proteins. This family of proteins is comprised of 5 members (Fig.

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1) that form two distinct subfamilies based upon structural homologies [8, 9]. The WASP subfamily includes the WASP protein which is associated with the Wiskott-Aldrich syndrome [4, 10], and its more widely-expressed homologue, NWASP [11]. The WAVE subfamily of proteins contains three members, WAVE 1, 2 and 3 [12, 13].

The WASP/WAVE proteins which function as effectors downstream of the Rho GTPases play a critical role in several cellular processes, such as cell motility and proliferation, by regulating actin polymerization and cytoskeleton organization. In response to extracellular signals, both the WASP and WAVE subfamilies activate the Arp2/3 complex, leading to the stimulation of actin polymerization and assembly of actin filaments. The involvement of the Arp2/3 complex in the formation of new branched actin filaments is dependent upon interactions with nucleation-promoting factors (NPFs). These NPFs consist of WASP [14], N-WASP [15, 16], WAVE1 [17, 18], WAVE2 [19, 20], WAVE3 [5, 8], and the newly identified WASH [21], WHAMM [22] and JMY [23]. Recent work has demonstrated that Abp1 [24], Pan1 and cortactin [25-27] can directly activate the Arp2/3 complex, while the NPFs need first to be activated by Cdc42 and Rac [28-30].

The activity of WASP and WAVE proteins is controlled by different mechanisms of regulation. Cdc42 is required for the activation of WASP and N-WASP, while the activity of the WAVE proteins is regulated through their association with multiprotein complexes, downstream of Rac.

Despite the considerable efforts that have been expended on the analysis of WASP, N-WASP, WAVE1 and WAVE2, up to recently, very little was known about the role of WAVE3 in the pathologies that are associated with defects in the remodeling of the actin cytoskeleton. Loss of function of either WAVE1 or WAVE2 has been shown to significantly affect mouse embryonic development and survival [17, 19, 20]. Moreover, although the WASP/WAVE proteins are believed to be equally involved in the mechanisms that regulate the actin-cytoskeleton organization, comparatively, little is known about the impact of WAVE3 deregulation on these processes. In this review we present the recent advances in the field about the involvement of WAVE3 in both normal physiology and in pathological conditions, including cancer.

2. Mechanisms of Regulation of WAVE3

2.1. Different mechanisms of regulation for WASPs and WAVEs

WAVE3, as a member of the WASP/WAVE family of structurally and functionally related proteins, plays a critical role in actin polymerization and cytoskeleton organization [1, 31]. The WASP and WAVE proteins share several domains in their protein structures (Fig. 1), which are believed to regulate their activity and sub-cellular localization, in response to a variety of extra-cellular signals [32]. WASP and WAVE proteins function as effectors downstream of the Rho GTPases to regulate the actin cytoskeleton [9]. Members of the WASP subfamily are activated through Cdc42 to induce filopodia, while the WAVE proteins function downstream of Rac to induce the formation of lamellipodia [9]. All members of the WASP and WAVE family of proteins share a tripartite VCA (**V**erprolin homology, **C**ofilin homology and **A**cidic) C-terminal domain. Activation of the WASP and

WAVE proteins leads to the exposure of the VCA domain, which can bind to the Arp2/3 complex and initiate the rapid polymerization of actin filaments [33, 34], ultimately leading to cytoskeletal remodeling, which is necessary for cell motility and migration [31].

WASP/WAVE proteins differ both in the signaling inputs that they receive, as well as in their mode of regulation (Fig. 3). A CRIB (Cdc42- and Rac-interactive binding) domain, which is present in the N-terminal half of both WASP and NWASP, and which was shown to specifically mediate binding of active GTP-bound Cdc42, is thought to regulate the activity of these two proteins [35, 36]. In the absence of extra-cellular signals, WASP and NWASP are auto-inhibited through the intramolecular interaction between the CRIB and the VCA domains [37, 38]. The binding of Cdc42 and/or phosphatidylinositol (4,5)P_2 (PtdIns(4,5)P_2) to the CRIB domain is required for their activation. This allows the release of the VCA domain which becomes exposed, and can then bind to the Arp2/3 complex and initiate actin polymerization [30, 39]. In contrast, the WAVE1 and WAVE2 proteins, which function downstream of Rac to induce actin polymerization [12, 40], were found to be regulated through the formation of multimeric protein complexes with other actin-cytoskeleton remodeling proteins [41-43]. WAVE1 and WAVE2 proteins were shown to be sequestered in an inactive state through the formation of a complex with four other proteins, PIR121, Nap125, HSPC300 and Abi1 [40-43]. WAVE3 has also been shown to be included in a similar multimeric protein complex [44].

In addition to the VCA domain, which is critical for the Arp2/3-mediated actin polymerization, the WASP/WAVE proteins have other functional domains (Fig. 1), that mediate interactions with several other proteins to regulate the activity of the WASP/WAVE proteins [9, 32]. Profilin, which has a high affinity to poly-L-proline, binds to the proline-rich domain (PRD) which is conserved in all WASP and WAVE proteins [12], and was shown to play a critical role in the enhancement of Cdc42-WASP-induced nucleation of actin polymerization [45]. On the other hand, the WISH protein, an SH3-containing protein, was found to activate N-WASP independently of either Cdc42 or PtdIns(4,5)P_2, by binding to the PRD of N-WASP [46]. Other proteins such as Syndapin, WIP and Nck have been shown to physically interact with the WASP protein through different motifs to induce actin polymerization [47-49]. While the regulation of WASP proteins is well documented, the regulation of the WAVE proteins, and WAVE3 in particular, has not been well elucidated. The best-characterized WAVE protein partner is IRSp53, which has been shown to be an essential intermediate between Rac and WAVE2 in the regulation of actin polymerization and membrane ruffling [50]. In addition to the involvement of WAVE3 in actin polymerization and cytoskeleton organization [1, 13, 44, 51], WAVE3was found to be associated with the development of low grade neuroblastoma [5].

2.2. Independent roles for the WAVE proteins

The expression profiles of the WAVE genes clearly show an overlap in the expression of all three WAVE transcripts in several embryonic and adult tissues [8]. Previous studies have also suggested co-localization of all three WAVE proteins in multimeric proteins complexes [43, 52], which suggests the involvement of the WAVE proteins in similar cellular pathways. However, although WAVE1 and WAVE2 are both expressed in mouse

embryonic fibroblasts, they were found to have differential roles in cell migration [53]. WAVE1 was found to be required for the formation of dorsal ruffles, while WAVE2 is required for the formation of peripheral ruffles, two membrane-based actin structures that are necessary for the initiation of cell migration [53], clearly suggesting independent roles for the WAVE proteins

2.3. Effect of Wasp and Wave genes on mouse development and actin-based motility

Except for Wave3, all the other 4 members of the Wasp and Wave family of genes have been disrupted in mouse by independent groups, using gene-targeting mutations [15, 17, 19, 20, 54]. Targeted disruption of the X-linked immunodeficiency Wiskott-Aldrich syndrome protein (WASP), although not leading to embryonic lethality [54], resulted in impairment in the proliferation of blood cells as a consequence of severe defects in the cytoskeleton organization [55]. On the other hand, disruption of Nwasp, Wave1 or Wave2, each resulted in embryonic lethality, associated with marked developmental delay and organ malformations [15, 17, 19, 20]. Analysis of mouse embryonic fibroblasts (MEFs) derived from mice embryos lacking either one of these genes also showed a defect in the actin-based motility, as a consequence of alterations in cytoskeleton organization [15, 17, 19, 20]. In addition to the striking and distinct phenotypes that result from the targeted-disruption of each Wasp/Wave gene, these genes are individually required for the normal development of the mouse embryo, and for normal cell movement. These studies also imply a crucial non-redundant role for each WASP and WAVE gene in mouse embryogenesis, for the formation specific actin-containing structures, and in the actin-based motility. Thus, generating a mouse lacking the expression of WAVE3 may help in revealing the specific role that WAVE3 plays in mouse development and in actin cytoskeleton organization. While several studies have focused on elucidating the involvement of WAVE1 and WAVE2 in remodeling the actin cytoskeleton and in cell motility, little was known about the impact of of WAVE3 in these processes. Our first encounter with WAVE3 more than a decade ago was somehow serendipitous. While we were looking for the causative defective gene in a patient with low-grade neuroblastoma, and a genomic defect described as a constitutive balanced translocation between chromosomes 1 and 13, we found that WAVE3 was truncated and inactivated as a result of this translocation [5]. We subsequently reported on the hypothesis that loss of WAVE3 function might be associated with the development of this type of malignancy, and probably other cancers [5]. Since then more than 30 manuscripts have been published from both our group and others dealing with the mechanisms that involve WAVE3 in both physiological and pathological conditions.

2.4. The WAVE3-mediated remodeling of actin cytoskeleton is driven by PDGF downstream of PI3K

As mentioned above the WASP and WAVE proteins differ in both the signaling inputs that they receive, as well as in their mode of regulation. WASP proteins are auto-inhibited by intramolecular interactions that mask the VCA domain in the absence of signaling molecules such as Cdc42 and phosphatidylinositol 4,5-biphosphate [37, 38]. On the other hand, WAVE1 and WAVE2 proteins are sequestered in a complex with four other proteins, PIR121, Nap125, HSPC300, and Abi1 [40-42, 56]. Extracellular stimuli result in the dissolution of this complex, which subsequently leads to Arp2/3-mediated actin

polymerization. Reports on the basal activity of this complex; i.e., in the absence of external stimuli, however, are not concordant. On one hand, the *in vitro* study reported by Innocenti and colleagues suggested that the WAVE2 complex is constitutively active [42]. On the other hand, a more recent study by Lebensohn et al., has clearly shown that the WAVE2 complex is basally inactive [56]. WAVE3 was later found to be included in the same protein complex as WAVE1 and WAVE2, suggesting a similar mode of regulation [44]. Regardless of which WAVE isoform is involved, the activity and functionality of the WAVE complex in resting conditions have yet to be definitely elucidated.

Platelet-derived growth factor (PDGF) induces a variety of cellular responses in several cell types, including proliferation, migration, invasion, and cell survival, via its receptor [57, 58]. PDGF treatment of MDA-MB-231 breast cancer cells was found to induce the formation of lamellipodia at the edge of migrating cells, and the accumulation of WAVE3 in these lamellipodia [59]. The same study showed that the WAVE3 regulation of cell migration and the formation of lamellipodia that is induced by PDGF, involves PI3K as an upstream modulator, where a direct interaction between WAVE3 and p85, the regulatory subunit of PI3K, is required [59]. Inhibition of PI3K activity with LY294002, in the PDGF-treated cells, resulted in the inhibition of both lamellipodia formation and cell migration, as well as a disruption of the accumulation of WAVE3 at the edge of migrating cells. Similar studies have previously reported that the formation of lamellipodia and cell migration of PDGF-treated fibroblasts, or the HGF-treated myogenic cells, require the production of phosphatidylinositol 3,4,5-triphosphate (PIP3) by PI3K, which subsequently leads to the recruitment of WAVE2 to the polarized plasma membrane [60, 61]. It is also possible that the production of PIP3, as a result of PI3K activity, is essential for WAVE3-mediated lamellipodia formation and cell migration, as it has been shown for WAVE2 [60, 61]. These results support the previously reported data on the involvement of PI3K in the formation of lamellipodia and cell migration in other cell types in response to specific growth factors [1, 20, 53, 60].

2.5. The WAVE3-p85 interaction is required for cell migration

PI3K is a heterodimer consisting of p110, a 110-kDa catalytic subunit and p85, an 85-kDa regulatory subunit. PI3K activity depends upon the production of PIP3, a messenger molecule produced in response to a variety of growth factors. The targeting of PI3K to specific locations within the cell leads to its activation and therefore to the remodeling of the cytoskeleton organization [62]. p85, is a critical component of the actin cytoskeleton regulatory pathway [62]. Although the downstream targets of PI3K are still not completely understood, it has been shown that the binding of the PI3K complex to activated PDGF receptor via the p85 Src homology 2 (SH2) domains leads to the internalization of the complex into endocytotic vesicles, where cellular trafficking is regulated [63]. However, in addition to the SH2 domains located in the C-terminal half of p85, p85 also has additional functional domains in its N-terminal half (Fig. 2), including an SH3 domain and a domain homologous to the breakpoint cluster region (BCR) gene product. These domains may function to target PI3K to distinct regions in the cell in order to initiate specific downstream response cascades or provide a mechanism by which specific protein-protein interactions are mediated [64]. A direct interaction between WAVE3 and p85 proteins was demonstrated by

both *in vitro* binding and co-immunoprecipitation assays [59]. WAVE3 was found to bind to the C-terminal SH2 domain of p85 via the basic region domain (BR) of WAVE3 (Fig. 2). Using immunocytochemistry, an in vivo interaction was demonstrated between these two proteins in the actin-based membrane cytoskeleton, supporting the involvement of p85 in the regulation of the activity of WAVE3 [59]. These observations clearly support the involvement of p85 in the WAVE3-mediated actin polymerization and cytoskeleton organization. However, the mechanisms of p85-mediated regulation of the WAVE3 activity are yet to be identified.

2.6. Phosphorylation of WAVE3 by c-Abl tyrosine kinase is required for the regulation of cell motility, cancer cell migration and invasion

Phosphorylation is a major mechanism whereby gene function is regulated, and the WASP/WAVE proteins are no exception [65-69]. The non-receptor tyrosine kinase ACK1 phosphorylates and enhances the WASP-mediated actin polymerization [70], while stimulation of cultured cells with growth factors increases the phosphorylation levels of the WAVE proteins [71]. Phosphorylation of WAVE1 by CDK5 [72], or of WAVE2 by the non-receptor tyrosine kinase c-Abl [73], leads to the stimulation of membrane ruffling and cell spreading [74]. Not surprisingly, the kinase activity of c-Abl is critical for the remolding of actin cytoskeleton, since cells lacking c-Abl expression show a severe deficiency in membrane ruffling in response to growth factors [75]. In WAVE3, c-Abl was also found to phosphorylate four tyrosine residues scattered throughout the functional domains of the WAVE3 protein [51]. Among the four tyrosine residues, Tyrosine 151 is conserved among all three WAVE proteins, both in human and mouse [8], and its equivalent in WAVE2 (Y150) is also targeted for phosphorylation by c-Abl [73]. Y337 is specific to WAVE3, whereas Y486 is conserved in both WAVE1 and WAVE3, but not in WAVE2 [8]. Therefore, phosphorylation of different tyrosine residues in the different WAVE proteins might account for the specific and independent roles of the WAVE proteins in the regulation of actin polymerization and cytoskeleton remodeling. We showed that the c-Abl-mediated phosphorylation of WAVE3 also results in the stimulation of lamellipodia formation and cell migration, which clearly supports the hypothesis that WAVE3 is also a downstream effector of c-Abl. Interestingly, because c-Abl can bind all WAVE isoforms, it is possible that it might exert its effect by targeting different tyrosine residues in the different WAVE isoforms to regulate their specific functions the in the regulation of actin cytoskeleton.

Other studies have shown that cell stimulation with PDGF results in an increase in cell motility and lamellipodia formation downstream of PI3K [59]. It was also suggested that the PI3K-mediated activation of WAVE3 required the binding of the regulatory subunit p85 to a tyrosine-phosphorylated WAVE3 [59] to promote lamellipodia formation and cell motility. Accordingly it has been demonstrated that tyrosine phosphorylation levels of WAVE3 were indeed increased after PDGF stimulation of cultured cells [51]. More importantly, phosphorylation of WAVE3 was found to require c-Abl kinase activity, further supporting the hypothesis that c-Abl phosphorylation of WAVE3 might be required to link WAVE3 to the PI3K complex, thus allowing for the PDGF-mediated activation of WAVE3. Those studies have, therefore, provided a novel mechanism by which WAVE3, and probably other

members of the WAVE family of proteins, is linked to the PDGF-induced cytoskeleton remodeling downstream of PI3K.

Probing further into the biological significance of WAVE3 phosphorylation by c-Abl it was found that the lack of WAVE3 phosphorylation resulted in a dramatic inhibition of lamellipodia formation and cell migration, even in the presence of extracellular stimuli, such as PDGF. It is, however, still not clear which of the four tyrosine residues is (are) the most critical for lamellipodia formation and cell migration.

3. WAVE3 and Cancer Invasion and Metastasis

3.1. WAVE3 and MMPs

Degradation of the extracellular matrix (ECM) via MMP activity is essential for many normal physiological processes, e.g., during development, cell migration, growth, and wound healing [76]. On the other hand, increased expression and activity of MMPs are also associated with tumor invasion, metastasis, and tumor angiogenesis [77-79]. Expression of most MMPs is normally low in tissues, and only induced when remodeling of the extracellular matrix is required. MMP expression is primarily regulated at the transcriptional level, although stabilization of MMP transcripts in response to growth factors, as well as the influence of cytokines, also plays a role in the regulation of MMP activity [80, 81].

The possible involvement of the WAVE proteins in the regulation of MMPs was initially reported by Suetsugu and colleagues who showed that downregulation of WAVE1, but not WAVE2, affected MMP-2 activity [53]. Conversely, our group, while seeking to investigate the functional consequences of loss of WAVE3 using RNA interference (RNAi), we identified a MAPK signaling axis, where WAVE3 was essential for the regulation of the expression of different MMPs [82]. We showed that knockdown of WAVE3 expression affects the activity of the p38 MAPK pathway, but not that of AKT or ERK1/2. WAVE3-mediated downregulation of p38 activity was found to be independent of both WAVE1 and WAVE2 expression, as WAVE3 knockdown did not alter the transcription levels of either WAVE1 or WAVE2. Loss of WAVE3 resulted in a significant decrease in the expression levels of MMP-1, -3, and -9. The inhibition of these MMPs as a result of WAVE3 loss led to a dramatic inhibition of cell migration and invasion using both in the *in vitro* wound closure and Matrigel assays [82]. The study provided for the first time a clear evidence for a novel role of WAVE3 in the regulation of MMP activity via the p38 MAPK pathway. In fact, the MAPK signaling pathway plays an important role in regulating many fundamental processes such as cell growth, migration, and differentiation [83-86], which is linked to MMPs. Activation of MAPK pathways results in alterations in the expression levels and activity of MMPs, which in turn, are responsible for the degradation of extracellular matrix, and as such, are required for cell migration [87]. A recent study has also confirmed the important role of WAVE3 in the expression and activity of MMPs [88].

3.2. WAVE3 and microRNAs

MicroRNAs are emerging as important regulators of cellular differentiation, and they are now known to often be dysregulated during carcinogenesis. MicroRNAs are small noncoding RNAs, usually 20- to 22-nucleotides long, which regulate gene expression at the

post-transcriptional level. So far, more than 1000 microRNAs have been identified in mammalian cells, and each microRNA has several target genes. The broad spectrum of genes that can be regulated by a single microRNA is attributed to the high level of conservation of the target motifs, known as seed sequences, within the 3′untranslated regions (UTR) of the target genes. micoRNAs are now widely regarded as the most powerful regulators of gene expression in complex cellular processes including cancer cell invasion and metastasis [89-96]. In fact, several microRNAs have been found to function as tumor suppressors, such as miR-15a, miR-16-1, and let-7 [91, 97-101], whereas others were found to possess oncogenic properties, such as miR-155, miR-17-5p, and miR-21 [91, 102-104]. In our previously published work, we reported a highly significant correlation between the expression levels of WAVE3 and advanced stages of breast cancer [105], clearly supporting the function of WAVE3 as a metastasis promoter protein [106, 107]. However, the mechanisms for regulation of WAVE3 expression levels during tumor progression remained unresolved. In an effort to dissect the role of WAVE3 in cancer cell invasion and metastasis, we were able to establish a significant role of microRNAs in the regulation of WAVE3 expression and activity at two distinct stages of cancer progression; (i) during the epithelial to mesenchymal transition (EMT) process downstream of miR200 [108], and (ii) during the invasion-metastasis cascade downstream of miR31 [109-111].

3.2.1. The WAVE3-mediated modulation of cancer cell invasion is regulated by miR200 during EMT—We found that this evolutionary conserved microRNA, miR200, which regulates key differentiation processes during development, is also involved in the regulation of WAVE3 [108]. The key findings from the miR200 study in the regulation of WAVE3 expression and activity *in vitro* are summarized as follows: (i) Expression of WAVE3 is inversely correlated to miR200 in epithelial versus mesenchymal-type cancer cells; (ii) miR200 targets the 3′UTR of WAVE3 at three different seed sequences and represses its expression; (iii) miR200-mediated downregulation of WAVE3 expression inhibits cancer cell invasion and induces an mesenchymal to epithelial transition (MET)-like phenotype *in vitro*, which the reverse process of the epithelial-to-mesenchymal transition (EMT); (iv) Re-expression of miR200-resistant WAVE3 reverses the inhibition cancer cell invasion imposed by miR200; and (v) Expression levels of miR200 correlate inversely with human breast cancer progression.

3.2.2. miR31 regulates WAVE3 expression and activity during the invasion-metastasis cascade of breast cancer—We have previously performed *in silico* analysis [112-114] to search for potential binding sites for microRNAs in WAVE3 [108] and noted that, in addition to miR200, miR31 has a single target site in the 3′UTR of WAVE3, with a perfect match to the seed sequence recognized by miR31 [111]. The potential relationship between miR31 and WAVE3 became particularly noteworthy in view of the recent reports of the major role of miR31 in cancer metastasis [115-117] and the role of WAVE3 in cancer progression and metastasis [82, 105-108, 111]. We therefore investigated whether the influence of miR31 on cancer cell invasion and metastasis is WAVE3 dependent. The key findings from the miR31 study on the regulation of WAVE3 expression and activity *in vitro* have been published [108-111] and are summarized as follows: (i) Expression of WAVE3 is inversely correlated to miR31 in epithelial versus mesenchymal

BC cells. (ii) miR31 targets the 3′UTR of WAVE3 at s specific seed sequence and represses its expression. (iii) miR31-mediated downregulation of WAVE3 expression inhibits cancer cell invasion [111]. (iv) Re-expression of miR31-resistant WAVE3 is sufficient to reverse the inhibition of cancer cell invasion imposed by miR31 [111]. (v) β1 integrins, which we have shown to be involved in WAVE3-mediated regulation of cancer invasion, are specifically targeted and by miR31 in metastatic triple-negative BC (TNBC) [109]. (vi) miR31 expression and activity are regulated by epigenetic modification of its promoter leading to its silencing, and therefore enhancement of aggressiveness of TNBCs [110].

The data from these *in vitro* analyses were reproduced in other invasive cancer cells from different origins demonstrating that the effect of miR31 is not restricted to a single cell line or a cancer, but appears to be a generalized effect that extends to other cancers. More importantly, our analyses of the non-invasive breast cancer MCF7 cells, which express high levels of miR31 and low levels of WAVE3, have shown that inhibition of endogenous miR31 results in increased invasion associated with increased WAVE3 expression levels [111]. Based on the findings summarized above, we have developed a model whereby the regulation of WAVE3 during cancer progression and metastasis is achieved at the post-transcriptional level by both miR200, during EMT, and by miR31, during the late steps of the invasion-metastasis cascade. WAVE3 is also regulated at the post-translational level by tyrosine phosphorylation downstream of c-Abl (Fig. 3).

Interestingly, we also found that both miR200 and miR31, which modulate the activity of WAVE3 during EMT and the invasion-metastasis cascade of BC, are also regulated by TGF-β, therefore, placing TGF-β-miR200-miR31-WAVE3 in the same signaling axis (unpublished data). As such, we hypothesized that TNBC metastasis comprises TGF-β to downregulate expression of miR200 and miR31, which in turn enhances WAVE3 expression and activity necessary to the acquisition of EMT and metastatic phenotypes in TNBCs.

The body of evidence presented above, which has been published from several independent research groups who used a diverse array of cell culture, in vitro and in vivo assays, convincingly points to the critical role of WAVE3 in cell motility and migration as well as cancer cell invasion and metastasis both *in vitro*, *in vivo* in mouse models and in human clinical settings. Surprisingly a recent report from Spence et al. [118], claimed that WAVE3 may not be essential for the invasive ability of MDA-MB-231 breast cancer cells. Interestingly this cancer cell line has been used by our group and other groups as a model to investigate WAVE3 function in cancer invasion [82, 105, 119, 120]. Given that WAVE3 was confirmed to be involved in cancer invasion in many cancer types (Breast, Prostate and Colon) using different cancer cell lines [82, 88, 105, 107, 120-123], we could suggest that cells used by Spence et al. [118] must be different. In support of this possibility, we note that the shape and phenotype of the WAVE3-knockdown cells reported in their study were strikingly different from those we routinely observe as well as from those frequently published by others. Different reagents and assays conducted in different labs, including the knockdown levels of WAVE3, also could account for these differences.

3.3. WAVE3 is required for cancer progression and metastasis in mouse

Given the central role that WAVE3 plays in the regulation of the actin cytoskeleton, cell motility and cancer cell invasion [51, 59, 82, 105], the suggestion that WAVE3 may also play a critical role in cancer progression and metastasis seems evident. In fact, at least one member of the WAVE2, a close relative to WAVE3 has been associated with the metastatic phenotype of murine melanoma cells [124, 125].

One of the early studies provided the first evidence for the role of WAVE3 in tumor cell invasion and metastasis [105]. In the lung colonization assay, also known as experimental metastasis model, where human BC cells were injected the blood circulation of SCID mice via tail vein, WAVE3 was found to be required for BC cells to form tumor colonies in the lungs. Although the BC cells, where WAVE3 was stably knocked-down with shRNAs, were able to be established in the lungs, they formed significantly smaller sized colonies compared to their control counterparts. On the other hand, in the orthotopic xenograft assay, also known as the spontaneous metastasis assay, where cancer cells are implanted in mammary fat pads of SCID mice, the WAVE3-deficient cells were significantly less tumorigenic in SCID mice. Tumor incidence in the mice injected with the WAVE3-knockdown BC cells was reduced by 60 to 80% compared to their control counterparts, and tumors that developed from the shWAVE3 cells grew more slowly and were less angiogenic [105]. Based on the CD31 staining, the tumors derived from the WAVE3-knockdown cells showed a significant reduction in the number of blood vessels and the size of the blood vessel lumens. VEGF expression was also reduced in the shWAVE3 tumors, suggesting that WAVE3 may regulate expression of VEGF and the recruitment of the blood vessel-forming cells. Although these findings suggest a unique role for WAVE3 in tumor cells, one cannot exclude the possibility that all three WAVE isoforms may play a specific role in some aspects of cancer metastasis, given that WAVE2 also contributes to metastasis of melanoma cells [124, 125]. In fact different WAVE isoforms were found to have independent functions in regulating Arp2/3-mediated actin polymerization and cell migration [20, 53, 124].

Two possible molecular mechanisms were suggested to as how WAVE3 may contribute to tumor metastasis. Previous studies suggest that p38 MAPK is a downstream effector molecule of WAVE3 [82]. Coincidently, a blockade of p38 MAPK signaling by dominant-negative p38 MAPK (DN-p38) partially recapitulated the effect of WAVE3 on metastasis. The DN-p38 cells showed a three-fold reduction in lung tumor formation compared with a six-fold reduction from cells expressing shWAVE3. As with the shWAVE3 tumors, the DN-p38 orthotopic tumors were also less angiogenic [105, 126]. Given that Rac1 can activate WAVE3 [71] as well as p38 MAPK in several cell lines [34, 127] including MDA-MB231 [128], it is suggestive that WAVE3 is required for the Rac1-mediated activation of p38 MAPK. It is still not clear, however, how WAVE3 affects p38 MAPK activity or its intermediate effectors. For example, WAVE3 may be involved in the Rac1-dependent activation of PAK1, an upstream kinase in the p38 MAPK cascade [129]. Although p38 MAPK may contribute to some of the WAVE3 effects, other factors such as Arp2/3 can also mediate WAVE3 effects on tumor cell motility, invasiveness, and metastasis. Cell motility is tightly linked to the regulation of the Arp2/3-mediated remodeling of actin cytoskeleton [130, 131], driven by the WASP/WAVE proteins, [9, 82, 132]. Previous studies have

indicated that WAVE3 is involved in the regulation of lamellipodia production, actin stress fibers formation, and focal adhesions assembly in MDA-MB-231 cells [59, 82]. Therefore it was suggested that WAVE3 may contribute to anchorage-independent cell growth and cell death associated with detachment from extracellular matrix (anoikis).

Recently more studies have reported on the critical role thatWAVE3 plays in other malignancies such as prostate [107, 121] and colon cancers [133]. WAVE3 was also found to be upregulated under hypoxia which is believed to be required to trigger the invasion and metastatic phenotype of cancer cells [119].

3.4. WAVE3 is a biomarker for breast cancer progression and metastasis

Breast cancer is the most common malignancy diagnosed in women and the second leading cause of cancer mortality after lung cancer [134-137]. Metastasis is responsible for ∼90% of deaths in patients with solid tumors [89, 138-142], including those originating in the breast [143-145]. The risk of developing distant metastasis and therefore prognosis in BC is associated with the presence of a number of pathologic characteristics: positive lymph node status, increasing tumor size and histologic grade. BC is a heterogeneous disease that is characterized by the presence of at least five genetically distinct subtypes. For instance, luminal BCs, which also tend to be estrogen receptor positive (ER+) and low grade, have the lowest risk of developing distant metastases and have the best prognosis. At the other end of the spectrum, the basal BC subtypes, which also include the Triple Negative BCs (TNBCs) exhibit dismal survival rates due to their highly aggressive and metastatic behavior, and to their propensity to rapidly recur [146-152]. Genetically, TNBCs are characterized by lack of expression of hormone receptors (ER-α and PR) and HER2, harbor BRCA1-defects and/or deficiencies, and may remain p53-positive [153], which makes them refractory to hormonal therapy, further contributing to the risk of aggressive relapse and dismal survival rates amongst women bearing TNBCs [138, 141, 142, 154, 155].

Given the clinical characteristics of high-grade breast cancers, WAVE3 was thought to be expressed in higher levels compared to low grade tumors and this elevated expression might contribute to the increased metastatic potential seen in the high-grade tumors compared to low-grade tumors. To answer this question, two retrospective studies were conducted using two different cohorts of BC patients, in an attempt to isolate the effect of the levels of expression of WAVE3 on BC progression and metastasis [123]. In the first study 122 BC patients were identified from two very different groups of patients modified Scarff-Bloom-Richardson (mSBR1) and mSBR3 and assessed for WAVE3 expression levels in the primary tumors using IHC. Correlation of WAVE3 expression levels to the patients' clinicopatholological characteristics and the disease outcome and led to the following findings: *(i)* WAVE3 was highly expressed in malignant vs. adjacent normal ductal epithelium, *(ii)* WAVE3 expression was positively correlated with adverse clinicopathologic parameters, *(iii)* WAVE3 expression was increased in the tumors of patients who developed distant metastases, *(iv)* WAVE3 expression levels are positively correlated with reduced distant recurrence free survival and with decreased disease specific survival (Fig. 4 and ref [123]).

In the second part of the same study the prognostic value of WAVE3 mRNA expression levels was evaluated in the circulating tumor cells in the peripheral blood of women with operable breast cancer, based on the unique characteristic of the lack of WAVE3 expression in the peripheral blood mononuclear cells (PBMCs). Analysis of WAVE3 expression levels in the blood of 200 BC patients and correlation with the patients' clinical data revealed that (*i*) WAVE3 mRNA was highly expressed in the peripheral blood of patients with metastatic breast cancer, and (*ii*) WAVE3 expression levels in the blood of BC patients correlates positively with the aggressive TNBC subtype (Fig. 4 and ref. [123]). Other studies have also found significant correlation between WAVE3 expression levels and the pathology of other cancers, notably, prostate and colon cancers [107, 133].

4. Conclusions and perspectives

The last decade has seen significant advances related to the function of WAVE3 in both physiological and pathological setting, therefore closing the gap of lack of knowledge between WAVE3 and its close sisters. Both the mechanistic and the clinical studies have now clearly cemented the position of WAVE3 as a critical player in cancer progression and metastasis. The clinical studies have now characterized WAVE3 as biomarker for BC progression and metastasis, and more importantly, have identified as a driving force behind the pathology of the most aggressive BC subtype, i.e., TNBC. Additionally, WAVE3 has now a potential therapeutic use in a non-invasive liquid bioassay for early detection of TNBCs. Detection of increased levels of WAVE3 in the blood of BC patients after the completion of an adjuvant systemic treatment could help identify those patients who may have a substantial clinical benefit from a ‘secondary’ adjuvant treatment before the occurrence of overt metastasis. Finally, despite these significant advances, several unanswered questions remain to be addressed, pertaining to the molecular mechanisms whereby the function of WAVE3 is being regulated in both physiological and pathological conditions.

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Abbreviation list

NPFs	Nucleating promoter factors
EMT	Epithelial-to-Mesenchymal transition
TNBC	Triple negative breast cancer

ECM	extracellular matrix
MMP	matrix metalloproteinase
PDGF	Platelet-derived growth factor
PI3K	phosphatidylinositol 3-kinase

Highlights

- Regulation of MMPs by WAVE3 is required for cancer cell invasion.
- c-Abl-mediated phosphorylation of WAVE3 is required for lamellipodia formation and cell migration.
- Metastasis-suppressor microRNAs regulate WAVE3 during EMT and the invasion-metastasis cascade.
- WAVE3, a metastasis promoter gene, regulates invasion and metastasis of breast and prostate cancer cells.
- WAVE3 is a biomarker for the triple negative breast cancer subtype.

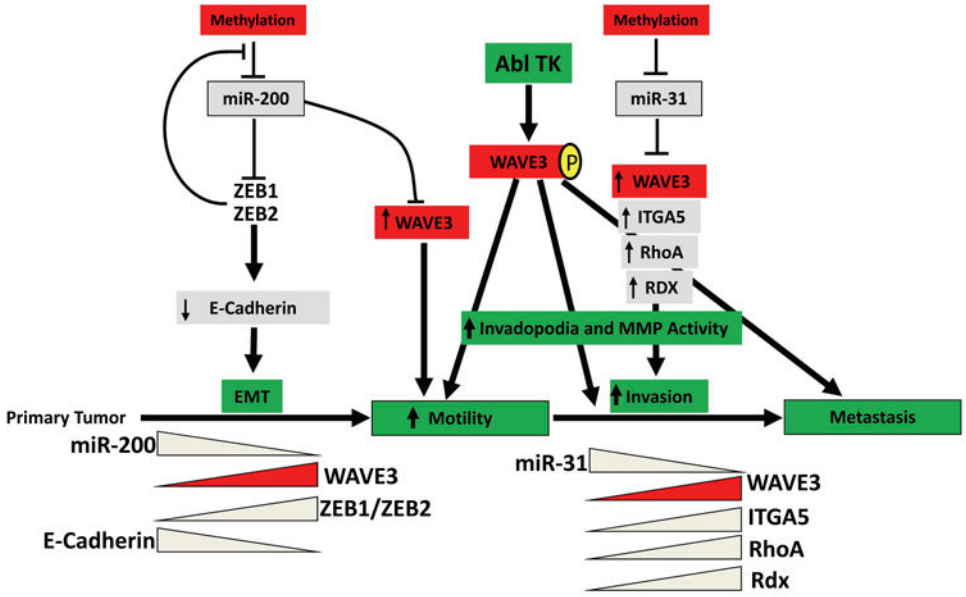

Figure 1. The WASP/WAVE protein family

A. Chromosome location in the human genome of each member of the WASP/WAVE family. **B.** Schematic representation of the functional domains of the WASP/WAVE proteins. SHD: Scar Homology Domain. WH: Wiskott Homology Domain. BR: Basic Region. CRIB: Cdc42- and Rac-Interactive Binding Domain. PRD: Proline Rich Domain. VCA: Verprolin homology, Cofilin homology and Acidic Domain.

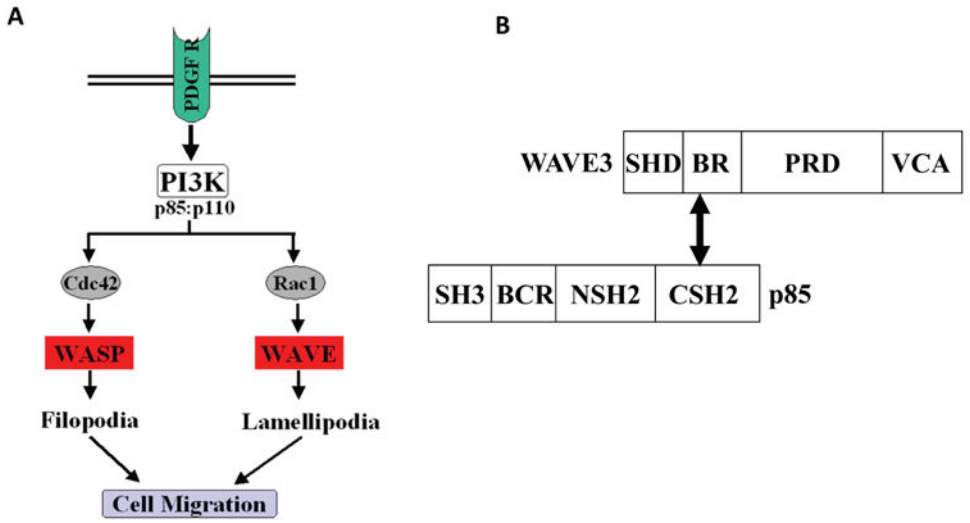

Figure 2. Role of p85 in the WASP- and WAVE-mediated actin-cytoskeleton organization

A. Stimulation of the receptor tyrosine kinase, PDGFR, leads to activation of the components of PI3K; p110 and p85. p85. By regulating the activity of Cdc42 and Rac1, PI3K regulates, in turn, the WASP- and WAVE-mediated actin polymerization. **B.** Schematic representation of the functional domains of WAVE3 and p85. The double-headed arrow points the interacting domains between WAVE3 (BR) and p85 (CSH2).

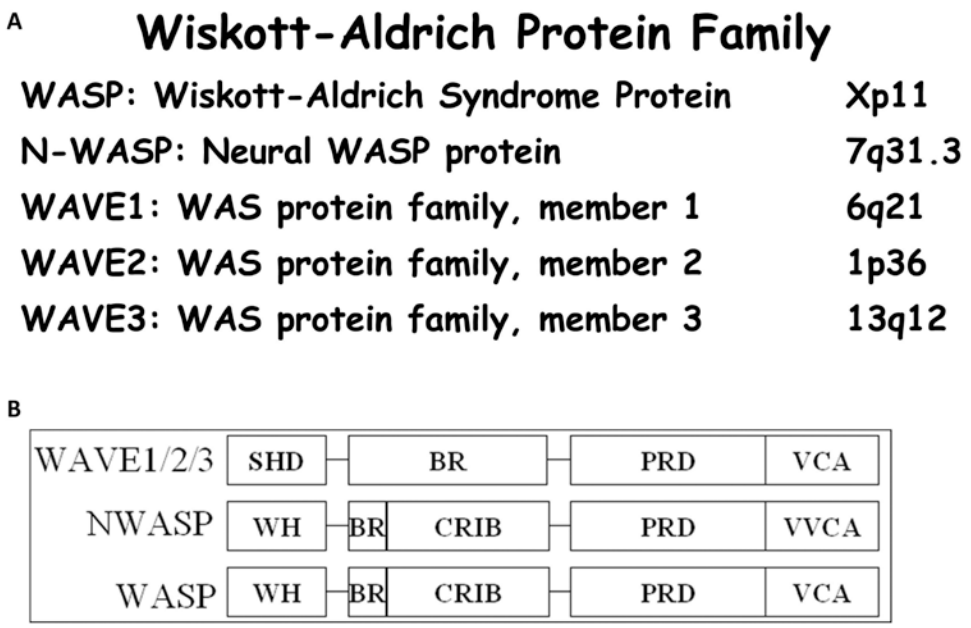

Figure 3. Mechanisms of WAVE3 regulation in cancer metastasis

WAVE3 is regulated by microRNA miR-200, where Epithelial-to mesenchymal transition (EMT) is critical during the early steps of cancer cell invasion. Loss of miR-31 which results in the re-activation of its metastasis promoter target genes, including WAVE3, is a defining step during the invasion-metastasis cascade. Phosphorylation of WAVE3 by c-Abl provides an additional checkpoint to modulate invadopodia formation and MMP activity.

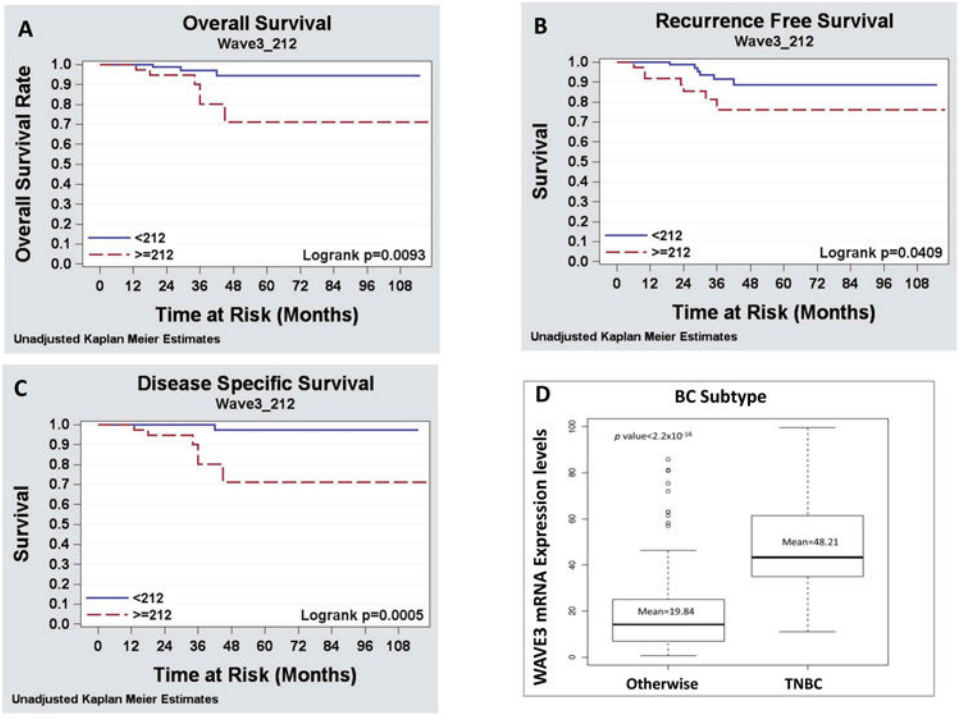

Figure 4. WAVE3 expression levels are positively correlated with reduced distant-recurrence-free-survival and with decreased disease-specific-survival

Kaplan-Meier analysis of **(A)** Overall survival, **(B)** Distant-recurrence-free-survival and **(C)** Disease-specific-survival. The WAVE3 score was dichotomized as positive or negative to determine the relative contribution of the WAVE3 score to each of the three disease outcome parameters, respectively. **(D)** WAVE3 expression levels are associated the TNBC subtype, the most aggressive subtype in breast cancer.