

Membrane targeting of WAVE2 is not sufficient for WAVE2-dependent actin polymerization: a role for IRSp53 in mediating the interaction between Rac and WAVE2

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Summary

Wiskott-Aldrich syndrome protein (WASP)-family verprolin homologous (WAVE) proteins play a major role in Rac-induced actin dynamics, but Rac does not bind directly to WAVE proteins. It has been proposed that either the insulin receptor substrate protein 53 (IRSp53) or a complex of proteins containing Abelson interactor protein 1 (Abi1) mediates the interaction of WAVE2 and Rac. Depletion of endogenous IRSp53 by RNA-mediated interference (RNAi) in a RAW/LR5 macrophage cell line resulted in a significant reduction of Rac1Q61L-induced surface ruffles and colony-stimulating factor 1 (CSF-1)-induced actin polymerization, protrusion and cell migration. However, IRSp53 was not essential for Fcγ-R-mediated phagocytosis, formation of podosomes or for formation of Cdc42V12-induced filopodia. IRSp53 was found to be present in an immunoprecipitable complex with WAVE2 and Abi1 in a Rac1-activation-dependent manner in RAW/LR5 cells in vivo. Importantly, reduction of endogenous IRSp53 or expression of IRSp53 lacking the WAVE2-binding site (IRSp53ΔSH3) resulted in a significant reduction in the association of Rac1 with WAVE2 and Abi1, indicating that the

association of Rac1 with WAVE2 and Abi1 is IRSp53 dependent. While it has been proposed that WAVE2 activity is regulated by membrane recruitment, membrane targeting of WAVE2 in RAW/LR5 and Cos-7 cells did not induce actin polymerization or protrusion, suggesting that membrane recruitment was insufficient for regulation of WAVE2. Combined, these data suggest that IRSp53 links Rac1 to WAVE2 in vivo and its function is crucial for production of CSF-1-induced F-actin-rich protrusions and cell migration in macrophages. This study indicates that Rac1, along with IRSp53 and Abi1, is involved in a more complex and tight regulation of WAVE2 than one operating solely through membrane localization.

Supplementary material available online at
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Introduction

Cell migration is a fundamental process required for normal functioning of the immune system, embryonic development and tissue repair. Nevertheless, this process also contributes to the pathogenesis of several diseases, such as chronic inflammatory disease and tumor cell invasion (Raftopoulou and Hall, 2004; Ridley et al., 2003). Macrophages and other leukocytes have been extensively used as a model system to study actin-based motility in response to various chemotactic factors. Macrophages represent a key component of the immune system through their ability to phagocytose foreign material and dying cells, release cytokines and function as antigen-presenting cells. Recruitment of macrophages at specific sites is therefore an important process in order to achieve a localized and efficient response and is dependent on their ability to undergo chemotaxis towards a variety of secreted molecules. Colony-stimulating factor 1 (CSF1, also known as macrophage CSF and hereafter referred to as CSF-1), is a major chemotactic factor for macrophages and is also essential for their differentiation,

survival and proliferation (Pixley and Stanley, 2004). However, expression of CSF-1 has also been correlated with the progression of several disease states. Enhanced production of CSF-1 and the associated macrophage recruitment have been shown to promote the progression of diseases such as rheumatoid arthritis (Bischof et al., 2000; Campbell et al., 2000), atherosclerosis (Rajavashisth et al., 1998; Smith et al., 1995) and breast cancer (Aharinejad et al., 2004; Lin et al., 2001). Treatment of macrophages with CSF-1 induces a massive reorganization of the actin cytoskeleton, leading to the formation of surface ruffles, followed by cell migration towards the source of CSF-1 (Allen et al., 1997; Boock et al., 1989; Webb et al., 1996).

The small Rho GTPases Rho, Rac and Cdc42 play an important role in transducing CSF-1 signals to the actin cytoskeleton and regulate the formation of different actin-filament-based structures, assembly of adhesion structures and cell migration (Allen et al., 1997; Allen et al., 1998; Cox et al., 1997). Members of the Wiskott-Aldrich syndrome protein (WAS,

hereafter referred to as WASP) family of proteins mediate the effects of the Rho GTPases on the actin cytoskeleton and stimulate de novo actin nucleation by the actin-related protein 2/3 (Arp2/3) complex, creating branched actin filaments and resulting in the formation of lamellipodia and cell motility (Millard et al., 2004; Stradal et al., 2004; Takenawa and Miki, 2001). Five WASP family proteins have been identified in mammalian cells: WASP, neural N-WASP and WASP-family verprolin homologous (WAVE or verprolin-homology-domain-containing protein) 1, 2 and 3 (Suetsugu et al., 1999; Takenawa and Miki, 2001). The common feature of these proteins is the presence at their C-terminal end of a verprolin-homology cofilin-homology acidic (VCA) module, which is necessary and sufficient to interact with the Arp2/3 complex and to stimulate actin nucleation and polymerization (Millard et al., 2004; Stradal et al., 2004; Takenawa and Miki, 2001). Adjacent to the VCA domain, a proline-rich region represents a binding site for proteins containing Src-homology 3 (SH3) domains and is also shared by all WASP family proteins. The N-termini are more divergent, with WASP and N-WASP bearing a WASP homology domain (WH1), followed by a short basic region and a GTPase-binding domain (GBD), whereas WAVE proteins possess their own N-terminal WAVE homology domain (WHD) immediately followed by a stretch of basic residues (Millard et al., 2004; Stradal et al., 2004; Takenawa and Miki, 2001). WAVE2 (also known as WASF2) and WASP, the only members of the WASP family to be predominantly expressed in macrophages, have been demonstrated to play a role in CSF-1-mediated reorganization of the actin cytoskeleton and cell migration (Abou Kheir et al., 2005; Jones et al., 2002; Zicha et al., 1998). While WASP has a GTPase-binding domain capable of directly binding to GTP-loaded Cdc42, WAVE2 has no domain comparable to the GBD and, therefore, lacks the ability to bind directly to small Rho GTPases, indicating the existence of distinct modes of regulation for WASP and WAVE2 proteins (Takenawa and Miki, 2001). WAVE2 activates the Arp2/3 complex downstream of activated Rac1 (Suetsugu et al., 2003), and several proteins have been suggested to mediate the interaction between Rac1 and WAVE2.

Recently, WAVE2 has been shown to exist in a constitutive multiprotein complex in various organisms, including mammalian cells, *Dictyostelium discoideum* and *Drosophila melanogaster*. Several reports noted that Abelson interactor protein 1 (Abi1) is an essential element, connecting WAVE2 to a complex between Nck-associated protein 1 (Nap1) and p53-inducible protein 121 (PIR121) (Gautreau et al., 2004; Innocenti et al., 2004; Kunda et al., 2003; Rogers et al., 2003; Steffen et al., 2004). In vitro studies showed that GTP-loaded Rac1 binds to PIR121 and Nap1 but not to Abi1 or WAVE2 in the aforementioned complex (Innocenti et al., 2004). Moreover, it has been proposed that WAVE2 is regulated by membrane translocation upon binding of activated Rac1 to the Abi1 complex (Innocenti et al., 2004; Stradal et al., 2004). Interestingly, Abi1 is involved in stabilizing WAVE2 protein, as functional removal of Abi1, or any member of the complex, resulted in decreased amounts of WAVE2 (Kunda et al., 2003; Rogers et al., 2003; Steffen et al., 2004). In macrophages, consistent with other results, WAVE2 binds to Abi1 in a constitutive fashion, and this is required for the stability and function of WAVE2 (Abou Kheir et al., 2005). The requirement for the Abi1 complex to stabilize WAVE2 protein complicates the study of the role of the Abi1 complex in mediating the Rac1-WAVE2 interaction.

The first protein described as a potential link between GTP-bound Rac1 and WAVE2 was the insulin receptor substrate protein 53 (BAIAP2, hereafter referred to as IRSp53), which was found to stimulate WAVE2-dependent activation of Arp2/3 in vitro (Miki et al., 2000). It was demonstrated that the SH3 domain of IRSp53 could bind to the proline-rich region of WAVE2, and the N-terminal Rac1-binding domain (RCB) of IRSp53 could bind to GTP-loaded Rac1 to mediate formation of lamellipodia and cell spreading in fibroblasts (Choi et al., 2005; Miki and Takenawa, 2002; Miki et al., 2000). By contrast, other groups showed that IRSp53 is downstream of Cdc42 rather than Rac1, where GTP-loaded Cdc42 binds to the Cdc42/Rac1-interactive binding (CRIB) motif of IRSp53, located between the RCB and SH3 domains, and mediates the formation of filopodia in fibroblasts and neuronal cells (Govind et al., 2001; Krugmann et al., 2001). Therefore, the relevance of the IRSp53-WAVE2 interaction in vivo remains unclear.

This study examines the role of IRSp53 in mediating the interaction of Rac1 with WAVE2 and the requirement for this interaction in macrophage function. The results provide evidence that IRSp53 exists in a complex with WAVE2 and Abi1 downstream of GTP-loaded Rac1 in vivo that is required for CSF-1-induced formation of protrusions and cell migration.

Results

Requirement for IRSp53 for CSF-1-induced cytoskeletal reorganization and cell migration

The presence of a transcript encoding IRSp53 in the murine monocyte/macrophage RAW/LR5 cell line and in primary murine macrophages was examined by reverse transcription PCR. Using primers specific for IRSp53, amplicons corresponding to mRNA were detected in both samples (supplementary material Fig. S1A). IRSp53 expression was evaluated by western blotting to determine whether the detected transcripts were indicative of protein expression. Using antibodies against IRSp53, a signal was detected in both RAW/LR5 cells and in murine bone-marrow-derived macrophages (BMMs) (supplementary material Fig. S1B). Moreover, we confirmed the presence of three other murine IRSp53 family members (splice variants), described previously (Miyahara et al., 2003), in macrophages (supplementary material Fig. S1C). Indirect immunofluorescence confocal microscopy was then used to examine the subcellular localization of IRSp53 in macrophages in the presence or absence of CSF-1 (Fig. 1). In resting cells (untreated), IRSp53 showed mainly a cytosolic staining in both BMM and RAW/LRS cells. Upon treatment with CSF-1, both BMM and RAW/LRS cells underwent a massive remodeling of their actin cytoskeleton, resulting in the formation of F-actin-rich membrane protrusions (ruffles). IRSp53 was localized in this compartment following CSF-1 addition, as shown by the significant colocalization with F-actin (Fig. 1, CSF-1). The presence of IRSp53 in CSF-1-elicited F-actin protrusions suggested a possible role for IRSp53 in the formation of these structures.

To investigate the possible involvement of IRSp53 in the formation of CSF-1 protrusions, IRSp53 expression was reduced using RNA-mediated interference (RNAi) and the effect of this on the reorganization of the actin cytoskeleton in response to CSF-1 was examined. Retroviral delivery of IRSp53-specific short-hairpin RNA (shRNA) in RAW/LR5 cells resulted in a 60–80% reduction of IRSp53 protein expression compared with mock-shRNA-treated cells, as determined by western blot analysis (Fig. 2A), as well as by immunofluorescence microscopy analysis (Fig.

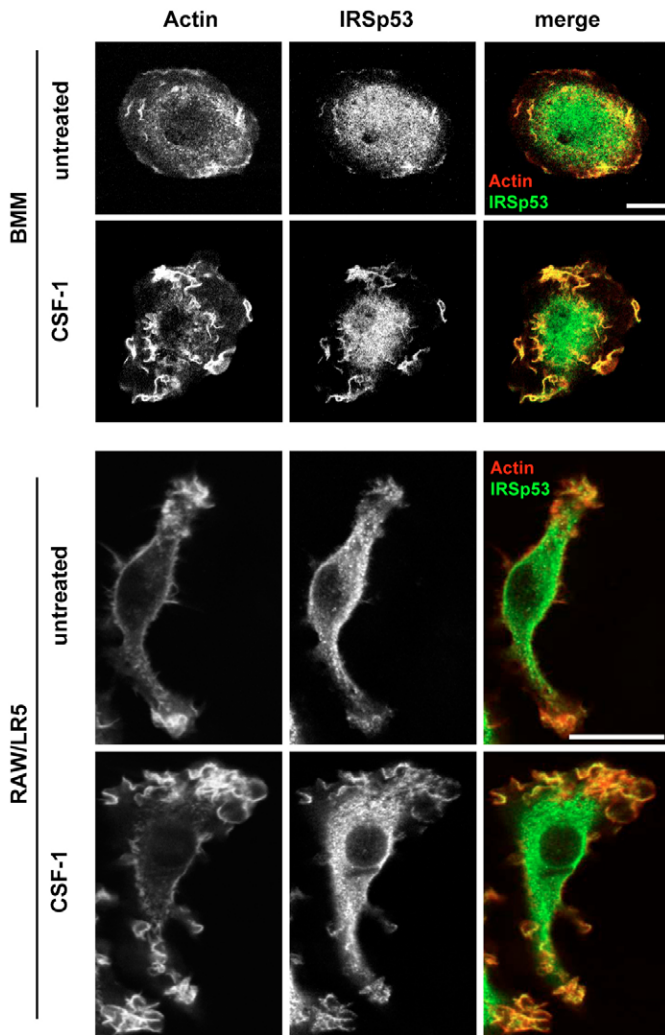


Fig. 1. The localization of IRSp53 in macrophages. BMM (top panel) or RAW/LR5 cells (bottom panel) were treated, or not, with 20 ng/ml CSF-1 for 5 minutes and then fixed and stained for F-actin using Alexa-568-phalloidin and for IRSp53 using a polyclonal goat antibody followed by Alexa-488-coupled anti-goat IgG. Bar, 10 μ m.

2B). Unlike the case in Abi1-shRNA-treated cells (Abou Kheir et al., 2005), WAVE2 protein levels were not altered in IRSp53-shRNA-treated RAW/LR5 cells (Fig. 2A). The ability of RAW/LR5 cells with reduced IRSp53 expression to exhibit F-actin rich membrane protrusions in response to CSF-1 was then evaluated and compared with that of mock-shRNA-treated cells. IRSp53-shRNA-treated cells showed a significant inhibition of CSF-1-induced F-actin-rich membrane protrusions when compared with mock-shRNA-treated cells, whereas the basal level of protrusion was not affected (Fig. 2C,D). To examine whether the defect in CSF-1-induced protrusions was due to alterations in actin polymerization, RAW/LR5 cells with reduced IRSp53 expression were analyzed with respect to actin polymerization in response to CSF-1. Quantitative measurement of the total F-actin cellular content showed a 35% increase of F-actin compared with that of unstimulated cells in mock-shRNA-treated cells, whereas the response to CSF-1 was significantly reduced in IRSp53-shRNA-treated cells (Fig. 2E). These data suggested that the defect

in protrusion in cells with reduced IRSp53 expression is due to reduced actin polymerization in response to CSF-1.

As cells with reduced IRSp53 levels showed a defect in the formation of CSF-1-induced protrusions and actin polymerization, it was then determined whether this defect was associated with a defect in the ability of the cells to migrate towards CSF-1. Cells were subjected to a transmigration chamber assay, as described in Materials and Methods. Consistent with previous results in RAW/LR5 cells with reduced levels of WAVE2 or Abi1 (Abou Kheir et al., 2005), mock-shRNA-treated cells showed an ~4.5-fold increase in chemotaxis in response to CSF-1, whereas IRSp53-shRNA-treated cells showed a significant reduction of ~60% in their ability to migrate towards CSF-1 (Fig. 2F and supplementary material Fig. S2). To determine whether IRSp53 was required for cell motility, a chemokinesis assay was performed. When CSF-1 was added to the upper and lower chambers, an ~2.5-fold increase in cell migration was observed in mock-shRNA-treated cells, however, the ability of IRSp53-shRNA-treated cells to migrate in response to CSF-1 was significantly reduced by ~50% (Fig. 2F and supplementary material Fig. S2). These experiments suggest that IRSp53-dependent actin polymerization is required for both chemotaxis and chemokinesis in response to CSF-1 in macrophages.

As actin polymerization in response to CSF-1 was altered in cells with reduced IRSp53 expression and this was consistent with a potential role for IRSp53 in interacting with WAVE2, the effect on different F-actin-based processes was investigated. Podosomes are prominent dot-like adhesion structures present in macrophages that are characterized by a core of F-actin surrounded by a complex of proteins such as vinculin and talin (Jones et al., 2002; Linder and Aeppelbacher, 2003). Consistent with the lack of localization of IRSp53, Abi1 and WAVE2 to podosomes (supplementary material Fig. S3A), the ability of IRSp53- or WAVE2-shRNA-treated cells to form podosomes (evident by F-actin and vinculin co-staining) was not affected compared with mock-shRNA-treated cells (supplementary material Fig. S3B-E). Therefore, IRSp53 and WAVE2 are not required for podosome formation in macrophages. In addition to podosomes, we determined the effect of RAW/LR5 cells possessing reduced IRSp53 on another F-actin-dependent process, namely Fc γ R-mediated phagocytosis (Cox and Greenberg, 2001). WASP-deficient macrophages or cells in which WASP function was inhibited by expression of a dominant-negative version of WASP showed reduced phagocytosis (Lorenzi et al., 2000), whereas RAW/LR5 cells with reduced levels of WAVE2 did not show a defect in phagocytosis (Abou Kheir et al., 2005). Consistent with RAW/LR5 cells with reduced WAVE2 levels, reducing the levels of Abi1 or IRSp53 had no significant effect on the ability of cells to ingest particles when compared with control cells (supplementary material Fig. S3F). Therefore, while IRSp53, WAVE2 and Abi1 are required for CSF-1-dependent actin remodeling, they are not required for all F-actin-mediated processes.

IRSp53 is involved in formation of Rac1-mediated ruffles but not in Cdc42-mediated formation of filopodia in macrophages. Whether IRSp53 interacts with Rac1 or Cdc42 remains controversial owing to the fact that IRSp53 has been shown to be a downstream effector for both GTP-bound Rac1 (Choi et al., 2005; Miki et al., 2000) and GTP-bound Cdc42 (Krugmann et al., 2001). To investigate whether IRSp53 acts functionally as a downstream effector for Rac1 or Cdc42 in macrophages, mock- or IRSp53-

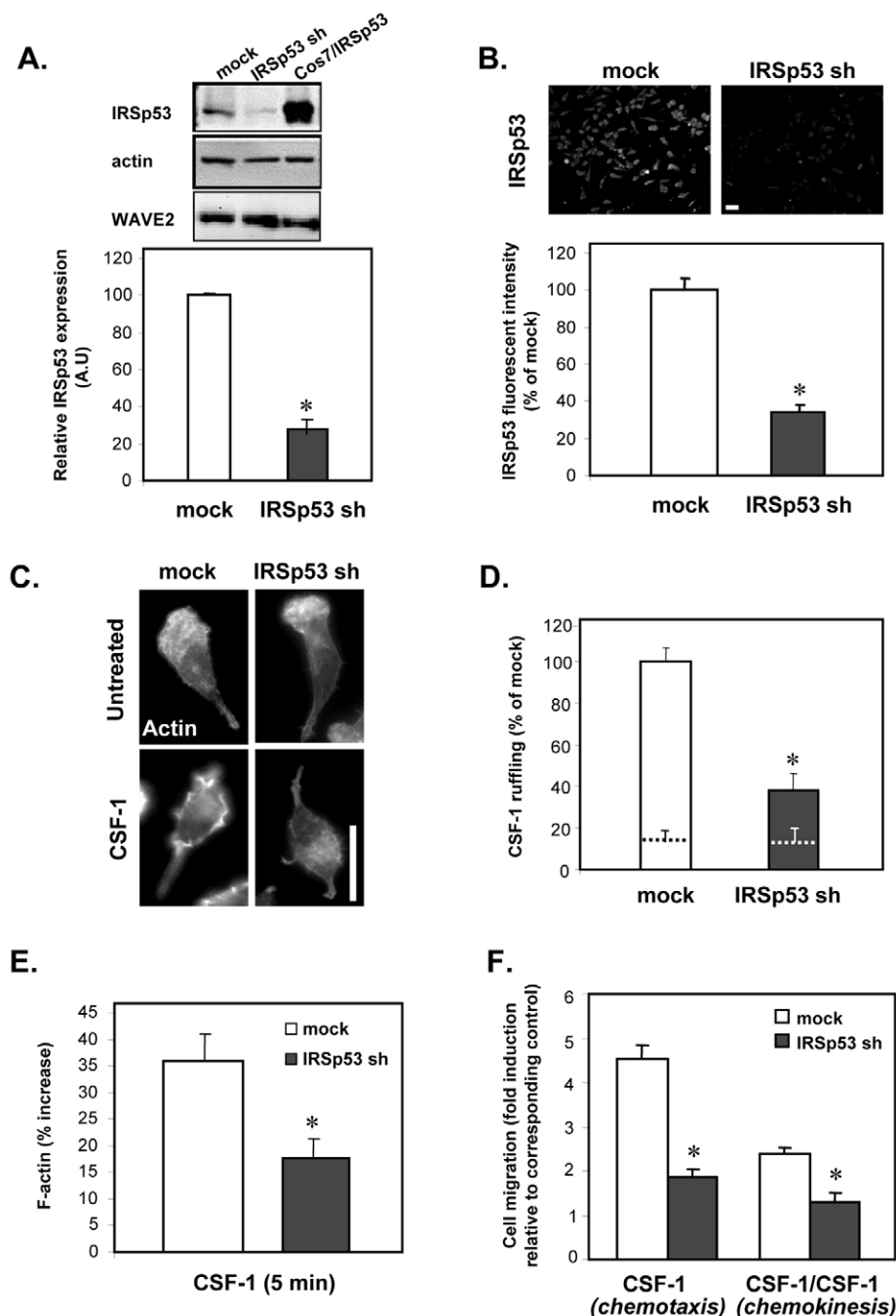


Fig. 2. IRSp53 is required for generation of CSF-1-induced F-actin rich membrane protrusions and cell migration. (A) The level of IRSp53 and WAVE2 present in IRSp53-shRNA-treated (IRSp53 sh) RAW/LR5 cells was analyzed by western blotting with the indicated antibodies and compared with mock-shRNA-treated cells. A representative blot and quantification of IRSp53:β-actin signal-intensity ratios are shown; $n=3$; *, $P<0.05$ compared with mock-shRNA-treated cells. (B) The level of IRSp53 present in IRSp53-shRNA-treated (IRSp53 sh) RAW/LR5 cells was analyzed by immunofluorescence with the indicated antibodies and compared with mock-shRNA-treated cells. Representative images and quantification of the IRSp53 signal intensity are shown; $n=3$; *, $P<0.05$ compared with mock-shRNA-treated cells. (C) Mock or IRSp53-shRNA-treated RAW/LR5 cells were treated with, or without, CSF-1 for 5 minutes, and F-actin-rich protrusions were visualized by Alexa-568-phalloidin staining. Bar, 10 μm . (D) The number of CSF-1-elicited protrusions in mock- (white bar) or IRSp53-shRNA-treated RAW/LR5 cells from C was quantified and expressed as a percentage of the CSF-1 stimulation observed in mock-shRNA-treated cells; $n=3$; *, $P<0.05$ compared with mock-shRNA-treated cells. The dotted line represents basal ruffling. (E) Mock- (white bar) or IRSp53-shRNA-treated (gray bar) RAW/LR5 cells were fixed after treatment with, or without, CSF-1, and the total F-actin content, normalized to the cell number, was quantitatively measured as described in Materials and Methods and compared with unstimulated control cells; $n=3$; *, $P<0.05$ compared with mock-shRNA-treated cells. (F) Chemotaxis and chemokinesis in response to CSF-1 in mock- (white bars) or IRSp53-shRNA-treated (gray bars) RAW/LR5 cells were evaluated using a transmigration chamber assay as described in Materials and Methods. The CSF-1-stimulated migration of each cell population was compared with the corresponding unstimulated condition and expressed as a fold induction. $n=3$; *, $P<0.05$ compared with mock-shRNA-treated cells.

shRNA-treated RAW/LR5 cells were transfected with either constitutively active Rac1Q61L or constitutively active Cdc42V12 constructs (Fig. 3A). Cells with reduced IRSp53 expression showed a significant reduction in Rac1Q61L-induced surface ruffling compared with mock-shRNA-treated cells (Fig. 3B), whereas formation of Cdc42V12-induced filopodia was unaffected (Fig. 3C). These data indicate that IRSp53 is a downstream effector for Rac1 and not Cdc42 in macrophages. These results were also confirmed biochemically using RAW/LR5 cells co-transfected with GFP-tagged IRSp53 and Myc-tagged Rac1Q61L or Cdc42V12. Immunoprecipitation experiments using antibodies against either Myc or GFP showed that activated Rac1, but not activated Cdc42, associates with IRSp53 in macrophages (Fig.

4A). These data indicate that IRSp53 interacts functionally with Rac1, and not Cdc42, in macrophages. By contrast, and consistent with a recent report (Disanza et al., 2006), immunoprecipitation experiments using antibodies against Myc in Cos-7 cells coexpressing Myc-tagged Cdc42V12 and GFP-tagged IRSp53 showed that IRSp53 interacts with Cdc42 in those cells (Fig. 4A), suggesting that the interaction between IRSp53 and Rac1 or Cdc42 is cell-type dependent.

IRSp53 interaction with Rac and WAVE2

As it has been proposed that IRSp53 mediates the interaction between Rac1 and WAVE2, and, as we showed that Rac1 interacts with IRSp53 (Fig. 3), and both WAVE2 and Abi1 exist as a stable

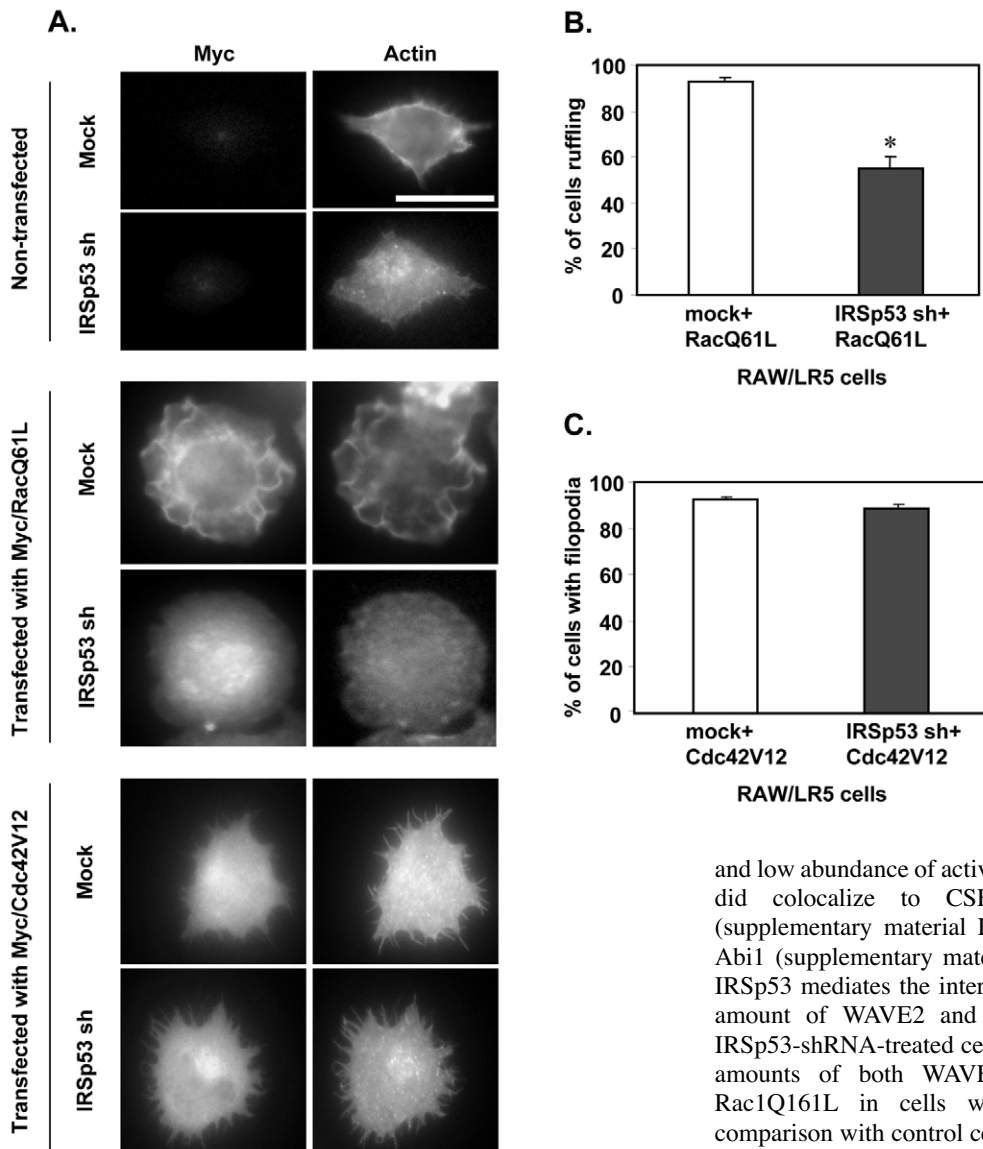


Fig. 3. IRSp53 is involved in Rac1-mediated ruffling but not Cdc42-mediated formation of filopodia in macrophages. (A) Representative images of F-actin-stained mock- or IRSp53-shRNA-treated nontransfected (top panel), or transfected to express either Myc/Rac1Q61L (middle panel) or Myc/Cdc42V12 (bottom panel), RAW/LR5 cells are shown where cells were stained for Myc to identify expressing cells. Bar, 10 μ m. The ability of mock- (white bars) or IRSp53-shRNA-treated (gray bars) cells to exhibit either ruffles following Rac1Q61L expression (B) or filopodia following Cdc42V12 expression (C) was quantified and expressed as a percentage of the total cells counted; $n=3$; *, $P<0.05$ compared with mock-shRNA-treated cells. Bar, 10 μ m.

unit in macrophages (Abou Kheir et al., 2005), these facts suggested that Rac1, IRSp53, WAVE2 and Abi1 might exist in the same complex in macrophages. To determine whether this was true, a biochemical approach was applied using RAW/LR5 cells co-transfected with GFP-tagged IRSp53 and Myc-tagged Rac1, Rac1N17, Rac1Q61L or Cdc42V12 constructs followed by immunoprecipitation using antibodies against either GFP or Myc. An association of IRSp53 with WAVE2 and Abi1 occurred only in the presence of activated Rac1, and not activated Cdc42 (Fig. 4A). No signal for IRSp53, WAVE2 and Abi1 was detected in the immunoprecipitates when either Myc-tagged wild-type Rac1 or Rac1N17 was transfected (Fig. 4A), indicating that IRSp53 exists in a complex with WAVE2 and Abi1 in a Rac1-activation-dependent manner. Furthermore, WAVE2 colocalized with GFP-tagged IRSp53 and Myc-tagged Rac1Q61L in RAW/LR5 cells (supplementary material Fig. S5A). Consistent with a report by Suetsugu and colleagues (Suetsugu et al., 2006a), we were unable to detect the interaction between the endogenous Rac, IRSp53 and WAVE2 or between overexpressed Rac and GFP-IRSp53 following CSF-1 stimulation, presumably owing to the transience

and low abundance of activated Rac1. However, IRSp53 and Rac1 did colocalize to CSF-1-induced protrusions in BMMs (supplementary material Fig. S4), as did IRSp53, WAVE2 and Abi1 (supplementary material Fig. S5B). To determine whether IRSp53 mediates the interaction between Rac1 and WAVE2, the amount of WAVE2 and Abi1 associated with Rac1Q61L in IRSp53-shRNA-treated cells was analyzed. Significantly reduced amounts of both WAVE2 and Abi1 were associated with Rac1Q61L in cells with reduced IRSp53 expression in comparison with control cells (Fig. 4B,C), indicating that IRSp53 mediates the interaction between Rac1 and WAVE2 in macrophages.

Previously, it has been shown that IRSp53 binds to WAVE2 through its SH3 domain in vitro (Miki and Takenawa, 2002; Miki et al., 2000). To determine whether the SH3 domain of IRSp53 is required for its interaction with WAVE2 in vivo, immunoprecipitation experiments were performed on RAW/LR5 cells coexpressing Myc-tagged Rac1Q61L and hemagglutinin (HA)-tagged IRSp53 Δ SH3 using an antibody against HA. As expected, Rac1Q61L associated with IRSp53 Δ SH3, similar to the results seen using GFP-IRSp53 in Fig. 4A (Fig. 4D). However, no WAVE2 or Abi1 signal was detected when HA-IRSp53 Δ SH3 was immunoprecipitated (Fig. 4D). These data suggested that IRSp53 lacking the SH3 domain lost its ability to bind to WAVE2 but not to activated Rac1. Furthermore, when Myc-tagged Rac1Q61L was sequentially immunoprecipitated from the same lysates (Fig. 4D sequential IP-Myc), no signal was detected for WAVE2, Abi1 or IRSp53, indicating that IRSp53 mediates the interaction of Rac1 with the stable WAVE2-Abi1 unit. To investigate the effect of IRSp53 Δ SH3 on the ability of cells to extend F-actin protrusions, the average protrusion index of cells in response to CSF-1 was scored, as described previously, in mock- and IRSp53-shRNA-

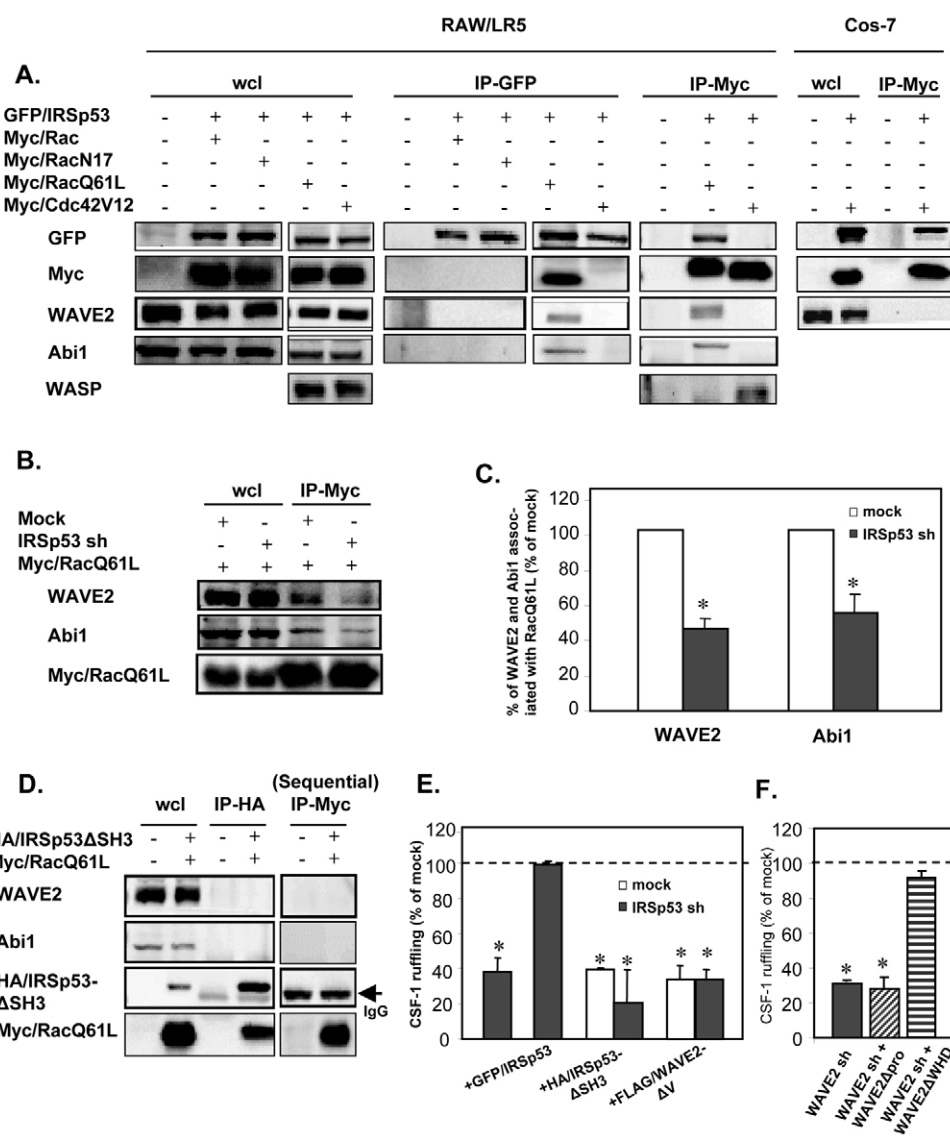
treated RAW/LR5 cells. While expression of human HA-IRSp53 Δ SH3 protein resulted in a significant reduction in CSF-1-induced F-actin-rich protrusions in both mock- and IRSp53-shRNA-treated RAW/LR5 cells, expression of human GFP-IRSp53 wild-type protein fully rescued the ability of cells with reduced IRSp53 expression to extend protrusions in response to CSF-1 (Fig. 4E). In addition, expression of a dominant-negative WAVE2 construct, FLAG-WAVE2 Δ V (Abou Kheir et al., 2005), had no additional effect on the inhibition of CSF-1-induced ruffling of IRSp53-shRNA-treated RAW/LR5 cells (Fig. 4E), suggesting that the effect of IRSp53 reduction on CSF-1 protrusions is mediated through its interaction with WAVE2. Consistent with the lack of association of Rac1 with the Abi1 complex, and with a role for IRSp53 in mediating the interaction between Rac1 and WAVE2 in macrophages, the expression of GFP-tagged WAVE2 Δ proline (lacking the IRSp53 binding site) was not able to rescue the ability of WAVE2-shRNA-treated cells to extend protrusions in response to CSF-1 (Fig. 4F). By contrast, expression of GFP-tagged WAVE2 Δ WHD (lacking the Abi1 binding site) almost fully rescued the ability of cells with reduced WAVE2 expression to extend CSF-1-induced protrusions, indicating a minor role for the

Abi1 complex in mediating Rac1 signaling to WAVE2 in macrophages (Fig. 4F).

Membrane translocation is not sufficient for WAVE2-dependent actin polymerization

It has been proposed that WAVE2 activity is regulated simply by its translocation to the membrane upon binding of activated Rac1 to WAVE2 (Innocenti et al., 2004; Stradal et al., 2004). To test this hypothesis, RAW/LR5 cells and Cos-7 cells were transfected with FLAG-tagged WAVE2 (WAVE2) or a membrane-targeted FLAG-tagged WAVE2 construct (WAVE2CAAX). When examined by indirect immunofluorescence confocal microscopy, WAVE2CAAX was found to localize to the membrane, whereas WAVE2 was localized diffusely (Fig. 5A). The presence of WAVE2CAAX in the membrane was also examined by subcellular fractionation. A significant increase of $\sim 2.0 \pm 0.1$ fold of FLAG-tagged WAVE2CAAX compared with FLAG-tagged WAVE2 was detected in the membrane fraction. Both methods confirmed that WAVE2CAAX was successfully targeted to the membrane. Interestingly, targeting of WAVE2 to the membrane did not induce F-actin protrusions (Fig. 5A), suggesting that membrane targeting

Fig. 4. IRSp53 exists in a complex with WAVE2 and Abi1 in a Rac1-dependent manner. (A) Lysates from nontransfected or GFP-tagged IRSp53 and Myc-tagged Rac1- or Rac1N17- or Rac1Q61L- or Cdc42V12-coexpressing RAW/LR5 cells and Cos7 cells were immunoprecipitated with antibodies against either GFP or Myc (IP) and subjected to western blotting using the indicated antibodies. A representative example of three independent experiments is shown. (B) Lysates from mock- or IRSp53-shRNA-treated RAW/LR5 cells expressing Myc-tagged Rac1Q61L were immunoprecipitated with antibodies against Myc (IP) and were then subjected to western blotting using the indicated antibodies. (C) Quantification of WAVE2 or Abi1 co-immunoprecipitated by Myc (from B) is shown; $n=3$; *, $P<0.05$ compared with mock-shRNA-treated cells expressing Rac1Q61L. (D) Lysates from nontransfected or Myc-tagged Rac1Q61L- and HA-tagged IRSp53 Δ SH3-coexpressing RAW/LR5 cells were incubated with antibodies against HA for immunoprecipitation (IP-HA), followed by sequential immunoprecipitation of Rac1Q61L using antibodies against Myc (IP-Myc). Immunoprecipitates were then subjected to western blotting using the indicated antibodies. Mock- or IRSp53-shRNA-treated (E) or WAVE2-shRNA-treated (F) RAW/LR5 cells either transiently transfected with the indicated constructs, or not, were stimulated with CSF-1 and their ability to form F-actin-rich protrusions in response to CSF-1 was analyzed as in Fig. 2D and expressed as a percentage of the CSF-1 stimulation observed in nontransfected mock-shRNA-treated cells; $n=3$; *, $P<0.05$ compared with nontransfected mock-shRNA-treated cells (represented by the dotted line).



did not induce actin polymerization. To test this, RAW/LR5 cells were transfected with either FLAG-tagged WAVE2 or WAVE2CAAX constructs and then stained for F-actin and FLAG, and the amount of F-actin was quantitated as described in Materials and Methods. No significant increase in total F-actin was detected in cells transfected with WAVE2CAAX in comparison with nontransfected cells or with cells transfected with WAVE2 (Fig. 5B). To exclude the possibility that WAVE2CAAX might be acting as a dominant negative, the ability of RAW/LR5 cells expressing FLAG-tagged WAVE2CAAX to exhibit F-actin protrusions in response to CSF-1 was evaluated and compared with non-expressing cells. Cells expressing WAVE2CAAX showed no significant inhibition or enhancement of CSF-1-induced F-actin protrusion in comparison with non-transfected cells (Fig. 5C, white bars). Moreover, basal ruffling in WAVE2CAAX-expressing cells was not affected in comparison with nontransfected cells (Fig. 5C, gray bars). Furthermore, expression of FLAG-tagged WAVE2CAAX did not inhibit Rac1Q61L-induced ruffles in either RAW/LR5 or Cos-7 cells (Fig. 5D). This indicated that

WAVE2CAAX was not acting as a dominant negative or as a constitutively active form of WAVE2 in cells. However, expressed WAVE2CAAX was functional as it was capable of rescuing the protrusion defect found in cells in which the levels of endogenous WAVE2 were reduced by RNAi (Fig. 5E). Together, these data demonstrate that membrane translocation of WAVE2 is not sufficient to activate WAVE2 *in vivo*.

Overall, our data identify a role for IRSp53 in linking Rac1 to the stable WAVE2-Abi1 unit that is required for cell migration and formation of protrusions but not for phagocytosis and formation of filopodia and podosomes in macrophages. Our results also suggest that the mechanism of WAVE2 activation by Rac through IRSp53 is complex and that membrane recruitment alone is insufficient for WAVE2-dependent actin polymerization.

Discussion

WAVE2 is an essential regulator of the actin cytoskeleton through its ability to stimulate Arp2/3-dependent actin polymerization downstream of activated Rac1 in different cell types (Suetsugu et al., 2003; Takenawa and Miki, 2001). The embryonic lethality in mice in which the gene encoding WAVE2 has been knocked out is associated with deficient lamellipodia and surface ruffling in cells and with impaired cell migration of embryonic fibroblasts and of endothelial cells lacking WAVE expression – this highlights the importance of WAVE-mediated actin polymerization (Yamazaki et al., 2003). Moreover, in a previous report, WAVE2 was reported to be important in CSF-1-induced actin reorganization and cell migration in macrophages (Abou Kheir et al.,

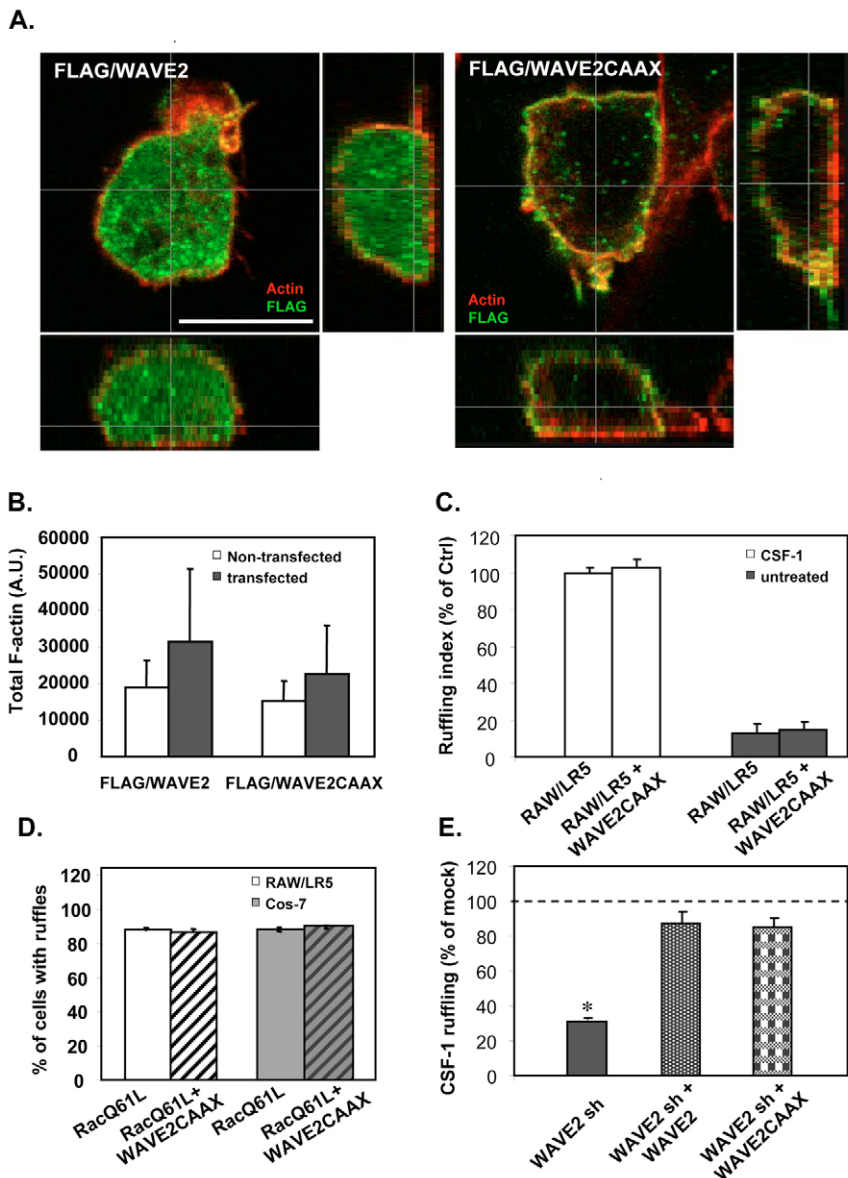


Fig. 5. Membrane targeting of WAVE2 is not sufficient for its regulation. (A) Confocal images of RAW/LR5 cells expressing either FLAG-tagged WAVE2 (left) or WAVE2CAAX (right) are shown with single XY, XZ and YZ cross-sections through different areas of the cell, as indicated by the cross-hair lines. Bar, 10 μm. (B) RAW/LR5 cells expressing either FLAG-tagged WAVE2 or WAVE2CAAX were fixed and stained for FLAG and F-actin and the total F-actin content was quantified as described in Materials and Methods and compared with nontransfected cells; $n=3$. (C) FLAG-tagged WAVE2CAAX-expressing RAW/LR5 cells were treated, or not, with CSF-1 for 5 minutes. The number of CSF-1-elicited protrusions was quantified as in Fig. 2D and expressed as a percentage of the CSF-1 stimulation observed in nontransfected cells on the same coverslip; $n=3$. (D) RAW/LR5 (white bars) and Cos-7 (gray bars) cells were either transfected with Myc-tagged Rac1Q61L (plain) or RacQ61L and FLAG-tagged WAVE2CAAX (striped) constructs. Coexpressing cells were identified by the Myc and FLAG staining, and the ability of cells to exhibit ruffles was quantified and expressed as a percentage of the total cells counted; $n=3$. (E) Mock- or WAVE2-shRNA-treated RAW/LR5 cells either transiently transfected with the indicated constructs, or not, were stimulated with CSF-1 and their ability to form F-actin-rich protrusions in response to CSF-1 was analyzed as in Fig. 2D and expressed as a percentage of the CSF-1 stimulation observed in nontransfected mock-shRNA-treated cells; $n=3$; *, $P<0.05$ compared with nontransfected mock-shRNA-treated cells (represented by the dotted line).

2005). Although WAVE2 has a well-documented function in cell migration, regulation of WAVE2 has proven to be very complicated as Rac1 does not bind directly to WAVE2, as is the case between Cdc42 and WASP/N-WASP proteins. Based mostly on in vitro studies, IRSp53 was reported to be an important candidate linking WAVE2 to Rac1 (Miki and Takenawa, 2002; Miki et al., 2000; Oda et al., 2005; Suetsugu et al., 2006a). However, whether IRSp53 functions as an essential intermediate between Rac1 and WAVE2 in vivo was not established.

In this study, IRSp53 was identified as an essential intermediate that links Rac1 to WAVE2 to mediate reorganization of the actin cytoskeleton and cell migration in macrophages. Consistent with WAVE2 being important in chemotaxis (Abou Kheir et al., 2005), inhibition of the CSF-1-induced rearrangement of the actin cytoskeleton in cells with reduced IRSp53 expression also resulted in a reduced ability of the cells to migrate towards CSF-1. Together, these data identified IRSp53 as an essential mediator of macrophage motility in response to CSF-1.

The small Rho GTPases Rac1 and Cdc42 act as regulators of actin reorganization and mediate formation of lamellipodia and filopodia, respectively, in different cell types (Aspenstrom et al., 1996). IRSp53 can bind to both Rac1 and Cdc42. The N-terminal RCB domain of IRSp53 binds to activated GTP-Rac1 and mediates the formation of lamellipodia in fibroblasts (Miki et al., 2000), whereas the CRIB motif of IRSp53 binds to activated GTP-Cdc42 and mediates formation of microspikes and filopodia in fibroblasts and neuronal cells (Govind et al., 2001; Krugmann et al., 2001). This study reports that, in macrophages, IRSp53 binds to GTP-Rac1 but not GTP-Cdc42 in vivo. Moreover, IRSp53 is involved in Rac1-mediated surface ruffling, which is consistent with recent findings by Suetsugu and colleagues in human carcinoma A431 cells (Suetsugu et al., 2006a). Consistent with a role for IRSp53 in mediating Rac1 and WAVE2 interaction and with the lack of interaction with Cdc42 in macrophages, formation of Cdc42-mediated filopodia was not affected in cells with reduced amounts of IRSp53. Notably, while others have reported that overexpression of IRSp53 induces formation of filopodia, this was not the case in macrophages (data not shown). Our results do not invalidate the recent findings that IRSp53, along with Cdc42 and Eps8, are required for formation of filopodia in Cos-7 and HeLa cells (Disanza et al., 2006), but instead they suggest that there might be some cell-type specificity that regulates whether IRSp53 interacts with either Rac1 or Cdc42. Connolly and colleagues have demonstrated that Tiam1 can mediate the interaction between IRSp53 and Rac1 by enhancing IRSp53 binding to both active Rac1 and the WAVE2 scaffold. Moreover, they show that Tiam1 promotes the localization of IRSp53 to Rac1-induced lamellipodia rather than Cdc42-induced filopodia (Connolly et al., 2005). Tiam1 is expressed in macrophages and was reported to play a role in Rac1-dependent events (Abell et al., 2004; Mizrahi et al., 2005). Therefore, these results might explain the differences observed in the interaction of IRSp53 with either Rac1 or Cdc42.

It is important to note that IRSp53 and the WAVE2-Abi1 unit are required for certain actin-mediated processes such as surface ruffling and cell migration (Fig. 2C,D,F) but not for others such as FcγR-mediated phagocytosis and formation of filopodia and podosomes (supplementary material Fig. S3), suggesting a specific and complex regulation of actin-dependent events in macrophages. The fact that none of the aforementioned proteins was found to localize at podosomes and to play a role in their formation is consistent with a unique role of WASP in those structures (Jones

et al., 2002; Linder and Aepfelbacher, 2003). Moreover, WAVE2, Abi1 and IRSp53 had no role in the actin-dependent phagocytosis of IgG-opsonized erythrocytes in macrophages (this study) (Abou Kheir et al., 2005). However, the lack of a role for these proteins in phagocytosis is perplexing as it has been shown that Rac1 is required for FcγR-mediated phagocytosis (Caron and Hall, 1998; Cox et al., 1997; Yamauchi et al., 2004). These results suggest that Rac1-dependent actin polymerization downstream of the Fcγ receptor is independent of WAVE2 and thereby must be mediated through a different mechanism. This alternative pathway of Rac-dependent actin polymerization might explain the lack of complete inhibition that is observed following inhibition of WAVE2 activity by multiple mechanisms (Abou Kheir et al., 2005). Further work is required to uncover the mechanism of this WAVE2-independent, yet Rac1-dependent, actin polymerization.

As mentioned previously, WAVE proteins differ from WASP and N-WASP by lacking the GTPase-binding domain that mediates the direct interaction with Rac1 (Takenawa and Miki, 2001). Although regulation of WASP appears to be mediated by an auto-inhibitory state that is relieved by Cdc42 binding and tyrosine phosphorylation in response to stimuli (Cory et al., 2002; Prehoda et al., 2000), the mechanism of regulation of WAVE is still under debate, and understanding how WAVE proteins can relay signals from activated Rac1 to the actin cytoskeleton represents a current area of active investigation. Recently, many groups showed that WAVE1/WAVE2 exists in a multiprotein complex that contains Nap1, PIR121/Sra1, Hspc300 and Abi1, in which Abi1 comprises the core of the complex (Eden et al., 2002; Gautreau et al., 2004; Innocenti et al., 2004; Steffen et al., 2004). Although the architecture of the WAVE complex was the same, contrasting models have been proposed regarding its regulation. Initial reports suggested that WAVE1 would be kept inactive in this multiprotein complex and that Rac1 binding to PIR121 might trigger a conformational change that induces the dissociation of the complex and the release of active WAVE1 (Eden et al., 2002). Subsequently, later reports suggested that the WAVE2 multiprotein complex remained intact when bound to Rac1 and became relocalized to active sites of actin assembly within the cell (Innocenti et al., 2004; Steffen et al., 2004). However, the fact that reducing the amount of any protein in the Abi1 complex alters the endogenous levels of WAVE2 makes it difficult to study the role of the Abi1 complex in mediating the interaction between Rac1 and WAVE2 in vivo. Shi and colleagues showed that WAVE2 was necessary for efficient invasion of epithelial cells (MDCK) by *Salmonella*. Although this entry promotes the formation of an IRSp53-WAVE2 complex, it is the Abi1-WAVE2 complex and not the IRSp53 that was required for the internalization of *Salmonella* (Shi et al., 2005). However, more recently, Suetsugu and colleagues suggested that IRSp53, along with Rac1 and phosphatidylinositol (3,4,5)-trisphosphate, is required for the optimization of WAVE2-dependent actin assembly (Suetsugu et al., 2006a). In macrophages, WAVE2 and Abi1 exist constitutively in the same molecular complex, where Abi1 is required for the stability and function of WAVE2 downstream of CSF-1 (Abou Kheir et al., 2005). While we cannot completely eliminate a role for the Abi1 complex in cooperating in the interaction between Rac1 and WAVE2, this report presents evidence that supports the role for IRSp53 as the major factor linking Rac to WAVE2. We demonstrate that IRSp53 binds to the stable WAVE2-Abi1 subcomplex in a Rac1-dependent manner, and cells with reduced amounts of IRSp53 were indistinguishable from cells with reduced amounts of WAVE2 or Abi1 in terms of their

ability to form F-actin-rich surface ruffles and to migrate in response to CSF-1. Furthermore, transient expression of dominant-negative WAVE2 protein (WAVE2 Δ V) in cells with reduced amounts of IRSp53 did not have an additive effect on the ability of cells to ruffle in response to CSF-1 (Fig. 4E). This observation, along with the observation that the level of WAVE2 and Abi1 associated with Rac1Q61L in IRSp53-shRNA-treated cells was significantly decreased and there was no interaction between Rac1 and WAVE2 when the WAVE2-binding site of IRSp53 was deleted (Fig. 4B,C,D), suggested that Rac1-IRSp53-WAVE2-Abi1 form a single complex and act along the same pathway to activate Arp2/3-mediated assembly of actin filaments. Presumably, this macromolecular complex contains PIR121 and Nap1, as shown by others (Innocenti et al., 2004; Steffan et al., 2004), and as indicated by Coomassie staining of immunoprecipitates from RAW/LR5 cell lysates coexpressing GFP-tagged IRSp53 and Myc-tagged Rac1Q61L using antibodies against GFP where two bands corresponding to the molecular masses of PIR121 and Nap1 were detected (supplementary material Fig. S5C). The fact that WAVE2 Δ WHD, but not WAVE2 Δ proline, fully rescued the CSF-1 ruffling ability of WAVE2-shRNA-treated cells indicates that IRSp53, but not the Abi1 complex (along with PIR121 and Nap1), plays a major role in mediating the interaction between Rac1 and WAVE2 in macrophages *in vivo*.

As WAVE2 has been shown to be constitutively active in a purified state, the regulation of WAVE2-dependent actin polymerization *in vivo* has proven to be very difficult. One of the proposed models suggests that WAVE2 activity might be regulated simply by membrane translocation upon binding of Rac1 to the stable WAVE2 complex (Innocenti et al., 2004; Stradal et al.,

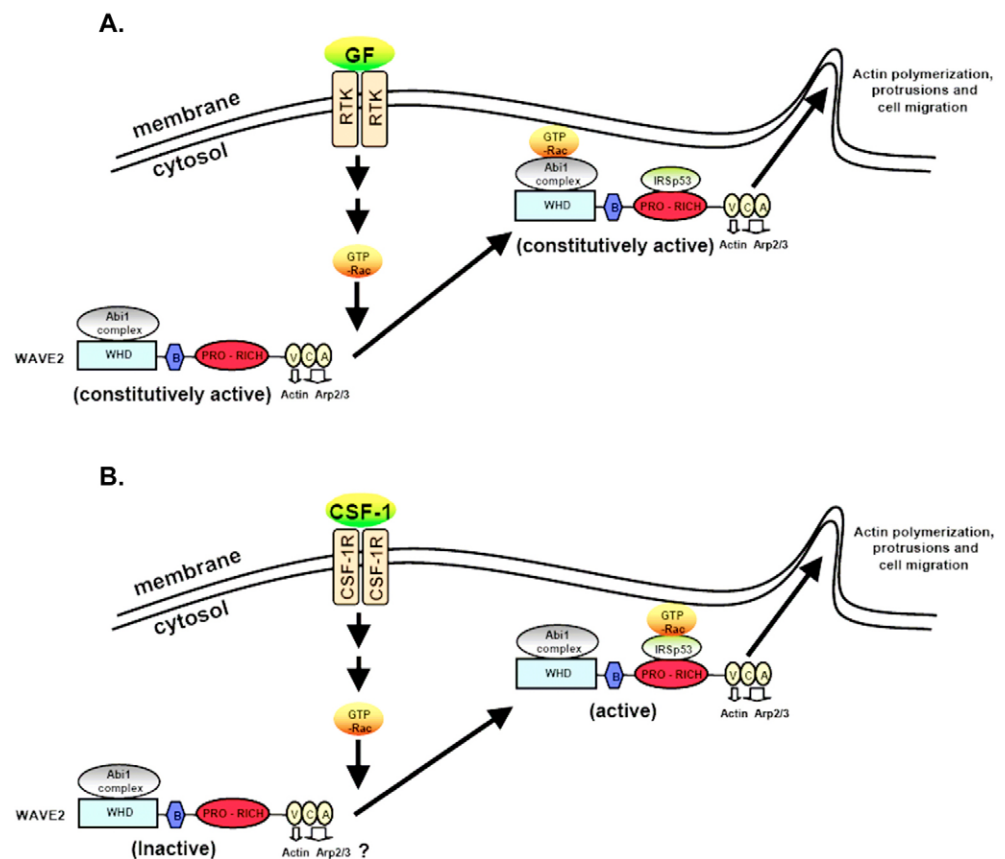
2004). Results from RAW/LR5 cells as well as from Cos-7 cells showed that membrane targeting of WAVE2 did not induce actin polymerization and protrusions (this report). This suggests that membrane recruitment by itself is insufficient and there are additional levels of WAVE2 regulation. One might envision a model whereby Rac1 binding to WAVE2 might induce a conformational change that would increase the affinity of Arp2/3 for the CA domain accompanied by membrane translocation that results in site-restricted actin polymerization. The dynamics of Arp2/3 binding to the CA domain of WAVE2 in quiescent or stimulated cells still needs to be examined. Furthermore, potential unidentified positive or negative regulators such as SH3-domain-containing or proline-rich proteins might play a role in regulating the activity of the WAVE2 complex.

In addition to its function in mediating its interaction with WAVE2, it might be possible that IRSp53 has additional functions that are required for the extension of actin-rich membrane protrusions. The Rac1-binding/IMD domain of IRSp53 shows homology to BAR domains but forms a 'zeppelin-shaped' dimer. When this domain is present in liposomes or expressed in cells, it induces outward membrane deformations in the direction opposite to that seen with BAR domains (Millard et al., 2005; Suetsugu et al., 2006b). These protrusions require activated Rac1 but can occur in the absence of actin polymerization. This outward membrane deformation might be a necessary component in the formation of an actin-rich protrusion. Therefore, IRSp53 might be required to initiate membrane protrusion as well as inducing the activation of WAVE2-dependent actin polymerization. Interestingly, the SH3 domain of IRSp53 has been shown to mediate the interaction with Eps8 and to enhance Eps8-Abi1-Sos-1 signaling to Rac1 (Funato et al., 2004).

Fig. 6. A model for WAVE2 interaction and regulation in macrophages.

(A) Based on several reports, a model for the regulation of WAVE2 has been proposed whereby the stable WAVE2-Abi1 complex is constitutively active in the cytoplasm *in vivo*. Upon receptor tyrosine kinase (RTK) stimulation by growth factor (GF), activated Rac1 binds to WAVE2, mediated by PIR121 in the Abi1 complex, and induces translocation of WAVE2 to the leading edge, where it stimulates the site-restricted actin polymerization necessary for cell protrusion and motility.

(B) Alternatively, the stable WAVE2-Abi1 complex is inactive in the cytoplasm *in vivo*. Upon stimulation of the CSF-1 receptor (CSF-1R) by CSF-1, activated Rac1 binds to WAVE2, mediated by IRSp53, and induces WAVE2 activation and translocation to the leading edge, where it stimulates the site-restricted actin polymerization required for cell protrusion and motility. Abbreviations: A, acidic region; B, basic region; C, cofilin-homology domain; Pro-rich, proline-rich region; V, verprolin-homology domain; WHD, WAVE-homology domain.



More recently, Yanagida-Asanuma and colleagues showed that synaptopodin, an actin-associated protein, binds directly to IRSp53 and suppresses Cdc42-IRSp53-Mena-initiated formation of filopodia by blocking the binding of Cdc42 and Mena to IRSp53 in kidney podocytes (Yanagida-Asanuma et al., 2007). This suggests that the interactions of IRSp53 alone are complex and warrant further investigation. It is worth mentioning that IMD proteins such as IRSp53 are not conserved in lower-eukaryotic organisms such as *Dictyostelium discoideum* and therefore the regulation of association between WAVE2, the Abi1 complex, Rac1 and Cdc42 is likely to be organism specific as well as cell-type dependent.

Posttranslational modifications such as phosphorylation have also been suggested to play a role in the regulation of WAVE2. Phosphorylation of WAVE2 by Abl kinase has been reported to play a role in WAVE2 regulation (Leng et al., 2005). However, no difference was detected in the phosphorylation status of WAVE2 upon CSF-1 stimulation of RAW/LR5 cells (data not shown), suggesting that phosphorylation of WAVE2 does not play a role in WAVE2 regulation in these cells. However, phosphorylation of WAVE2 might be involved in other cell types. This highlights how complex the regulation of WAVE proteins is and how it might vary between different experimental conditions and different cell types. Based on our *in vivo* results in macrophages, we propose that the stable WAVE2-Abi1 subcomplex exists in an inactive state in the cytoplasm. After extracellular stimulation (for example by CSF-1) and Rac1 binding to WAVE2, mediated by IRSp53, the now-active complex is recruited to the leading edge of the cell and induces the site-specific actin polymerization required for cell protrusion and motility (Fig. 6B).

In conclusion, our results suggest that IRSp53 is the major mediator that links Rac1 to WAVE2 *in vivo* and its function is crucial for the formation of CSF-1-induced F-actin-rich protrusions and cell migration in macrophages. Our data also suggest that the mechanism of WAVE2 activation by Rac1 through IRSp53 is complex and that membrane recruitment alone is insufficient for WAVE2-dependent actin polymerization. The details of the regulation of WAVE function have not been fully delineated, and this issue awaits further investigation.

Materials and Methods

Cells, antibodies and reagents

RAW/LR5 cells, derived from the murine monocyte/macrophage RAW 264.7 cell line (Cox et al., 1997), and Cos-7 cells were grown in RPMI (Mediatech) containing 10% newborn calf serum (Cambrex), 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma, St Louis, MO). Murine bone marrow-derived macrophages (BMM) were isolated as described previously (Stanley, 1997) and were maintained in α -MEM containing 15% FBS, 360 ng/ml recombinant human CSF-1 (Chiron) and antibiotics. Antibodies and stains used in this study were as follows: goat polyclonal anti-BAIAP2/IRSp53 (Ab CAM, Cambridge, MA), mouse monoclonal anti- β -actin and anti-vinculin (Sigma), rabbit polyclonal anti-WAVE2 (Yamazaki et al., 2003), goat polyclonal anti-Abi1 and anti-Myc (Santa Cruz Biotechnology, CA), mouse monoclonal anti-Myc and anti-HA (Cell Signaling Technology, Beverly, MA), horseradish peroxidase (HRP)-conjugated antibodies against goat, mouse or rabbit IgG, biotin-conjugated anti-rabbit IgG and the AMCA-conjugated donkey anti-goat IgG (Jackson Immuno research, West Grove, PA), Alexa Fluor 488 and Alexa 568-phalloidin (Molecular Probes, Eugene, OR). Protein A/G plus-agarose beads were from Santa Cruz. Transfection reagents were SuperFect (Qiagen, Valencia, CA) and FuGENE HD (Roche, Indianapolis, IN). Murine CSF-1 was from R&D systems (Minneapolis, MN).

Constructs and cell transfection

FLAG-tagged IRSp53 and WAVE2, Myc-tagged Cdc42V12 and FLAG-tagged WAVE2 Δ V (Miki et al., 1998; Miki and Takenawa, 2002; Suetsugu et al., 1999; Suetsugu et al., 2003), as well as the GFP-tagged IRSp53 and WAVE2 Δ proline and Myc-tagged WAVE2 Δ WHD, were a gift from Tadaomi Takenawa (University of Tokyo, Japan). Myc-tagged Rac1Q61L was a gift from Alan Hall (Memorial Sloan-Kettering Cancer Center, USA). HA-tagged IRSp53 Δ SH3 (Shi et al., 2005) was a

gift from Jim Casanova (University of Virginia Health Sciences Center, USA). Construction of FLAG-tagged WAVE2CAAX was performed as follows. The stop codon of FLAG-tagged WAVE2 was removed and an *Xba*I site was introduced by PCR using primers GAA TTC TCT AGA GCC ACC ATG and CAC TCT AGA ATC GGA CCA GTC GTC CTC-3. The product was then subcloned into pcDNA3 containing the membrane-localization sequence of H-Ras (CAAX). Transient transfections were performed using the SuperFect or FuGENE HD as indicated, according to the manufacturer's instructions.

Immunofluorescence microscopy

Adherent RAW/LR5 cells, plated on 12 mm glass coverslips, were serum starved for at least 1 hour at 37°C, followed by incubation for 10 minutes in BWD buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 5 mM glucose, 10 mM NaHCO₃, 1 mM MgCl₂, 1 mM CaCl₂, pH 7.4) for equilibration. BMM were deprived of CSF-1 overnight to upregulate CSF-1 receptor expression before equilibration in BWD. Cells were stimulated with 20 ng/ml CSF-1 in BWD for 5 minutes at 37°C followed by fixation with 3.7% formaldehyde and then permeabilized in 0.2% Triton X-100 (in BWD) for 5 minutes and blocked with 1% BSA (in TBS). F-actin was visualized by staining with Alexa-568 phalloidin. IRSp53 was detected using goat anti-IRSp53 antibody and Alexa 488-donkey anti-goat IgG. Expression of epitope tagged constructs was detected using the mouse anti-Myc, anti-FLAG or anti-HA antibodies as appropriate followed by Alexa 488 donkey anti-mouse IgG. In order to retain the membrane, RAW/LR5 cells expressing FLAG-tagged WAVE2 or WAVE2CAAX were simultaneously fixed and permeabilized with saponin, as described previously (Eddy et al., 2000). F-actin was visualized by staining with Alexa-568 phalloidin. Images in Figs 1 and 5 were taken with a confocal laser-scanning microscope (Model radiance 2000, Bio-Rad Laboratories), and images in Figs 2 and 3 were taken with an Olympus microscope equipped with a cooled CCD camera.

Quantification of F-actin-rich membrane protrusions (membrane ruffles) was performed as described previously (Abou Kheir et al., 2005). The extent of cells exhibiting F-actin-rich membrane protrusions in response to CSF-1 was scored using a scale of 0-3 [modified from Cox et al. (Cox et al., 1997)], where 0=no protrusions, 1=protrusions in one area of the cell, 2=protrusions in two distinct areas of the cell, 3=protrusions in more than two distinct areas of the cell. The protrusion index was calculated as the average of at least 60 cells and expressed as a percentage of that of a control.

Immunoprecipitation and western blotting

Cells were lysed in ice-cold lysis buffer (1% Triton X-100, 25 mM Tris, 137 mM NaCl, 2 mM EDTA, 1 mM orthovanadate, 1 mM benzamide, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, pH 7.4). Clarified whole-cell lysates were used for immunoprecipitation (IP), as described below, or mixed with 5× Laemmli buffer. IPs were carried out by incubating at 4°C with the specific antibody prebound to protein A/G agarose beads. Samples were resolved by SDS-PAGE and proteins were transferred onto PVDF membranes (Immobilon-P, Millipore) and western blotted with the indicated antibodies. Signals were detected using the Super Signal West Pico chemiluminescent substrate (Pierce) and images were acquired and analyzed using a Kodak image station 440.

The cell-fractionation procedure was adapted and modified from Suetsugu and colleagues (Suetsugu et al., 2006a). The cleared supernatants were then subjected to ultracentrifugation at 100,000 *g* for 5 hours to obtain the cytosol fraction and membrane pellet, and the proteins were then analyzed by western blotting.

RNA-mediated interference (RNAi)

Reduction of endogenous IRSp53, WAVE2 and Abi1 expression in RAW/LR5 cells was performed using the pSUPER RNAi system (Oligoengine, Seattle, WA), according to the manufacturer's instructions and as described previously (Abou Kheir et al., 2005). Oligonucleotides within the mouse gene encoding IRSp53 (1102-1120) or the mouse genes encoding WAVE2 and Abi1 (Abou Kheir et al., 2005) were used as target sequences, and heterogeneous cell populations were isolated.

F-actin measurements

The total F-actin content was measured as described previously (Cox et al., 1996). Serum-starved cells were incubated with BWD in the presence or absence of CSF-1 for 5 minutes at room temperature, then fixed and permeabilized and stained with saturating concentrations of rhodamine-phalloidin and YO-PRO-1 (both from Molecular Probes) to stain F-actin and nucleic acids, respectively. The fluorescence intensities of rhodamine and YO-PRO-1 were measured using a plate reader (Polarstar Optima), and the normalized F-actin cellular content (the ratio of rhodamine to YO-PRO-1 signal) was expressed as the percentage increase in response to CSF-1 compared with the unstimulated condition.

For quantification of the total F-actin content of single cells, FLAG-tagged WAVE2- or WAVE2CAAX-expressing RAW/LR5 cells were fixed and stained for F-actin using Alexa-568 phalloidin. FLAG was detected using mouse anti-FLAG antibody and Alexa 488-donkey anti-mouse IgG. The fluorescent intensity of phalloidin was measured by tracing cells expressing WAVE2 or WAVE2CAAX (as evident by the FLAG staining) using ImageJ software.

Cell migration assay

The ability of cells to migrate towards a source of CSF-1 was measured using a transmigration chamber assay with 8 μ m pore size inserts (Falcon) according to the manufacturers' instructions. Briefly, the inserts were placed into 24-well plates containing RPMI in the presence or absence of 20 ng/ml CSF-1. 500,000 serum-starved cells were then loaded onto the inserts and incubated at 37°C for 4 hours. CSF-1 was either added to the lower chamber only (chemotaxis, directional cell motility) or to the upper and the lower chambers (chemokinesis, random cell motility). Phase microscopy was used to count cells that have migrated through the inserts, and the average number of cells in 15–20 different fields was calculated. Cell migration in response to CSF-1 was expressed as the fold induction compared with the corresponding condition in the absence of CSF-1 for both mock- and IRSp53-shRNA-treated RAW/LR5 cells.

Data analysis

The significance of the data was analyzed using a Student's *t*-test, and differences between two means with a *P* < 0.05 were considered significant. Error bars represent the standard error of the mean (s.e.m.).

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References

- Abell, A. N., DeCathelineau, A. M., Weed, S. A., Ambruso, D. R., Riches, D. W. and Johnson, G. L. (2004). Rac2D57N, a dominant inhibitory Rac2 mutant that inhibits p38 kinase signaling and prevents surface ruffling in bone-marrow-derived macrophages. *J. Cell Sci.* **117**, 243–255.
- Abou Kheir, W., Gevrey, J. C., Yamaguchi, H., Isaac, B. and Cox, D. (2005). A WAVE2-Abi1 complex mediates CSF-1-induced F-actin-rich membrane protrusions and migration in macrophages. *J. Cell Sci.* **118**, 5369–5379.
- Aharinejad, S., Paulus, P., Sioud, M., Hofmann, M., Zins, K., Schafer, R., Stanley, E. R. and Abraham, D. (2004). Colony-stimulating factor-1 blockade by antisense oligonucleotides and small interfering RNAs suppresses growth of human mammary tumor xenografts in mice. *Cancer Res.* **64**, 5378–5384.
- Allen, W. E., Jones, G. E., Pollard, J. W. and Ridley, A. J. (1997). Rho, Rac and Cdc42 regulate actin organization and cell adhesion in macrophages. *J. Cell Sci.* **110**, 707–720.
- Allen, W. E., Zicha, D., Ridley, A. J. and Jones, G. E. (1998). A role for Cdc42 in macrophage chemotaxis. *J. Cell Biol.* **141**, 1147–1157.
- Aspenstrom, P., Lindberg, U. and Hall, A. (1996). Two GTPases, cdc42 and rac, bind directly to a protein implicated in the immunodeficiency disorder Wiskott-Aldrich syndrome. *Curr. Biol.* **6**, 70–75.
- Bischof, R. J., Zafropoulos, D., Hamilton, J. A. and Campbell, I. K. (2000). Exacerbation of acute inflammatory arthritis by the colony-stimulating factors CSF-1 and granulocyte macrophage (GM)-CSF: evidence of macrophage infiltration and local proliferation. *Clin. Exp. Immunol.* **119**, 361–367.
- Boocock, C. A., Jones, G. E., Stanley, E. R. and Pollard, J. W. (1989). Colony-stimulating factor-1 induces rapid behavioural responses in the mouse macrophage cell line, BAC1.2F5. *J. Cell Sci.* **93**, 447–456.
- Campbell, I. K., Rich, M. J., Bischof, R. J. and Hamilton, J. A. (2000). The colony-stimulating factors and collagen-induced arthritis: exacerbation of disease by M-CSF and G-CSF and requirement for endogenous M-CSF. *J. Leukoc. Biol.* **68**, 144–150.
- Caron, E. and Hall, A. (1998). Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* **282**, 1717–1721.
- Choi, J., Ko, J., Racz, B., Burette, A., Lee, J. R., Kim, S., Na, M., Lee, H. W., Kim, K., Weinberg, R. J. et al. (2005). Regulation of dendritic spine morphogenesis by insulin receptor substrate 53, a downstream effector of Rac1 and Cdc42 small GTPases. *J. Neurosci.* **25**, 869–879.
- Connolly, B. A., Rice, J., Feig, L. A. and Buchsbaum, R. J. (2005). Tiam1-IRSp53 complex formation directs specificity of Rac-mediated actin cytoskeleton regulation. *Mol. Cell. Biol.* **25**, 4602–4614.
- Cory, G. O. C., Garg, R., Cramer, R. and Ridley, A. J. (2002). Phosphorylation of tyrosine enhances the ability of WASp to stimulate actin polymerization and filopodium formation. *J. Biol. Chem.* **277**, 45115–45121.
- Cox, D. and Greenberg, S. (2001). Phagocytic signaling strategies: Fc(gamma)receptor-mediated phagocytosis as a model system. *Semin. Immunol.* **13**, 339–345.
- Cox, D., Chang, P., Kurosaki, T. and Greenberg, S. (1996). Syk tyrosine kinase is required for immunoreceptor tyrosine activation motif-dependent actin assembly. *J. Biol. Chem.* **271**, 16597–16602.
- Cox, D., Chang, P., Zhang, Q., Reddy, P. G., Bokoch, G. M. and Greenberg, S. (1997). Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. *J. Exp. Med.* **186**, 1487–1494.
- Disanza, A., Mantoani, S., Hertzog, M., Gerboth, S., Frittoli, E., Steffen, A., Berhoerster, K., Kreienkamp, H. J., Milanesi, F., Fiore, P. P. et al. (2006). Regulation of cell shape by Cdc42 is mediated by the synergic actin-bundling activity of the Eps8-IRSp53 complex. *Nat. Cell Biol.* **8**, 1337–1347.
- Eddy, R. J., Pierini, L. M., Matsumura, F. and Maxfield, F. R. (2000). Ca²⁺-dependent myosin II activation is required for uropod retraction during neutrophil migration. *J. Cell Sci.* **113**, 1287–1298.
- Eden, S., Rohatgi, R., Podtelejnikov, A. V., Mann, M. and Kirschner, M. W. (2002). Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* **418**, 790–793.
- Funato, Y., Terabayashi, T., Suenaga, N., Seiki, M., Takenawa, T. and Miki, H. (2004). IRSp53/Eps8 complex is important for positive regulation of Rac and cancer cell motility/invasiveness. *Cancer Res.* **64**, 5237–5244.
- Gautreau, A., Ho, H. Y., Li, J., Steen, H., Gygi, S. P. and Kirschner, M. W. (2004). Purification and architecture of the ubiquitous Wave complex. *Proc. Natl. Acad. Sci. USA* **101**, 4379–4383.
- Govind, S., Kozma, R., Monfries, C., Lim, L. and Ahmed, S. (2001). Cdc42Hs facilitates cytoskeletal reorganization and neurite outgrowth by localizing the 58-kD insulin receptor substrate to filamentous actin. *J. Cell Biol.* **152**, 579–594.
- Innocenti, M., Zucconi, A., Disanza, A., Frittoli, E., Arcesi, L. B., Steffen, A., Stradal, T. E. B., Di Fiore, P. P., Carlier, M. F. and Scita, G. (2004). Abi1 is essential for the formation and activation of a WAVE2 signalling complex. *Nat. Cell Biol.* **6**, 319–327.
- Jones, G. E., Zicha, D., Dunn, G. A., Blundell, M. and Thrasher, A. (2002). Restoration of podosomes and chemotaxis in Wiskott-Aldrich syndrome macrophages following induced expression of WASp. *Int. J. Biochem. Cell Biol.* **34**, 806–815.
- Krugmann, S., Jordens, I., Gevaert, K., Driessens, M., Vandekerckhove, J. and Hall, A. (2001). Cdc42 induces filopodia by promoting the formation of an IRSp53:Mena complex. *Curr. Biol.* **11**, 1645–1655.
- Kunda, P., Craig, G., Dominguez, V. and Baum, B. (2003). Abi, Sra1, and Kette control the stability and localization of SCAR/WAVE to regulate the formation of actin-based protrusions. *Curr. Biol.* **13**, 1867–1875.
- Leng, Y., Zhang, J. Y., Badour, K., Arpaia, E., Freeman, S., Cheung, P., Siu, M. and Siminovich, K. (2005). Abelson-interactor-1 promotes WAVE2 membrane translocation and Abelson-mediated tyrosine phosphorylation required for WAVE2 activation. *Proc. Natl. Acad. Sci. USA* **102**, 1098–1103.
- Lin, E. Y., Nguyen, A. V., Russell, R. G. and Pollard, J. W. (2001). Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J. Exp. Med.* **193**, 727–740.
- Linder, S. and Aeplfelbacher, M. (2003). Podosomes: adhesion hot-spots of invasive cells. *Trends Cell Biol.* **13**, 376–385.
- Lorenzi, R., Brickell, P. M., Katz, D. R., Kinnon, C. and Thrasher, A. J. (2000). Wiskott-Aldrich syndrome protein is necessary for efficient IgG-mediated phagocytosis. *Blood* **95**, 2943–2946.
- Miki, H. and Takenawa, T. (2002). WAVE2 serves a functional partner of IRSp53 by regulating its interaction with Rac. *Biochem. Biophys. Res. Commun.* **293**, 93–99.
- Miki, H., Suetsugu, S. and Takenawa, T. (1998). WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO J.* **17**, 6932–6941.
- Miki, H., Yamaguchi, H., Suetsugu, S. and Takenawa, T. (2000). IRSp53 is an essential intermediate between Rac and WAVE in the regulation of membrane ruffling. *Nature* **408**, 732–735.
- Millard, T. H., Sharp, S. J. and Machesky, L. M. (2004). Signalling to actin assembly by the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex. *Biochem. J.* **380**, 1–17.
- Millard, T. H., Bompard, G., Heung, M. Y., Dafforn, T. R., Scott, D. J., Machesky, L. M. and Futterer, K. (2005). Structural basis of filopodia formation induced by the IRSp53/MIM homology domain of human IRSp53. *EMBO J.* **24**, 240–250.
- Miyahara, A., Okamura-Oho, Y., Miyashita, T., Hoshika, A. and Yamada, M. (2003). Genomic structure and alternative splicing of the insulin receptor tyrosine kinase substrate of 53-kDa protein. *J. Hum. Genet.* **48**, 410–414.
- Mizrahi, A., Molshanski-Mor, S., Weinbaum, C., Zheng, Y., Hirshberg, M. and Pick, E. (2005). Activation of the phagocyte NADPH oxidase by Rac guanine nucleotide exchange factors in conjunction with ATP and nucleoside diphosphate kinase. *J. Biol. Chem.* **280**, 3802–3811.
- Oda, A., Miki, H., Wada, I., Yamaguchi, H., Yamazaki, D., Suetsugu, S., Nakajima, M., Nakayama, A., Okawa, K., Miyazaki, H. et al. (2005). WAVE/scars in platelets. *Blood* **105**, 3141–3148.
- Pixley, F. J. and Stanley, E. R. (2004). CSF-1 regulation of the wandering macrophage: complexity in action. *Trends Cell Biol.* **14**, 628–638.
- Prehoda, K. E., Scott, J. A., Mullins, R. D. and Lim, W. A. (2000). Integration of multiple signals through cooperative regulation of the N-WASP-Arp2/3 complex. *Science* **290**, 801–806.
- Raftopoulos, M. and Hall, A. (2004). Cell migration: Rho GTPases lead the way. *Dev. Biol.* **265**, 23–32.
- Rajavashisth, T., Qiao, J. H., Tripathi, S., Tripathi, J., Mishra, N., Hua, M., Wang, X. P., Loussarian, A., Clinton, S., Libby, P. et al. (1998). Heterozygous osteopetrotic (op) mutation reduces atherosclerosis in LDL receptor-deficient mice. *J. Clin. Invest.* **101**, 2702–2710.

- Ridley, A. J., Schwartz, M. A., Burridge, K., Firtel, R. A., Ginsberg, M. H., Borisy, G., Parsons, J. T. and Horwitz, A. R. (2003). Cell migration: Integrating signals from front to back. *Science* **302**, 1704-1709.
- Rogers, S. L., Wiedemann, U., Stuurman, N. and Vale, R. D. (2003). Molecular requirements for actin-based lamella formation in *Drosophila* S2 cells. *J. Cell Biol.* **162**, 1079-1088.
- Shi, J., Scita, G. and Casanova, J. E. (2005). WAVE2 signaling mediates invasion of polarized epithelial cells by *Salmonella typhimurium*. *J. Biol. Chem.* **280**, 29849-29855.
- Smith, J. D., Trogan, E., Ginsberg, M., Grigaux, C., Tian, J. and Miyata, M. (1995). Decreased atherosclerosis in mice deficient in both macrophage colony-stimulating factor (op) and apolipoprotein E. *Proc. Natl. Acad. Sci. USA* **92**, 8264-8268.
- Stanley, E. R. (1997). Murine bone marrow-derived macrophages. *Methods Mol. Biol.* **75**, 301-304.
- Steffen, A., Rottner, K., Ehinger, J., Innocenti, M., Scita, G., Wehland, J. and Stradal, T. E. (2004). Sra-1 and Nap1 link Rac to actin assembly driving lamellipodia formation. *EMBO J.* **23**, 749-759.
- Stradal, T. E., Rottner, K., Disanza, A., Confalonieri, S., Innocenti, M. and Scita, G. (2004). Regulation of actin dynamics by WASP and WAVE family proteins. *Trends Cell Biol.* **14**, 303-311.
- Suetsugu, S., Miki, H. and Takenawa, T. (1999). Identification of two human WAVE/SCAR homologues as general actin regulatory molecules which associate with the Arp2/3 complex. *Biochem. Biophys. Res. Commun.* **260**, 296-302.
- Suetsugu, S., Yamazaki, D., Kurisu, S. and Takenawa, T. (2003). Differential roles of WAVE1 and WAVE2 in dorsal and peripheral ruffle formation for fibroblast cell migration. *Dev. Cell* **5**, 595-609.
- Suetsugu, S., Kurisu, S., Oikawa, T., Yamazaki, D., Oda, A. and Takenawa, T. (2006a). Optimization of WAVE2 complex-induced actin polymerization by membrane-bound IRSp53, PIP3, and Rac. *J. Cell Biol.* **173**, 571-585.
- Suetsugu, S., Murayama, K., Sakamoto, A., Hanawa-Suetsugu, K., Seto, A., Oikawa, T., Mishima, C., Shirouzu, M., Takenawa, T. and Yokoyama, S. (2006b). The RAC binding domain/IRSp53-MIM homology domain of IRSp53 induces RAC-dependent membrane deformation. *J. Biol. Chem.* **281**, 35347-35358.
- Takenawa, T. and Miki, H. (2001). WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J. Cell Sci.* **114**, 1801-1809.
- Webb, S. E., Pollard, J. W. and Jones, G. E. (1996). Direct observation and quantification of macrophage chemoattraction to the growth factor CSF-1. *J. Cell Sci.* **109**, 793-803.
- Yamauchi, A., Kim, C., Li, S. J., Marchal, C. C., Towe, J., Atkinson, S. J. and Dinanier, M. C. (2004). Rac2-deficient murine macrophages have selective defects in superoxide production and phagocytosis of opsonized particles. *J. Immunol.* **173**, 5971-5979.
- Yamazaki, D., Suetsugu, S., Miki, H., Kataoka, Y., Nishikawa, S., Fujiwara, T., Yoshida, N. and Takenawa, T. (2003). WAVE2 is required for directed cell migration and cardiovascular development. *Nature* **424**, 452-456.
- Yanagida-Asanuma, E., Asanuma, K., Kim, K., Donnelly, M., Young Choi, H., Hyung Chang, J., Suetsugu, S., Tomino, Y., Takenawa, T., Faul, C. et al. (2007). Synaptopodin protects against proteinuria by disrupting Cdc42:IRSp53:mena signaling complexes in kidney podocytes. *Am. J. Pathol.* **171**, 415-427.
- Zicha, D., Allen, W. E., Brickell, P. M., Kinnon, C., Dunn, G. A., Jones, G. E. and Thrasher, A. J. (1998). Chemotaxis of macrophages is abolished in the Wiskott-Aldrich syndrome. *Br. J. Haematol.* **101**, 659-665.