The Cdc42 effector IRSp53 is a strong inducer of filopodia formation and consists of an Src homology domain 3 (SH3), a potential WW-binding motif, a partial-Cdc42/Rac interacting binding region motif, and an Inverse-Bin-Amphiphysins-Rvs (I-BAR) domain. We show that IRSp53 interacts directly with neuronal Wiskott-Aldrich syndrome protein (N-WASP) via its SH3 domain and furthermore that N-WASP is required for filopodia formation as IRSp53 failed to induce filopodia formation in N-WASP knock-out (KO) fibroblasts. IRSp53-induced filopodia formation can be reconstituted in N-WASP KO fibroblasts by full-length N-WASP, by N-WASPΔWA (a mutant unable to activate the Arp2/3 complex), and by N-WASPΔ208D (a mutant unable to bind Cdc42). IRSp53 failed to induce filopodia in mammalian enabled (Mena)/VASP KO cells, and N-WASP failed to induce filopodia when IRSp53 was knocked down with RNA interference. The IRSp53 I-BAR domain alone induces dynamic membrane protrusions that lack actin and are smaller than normal filopodia (“partial-filopodia”) in both wild-type N-WASP and N-WASP KO cells. We propose that IRSp53 generates filopodia by coupling membrane protrusion through its I-BAR domain with actin dynamics through SH3 domain binding partners, including N-WASP and Mena.

Filopodia and lamellipodia are ubiquitous and dynamic actin-based structures at the leading edge of cells that play important roles in processes such as cell invasion, cell migration, phagocytosis, and axonal guidance. Thus, understanding how the formation of filopodia and lamellipodia is regulated will give us insight into the fundamental aspects of cell biology what goes wrong in disease states such as cancer. Work over the last few years has revealed many of the important players involved in cell signaling events that regulate actin dynamics associated with filopodia and lamellipodia formation (1). Rho GTPases (Cdc42, Rac1, and RhoA) are intimately involved in communication between cell surface receptors and proteins that control actin dynamics (2). Cdc42 is a major regulator of filopodia formation in mammalian cells. The isolation and identification of Cdc42 effectors have opened up the possibility of defining the molecular mechanisms responsible for the regulation and formation of filopodia. To date the Cdc42 effectors N-WASP,2 IRSp53, PAK, and MRCK have been implicated in filopodia formation. In this study we focus on the role of Cdc42 effector IRSp53 and the mechanism by which it induces filopodia formation.

The WASP/N-WASP (Wiskott-Aldrich syndrome protein and neuronal-Wiskott-Aldrich syndrome protein) and WAVE1–3 (Wasp family verproline-homologue) family protein complexes are major downstream targets for Rho GTPase (3). WASP and WAVE proteins are activators of actin nucleation in vitro and adaptor proteins composed of a number of distinct domains as follows: WH1 (WASP Homology 1) and WH2 (WASP Homology 2), a basic stretch binding phosphatidylinositol 4,5-biphosphate, and the WA domain (W (verprolin and coflin) and Acidic region). The latter domain is involved in binding to the Arp2/3 complex (Actin-related proteins 2 and 3). Rac1 interacts directly with one of the proteins present in the WAVE complex, p140 Sra-1, although the function of this interaction is unclear (4, 5). WAVE1 and -2 complex proteins include Abi-1, p125 Nap-1, p140 Sra-1, and HSPC300 (6), respectively. Phosphatidylinositol 4,5-biphosphate and Toca-1 binding to N-WASP unfolds the protein to make the WA domain available to interact with the Arp2/3 complex (7, 8). How the activities of “WASP” family proteins are coordinated both spatially and temporally is under intense study (3).

The adaptor protein IRSp53 was identified in a yeast two-hybrid screen using the WAVE1 polyproline sequence as bait (9). Subsequent analysis suggested IRSp53 interacted with WAVE2 and might be involved in linking Rac1 to WAVE proteins (9, 10). IRSp53 was also identified in a yeast two-hybrid screen using Cdc42 and shown to be an effector for Cdc42 (11, 12). IRSp53 consists of an I-BAR3 domain (Inverse-Bin-Amphiphysin-Rvs) and sohail.ahmed@imbf.a-star.edu.sg.

2 The abbreviations used are: N-WASP, neuronal Wiskott-Aldrich syndrome protein; BAR, Bin-Amphiphysin-Rvs domain; CRIB, Cdc42/Rac interacting binding region; I-BAR, Inverse-Bin-Amphiphysin-Rvs domain; IMD, IRSp53-MIM homology domain; Mena, mammalian enabled; SH3, Src homology domain 3; GFP, green fluorescent protein; KO, knock-out; WT, wild type; RNAi, RNA interference; oligo, oligonucleotides; ROI, region of interest; FRET, fluorescence resonance energy transfer; GST, glutathione S-transferase; FCS, fluorescence correlation spectroscopy; DIC, differential interference contrast; mRFP, monomer red fluorescent protein; KD, knockdown.

3 For the I-BAR domain, the N-terminal 250 amino acid residues of IRSp53 have weak protein sequence similarity to the BAR domain (14, 41). Structural analysis of the N terminus of IRSp53 shows that it dimerizes, forms a cigar shape, and has a stronger relationship to the BAR domain (2). Functionally, the BAR domain of IRSp53 deforms membranes with a curvature opposite that seen by other BAR domains (17). Here we designate this domain of IRSp53 the term Inverse-BAR or i-BAR.
IRSp53 Couples Membrane Protrusion and Actin Dynamics

Mena via its SH3 domain. actin dynamics through proteins, including N-WASP and phophydysrin-β, also referred to as the IMD4 [IRSp53 and Missing in metastasis homology Domain] see Refs. 13, 14), a partial-CRIB motif interrupted by an SH3-binding site, an SH3 domain (11, 12), a potential WW domain binding site, and a PDZ domain binding site in some isoforms. The I-BAR domain has been suggested to play a role in F-actin bundling (14, 15). It has also been suggested that the N-terminal region of IRSp53 incorporating the I-BAR domain is able to bind Rac1 directly (10).

Recent work has suggested that the IRSp53 I-BAR domain is linked with membrane deformation/curvature of lipids in vitro but not F-actin bundling (16, 17). Suetsugu et al. (18) have shown that the IRSp53 I-BAR domain can produce membrane protrusion in cells. The partial-CRIB has high affinity for Cdc42, binds Rac1 extremely weakly, but does not bind RhoA (11, 12). To date, the SH3 domain of IRSp53 has been shown to bind a number of proteins, including dentatorubral pallidoluyisian, WAVE1 and -2, mDia1 (mouse Diaphanosus1), Mena, Espin, Eps8, Pro/Shank, and bovine angiogenesis inhibitor (9, 12, 19–24). IRSp53 interacts with the MALs (mammalian Lin7 homologue) protein through its PDZ domain (25). IRSp53 has recently been shown to bind the Rac1 exchange factor Tiam1 at a site near the partial-CRIB domain (26). IRSp53 has five splice variants, and two of them contain a functional actin monomer binding WH2 domain at the C terminus (27–29).

We identify N-WASP as an essential mediator of IRSp53-induced filopodia formation. The SH3 domain of IRSp53 can bind N-WASP directly, and IRSp53 fails to induce filopodia in N-WASP KO fibroblasts (30) but does induce lamellipodia formation and membrane ruffling in these cells. In N-WASP reconstitution experiments of N-WASP KO fibroblasts, IRSp53 regains its ability to induce filopodia formation. Intriguingly, the WA domain or the Cdc42-binding site of N-WASP is not required to allow IRSp53 to induce filopodia formation, but the WA domain may play a role in filopodia turnover. IRSp53 also fails to induce filopodia in Mena/VASP KO cells, and N-WASP failed to induce filopodia when IRSp53 was knocked down with RNAi. The I-BAR domain alone induces dynamic membrane protrusions that lack actin and are smaller than normal filopodia (“partial-filopodia”) in both N-WASP WT and N-WASP KO cells. We propose that IRSp53 generates filopodia by coupling membrane protrusion through its I-BAR domain with actin dynamics through proteins, including N-WASP and Mena via its SH3 domain.

**MATERIALS AND METHODS**

**Cell Culture and Transfection**

N1E115 neuroblastoma cells were grown as described in Ref. 11. The N-WASPfloxflox control fibroblast (WT) and N-WASPdel/del (KO) were derived from N-WASPfloxflox mice prepared on E14 cells and immortalized with retrovirus. Cells were plated out at approximately ~10⁵ cells per glass-bottom dish and grown overnight at 32 °C in Dulbecco’s modified Eagle’s medium low with supplements. cDNA of required constructs were prepared at 50 ng/µl in double distilled H₂O and centrifuged at 16,000 × g at 4 °C for 30 min. 6 µl of DNA mix was loaded into a microinjection needle, and cells were injected at a constant pressure of 20 p.s.i. for 100-ms duration. Microinjection was performed on a custom microinjection setup and Olympus microscope (IMT-10). Between 100 and 150 cells were injected per dish, and cells were left to express protein for 1–6 h before they were imaged or fixed and stained. For DIC/fluorescence time-lapse analysis, cells were incubated on a heated stage at 37 °C and imaged with a monochromator on a Zeiss Axiowert 200 microscope enclosed in an incubator with CoolSNAP CCD camera. Generally, images were taken over a period of 10 min at 10-s intervals. The supplemental movies were compiled using the Metamorph software.

**Knockdown of IRSp53 Protein by RNAi**

The IRSp53 oligos and the negative control oligo were obtained from Invitrogen. The oligos were transfected using HiPerfect (Qiagen), according to the manufacturer’s protocol. The oligos were transfected at a final concentration of 5 nM. Cells were harvested 27 and 48 h after transfection and lysed for Western blots to determine the level of IRSp53 knockdown. Oligo sequences were as follows: 5’ AUG GUA AGC AGC AGA GUU CUU GGC C 3’, 5’ AUU GGU AUU GGC CAU CUG UUA CGA C 3’, and 5’ AUU CAU GAC AGG UAC UAU CUC A C 3’.

**Recombinant Protein Preparations**

pGEX-GST, pGEX-Cdc42Q61L, and pGEX-IRSp53 proteins (SH3 domain, residues 400–469; ΔSH3, residues 1–295; I-BAR, residues 1–250) were prepared using standard procedures (11).

**In Vitro Transcription/Translation and Binding Assay**

N-WASP and GFP were in vitro transcribed and translated using the TNT T7-coupled reticulocyte lysate systems (Promega, L4610) with pXJ40-N-WASP-HA as the template for N-WASP and pXJ40-GFP as the template for GFP following the manufacturer’s protocol.
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**IRSp53 and N-WASP Constructs**

For mRFP-IRSp53, IRSp53 was subcloned from HA-IRSp53 in pXJ40 into mRFP-pXJ40 vector between the BamHI and NotI site. For mRFP-N-WASP, N-WASP was subcloned from HA-N-WASP in pXJ40 into mRFP-pXJ40 vector between the HindIII and NotI site. The 4K mutants were generated using a site-directed mutagenesis kit (Stratagene) as per the manufacturer’s protocol. The mutants are as follows: (i) IRSp53-4K, (ii) GFP-I-BAR-4K, and (iii) GST-I-BAR-4K.

**Mass Spectrometry Analysis**

Proteins associated with the SH3 domain of IRSp53 were isolated by affinity purification from lysates of adult rat brain with the GST fusion protein of the SH3 domain of IRSp53 immobilized on Sepharose beads. The protein complex was eluted and resolved by 10% SDS-PAGE and detected by colloidal Coomassie Blue (Pierce). Protein bands detected by colloidal Coomassie Blue were excised and subjected to in-gel reduction, S-alkylation, and trypsin hydrolysis. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of the peptides was performed on a Finnigan LCQ Deca ion trap mass spectrometer (Thermo Finnigan) fitted with a nanospray source (MDS Proteomics). Chromatographic separation was conducted using a Famos autosampler and an Ultimate gradient system (LC Packings) over Zorbas SB-C18 reverse phase resin (Agilent) packed into 75 μm inner diameter PicoFrit columns (New Objective). Protein identifications were made using the search engines Mascot (Matrix Sciences) and Sonar (ProteoMetrics). 44 peptides were obtained for N-WASP, with 60% coverage of the protein.

**Yeast Two-hybrid**

Yeast two-hybrid analysis was done by mating AH109 and Y187 strains carrying the appropriate plasmids as described in the Clontech manuals.

**FRET Measurement**

FRET was measured by acceptor photobleaching method (32) by making necessary settings in a Zeiss LSM 510 confocal microscope with a C-Apochromat 63 × 1.2-water objective. The fusion proteins of GFP/mRFP were excited using 488 and 561 nm laser line as excitation source, by selecting 405/488/561 filters to record the fluorescence intensity. ROI was selected and by visualization of F-actin bundles with fluorescence microscopy (FCS) was used to measure GFP-IRSp53 concentrations in vivo. For FCS analyses, the fluorescence intensity fluctuations arise from single molecules that are diffusing in and out of a defined confocal volume. Details of the instrumentation and method can be found in Ref. 34.

**RESULTS**

Filopodia are a ubiquitous but diverse group of cell structures making them difficult to define (35). However, in mammalian cells filopodia are more homogeneous. In studies examining the ability of the IRSp53 or its I-BAR domain to induce filopodia in mammalian cells, filopodia have not been defined (14, 15, 36). In addition, in most studies on filopodia measurement of...
filopodial dynamics have not been done, and this makes interpretation and comparison of the data difficult. Thus we felt it important to define mammalian filopodia at the outset and determine which domains of IRSp53 were essential for this activity using dynamic measurements.

**Definition of Filopodia**—To define filopodia more accurately, we measured their formation in a range of mammalian cells. N1E115, HeLa, COS7, and B16F1 cells were transfected with GFP-actin, and by using sequential time-lapse wide field fluorescence/DIC imaging, we scored the protrusions for length and lifetime. Mammalian cell line filopodia are actin-based structures with lengths between 8 and 15 μm and lifetimes between 79 and 142 s (Table 1). In addition, we noticed the following features of filopodia: (i) they have a width between 0.6 and 1.2 μm (see Table 1 legend); (ii) are unbranched; and (iii) rarely protrude together. In contrast to filopodia, retraction fibers are nondynamic, tapered, sometimes branched, and can protrude as clusters. Thus for structures to be designated *bona fide* filopodia in mammalian cells, they must be dynamic actin-based structures with characteristics similar to those presented in Table 1.

**IRSp53-SH3 and I-BAR Domains Are Required for Filopodia Formation**—IRSp53 induces filopodia in a Cdc42-dependent manner as a Cdc42-binding mutant IRSp531267N is unable to induce these structures. Induction of filopodia is also significantly reduced in an IRSp53 mutant deleted of its C-terminal domain comprising the SH3 domain (11). The C terminus of IRSp53 contains at least three protein binding domains, the SH3 domain, a potential WW domain binding motif, and a PDZ domain binding motif that could be responsible for filopodia formation (Fig. 1).

The I-BAR domain of IRSp53 has been reported to bundle F-actin, and this activity is reduced by mutation of four lysine residues (142, 143, 146, and 147) that form a potential actin-binding site (14). Using time-lapse analysis of GFP-

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**TABLE 1**

**Filopodia characteristics of mammalian cells**

Cells were transfected with GFP-actin and left for 18–24 h to allow for expression. GFP-actin-expressing cells were picked randomly, and morphological activity was then followed by sequential time-lapse DIC and fluorescence imaging. Filopodia were followed for 10 min at 6 frames/min. Filopodia scored positive for GFP-actin. Filopodia width was measured using GFP-actin-transfected cells. Measurements were taken at the base of the filopodia. The average width for the cells examined ranged between 0.6 and 1.2 μm and was affected by growth conditions. We attempted to measure filopodial characteristics of CHO-1 cells but failed to detect any endogenous filopodia formation and so were unable to make these measurements. All measurements are presented as an average ± S.D., *n* = 15 per experiment, from three experiments.

| Cell line (endogenous filopodia) | Length ± S.D. (μm) | Lifetime ± S.D. (s) |
|----------------------------------|---------------------|---------------------|
| N1E115                           | 15 ± 7.68           | 142 ± 101           |
| HeLa                             | 14 ± 8.34           | 131 ± 76            |
| COS 7                            | 10 ± 5.12           | 123 ± 77            |
| B16F1                            | 8 ± 5.25            | 79 ± 42             |

**cDNA transfections**

| Cdc42V12/Rac1N17 (N-WASP WT)    | 8.4 ± 1.59          | 157 ± 30            |
| IRSp53 (N1E115)                 | 6.8 ± 1.88          | 187 ± 38            |
| N-WASP (N1E115)                 | 7.4 ± 0.97          | 154 ± 20            |

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**FIGURE 1. Phenotypes of IRSp53, IRSp53-FP/AA, and IRSp53-4K mutants.** A, schematic of IRSp53 domain structure with amino acid numbers above. Positions of mutations are indicated by an arrow. B, DIC and GFP-actin images taken from a cell transfected with IRSp53, IRSp53-FP/AA, and IRSp53-4K. N-WASP WT cells were transfected with IRSp53, IRSp53-FP/AA, and IRSp53-4K and left between 18 and 24 h. Actin dynamics were followed with GFP-actin or mRFP-actin. Bar = 5 μm. C and D, scoring for morphological structures: filopodia per cell and % lamellipodia/membrane ruffling per cell was as described under “Materials and Methods.” Data are presented as means ± S.D. from three independent experiments (with *n* = 7–10 per experiment). Background morphological activity of N-WASP KO cells can be found in Fig. 4B and supplemental Fig. S2.
actin-transfected cells, we found that the full-length IRSp53-K142E/K143E/K146E/K147E (IRSp53-4K) mutant was unable to induce filopodia (Fig. 1). Thus, three distinct IRSp53 domains are required for filopodia formation as follows: partial-CRIB (11), the SH3, and the I-BAR domain. Krugmann et al. (12) also found the SH3 domain of IRSp53 to be important for filopodia formation in Swiss 3T3 cells. In the following sections of this study we investigate the role played by the IRSp53 SH3 domain and the I-BAR domain in filopodia formation.

**IRSp53 SH3 Domain Associates with N-WASP and Other Proteins, Including WAVE1/2, Mena, and mDia Protein Complexes**—Because the SH3 domain is essential for filopodia formation, we wanted to identify novel binding partners and thus carried out an IRSp53 GST SH3 domain affinity purification of interacting proteins from brain lysates followed by mass spectrometry analysis of the peptides to identify interactors. Interestingly, WAVE1 and the WAVE1 complex proteins, Abi-2b, p125 Nap-1, and p140 Sra-1, were present on the GST-SH3 column (Fig. 2). Actin, tubulin, dynamin, and mDia1 were also present on the column. In similar experiments using T-cell lysates instead of brain, we detected Mena, mDia2, and WAVE2 as found previously (9, 12 and data not shown). In addition to the WAVE1/2 complex, we found N-WASP and CR-16 binding to the affinity column (Fig. 2). From the proteins that bound to the IRSp53 SH3 domain column, N-WASP was unique in that it had been linked to filopodia formation previously (37, 38) but not with IRSp53. Thus we decided to investigate the relationship between IRSp53 and N-WASP in filopodia formation further.

**IRSp53 Interacts with N-WASP Directly**—The presence of N-WASP and CR-16 on SH3 domain affinity columns suggested that IRSp53 might induce filopodia formation by direct interaction with N-WASP. To investigate this, we used in vitro transcription/translation to produce N-WASP labeled with 35S-labeled methionine and GST-IRSp53-SH3 in pulldown experiments. GST-Cdc42 binding was used as positive control (Fig. 3A, lane 3) and GST-IRSp53-SH3/35S-GFP as a negative control (Fig. 3A, lane 5). We show that the SH3 domain of IRSp53 interacted with N-WASP specifically (Fig. 3A, lane 4), and the FP/AA mutant failed to interact (Fig. 3A, lane 6).

The IRSp53 SH3 domain interaction with N-WASP was also analyzed by using the yeast two-hybrid system. The SH3 domain was cloned into the bait vector (Clontech, system 3) and mated with a strain carrying the N-WASP cDNA cloned in the prey vector or with a strain carrying an empty prey vector (pACT2) as a control. IRSp53-N-WASP were found to interact as diploids grew on quadruple dropout plates and possessed significant β-galactosidase activity (Fig. 3B, panels i and ii). The IRSp53 FP/AA fails mutant to interact with N-WASP in similar yeast two-hybrid experiments (Fig. 3B, panels iii and iv).

To determine whether the IRSp53-N-WASP interaction occurred in vivo we used a FRET approach to measure protein-protein interactions (Fig. 3C). mRFP-IRSp53 and GFP-N-WASP were cotransfected into N1E115 and CHO-1 cells and allowed to express for 36 h (to allow proper folding of the fluorescent moieties). We used the acceptor photobleaching method to determine FRET. Briefly, FRET was measured in the following way. ROI were chosen, and mRFP-IRSp53 (acceptor) was bleached, and changes in GFP-N-WASP (donor) and acceptor fluorescence were measured. If acceptor bleaching induces an increase in donor fluorescence, FRET is occurring and can be quantitated as a percentage efficiency, %FE (see under “Materials and Methods”). The %FE is a measure of the distance between mRFP-IRSp53 and GFP-N-WASP. For FRET to occur the distance between donor and acceptor has to be 10 nm or less. We also reasoned that if FRET is present then there should be a negative correlation between rates of change of acceptor/donor fluorescence. This we define with a correlation coefficient (CC; see under “Materials and Methods” and supplemental Fig. S1 for details). We used four controls in our FRET experiments; cytoplasmic GFP and mRFP, mRFP-IRSp53 and GFP, mRF and GFP-N-WASP, and a tandem GFP-mRFP. Positive FRET values reached a maximum of 28% with CC = −0.99, whereas background %FE varied between 0.96 and 2.12% with CC = 0.17 to 0.34. Any FRET efficiency above 3% with CC = −1.0 to −0.7 is defined as positive FRET and indicates that protein-protein interaction was occurring.

In both CHO-1 cells and N1E115 cells, we observed positive FRET between IRSp53 and N-WASP in filopodia-like structures, neurites, neurite-like processes, and the cell body. Fig. 3C, panel b, shows an example of FRET occurring between mRFP-IRSp53 and GFP-N-WASP in a filopodia-like tip complex. The IRSp53 SH3 domain mutant IRSp53-4K/AA with N-WASP failed to give a positive FRET signal showing the specificity of the technique through point mutation (Fig. 3C, panel h).

**IRSp53 Requires N-WASP for Filopodia Formation**—To examine the functional consequence of the IRSp53-N-WASP interaction, we used N-WASP wild-type (WT) and N-WASP knock-out (KO) fibroblast cell lines (for details of N-WASP WT and KO fibroblasts see Ref. 30). In the first set of experiments
we compared the effect of IRSp53 cDNA microinjection on the morphology of the N-WASP WT and KO cell lines. cDNA for GFP-actin was included in the microinjection to identify expressing cells and to facilitate the imaging of actin dynamics (Fig. 4). When cells were injected with GFP-actin cDNA alone, and scored for filopodia, neurite-like processes, and membrane ruffling, there was no difference between N-WASP WT and N-WASP KO cells (Fig. 4A, panels a and b). For the basal morphological activity of N-WASP WT and KO cells, see supplemental Fig. S2A. IRSp53 induced filopodia formation in the N-WASP WT cells and caused the formation of neurite-like processes (Fig. 4A, panels d, e, and f; see supplemental Movie 1). In N-WASP KO cells no filopodia or neurite-like processes were seen. However, membrane ruffling (and lamellipodia) formation was strongly stimulated when IRSp53 was expressed in N-WASP KO cells (Fig. 4A, panels c and g; see supplemental Movie 2). N-WASP KO cells expressing IRSp53 appeared to be thicker than WT cells possibly because of dorsal ruffling (Fig. 4A, panel h).

IRSp53 induced membrane ruffling and lamellipodia formation in N-WASP KO cells. To rule out the possibility that these morphological activities were masking filopodia formation, we used Rac1N17. The presence of Rac1N17 with IRSp53 in N-WASP WT cells increased the observed filopodia formation (Fig. 5A, compare panels a and c). However, Rac1N17 did not affect the ability of IRSp53 to induce filopodia formation in N-WASP KO cells (Fig. 5A, compare panels b and d), although membrane ruffling/lamellipodia formation were reduced significantly (Fig. 5A, panel f).

Membrane ruffling and lamellipodia formation can mask filopodia formation. Therefore, to prevent this masking, Rac1N17, an inhibitor of membrane ruffling and lamellipodia, can be used.
Cdc42/Rac1N17 Fails to Induce Filopodia Formation in N-WASP KO Cells—Cdc42 has been reported to induce filopodia formation in N-WASP KO cells (30) using Cdc42L61/Rac1N17/C3 toxin microinjections. In our hands, cells retracted and died when injected with Cdc42L61/Rac1N17/C3 toxin (possibly through collapse of significant numbers of focal adhesions/complexes; data not shown). So we re-examined the ability of Cdc42 to induce filopodia formation in N-WASP KO cells. Cdc42V12 alone was found to induce membrane ruffling/lamellipodia formation as the main phenotype, and few filopodia were seen in N-WASP WT cells and none in KO cells (Fig. 5, compare panels a and b).

When Cdc42V12 was microinjected with Rac1N17, membrane ruffling/lamellipodia formation was strongly inhibited in both cell types (Fig. 5B, compare panels a and c, and b and d) and filopodia were induced in N-WASP WT cells but not in N-WASP KO cells. N-WASP was able to reconstitute Cdc42V12/Rac1N17-induced filopodia formation in KO cells showing that the N-WASP KO cells were competent to generate filopodia (Fig. 5B, compare panels e and f; statistics are presented in panels g–j). Expression of N-WASP alone induced membrane ruffling in KO cells (Fig. 5B, panel j). Taken together, these results suggest that N-WASP is essential for IRSp53- and Cdc42-induced filopodia formation.

Mena/VASP KO Cells and IRSp53 Knockdown (KD)—Mena has been linked to IRSp53-mediated filopodia formation (12). To examine this further, we used Mena/VASP KO cells. IRSp53 was unable to induce filopodia formation in Mena/VASP KO cells and IRSp53 failed to induce filopodia formation in Mena/VASP KO cells (Fig. 5B, compare panels a and b).

7 Cdc42 Q61L and G12V are mutant forms of the protein that have low intrinsic GTPase activity such that the protein remains in the GTP-bound form. The intrinsic GTPase activity Q61L is slightly lower than G12V; however, both are in the “on” state and have similar phenotypes and can be used interchangeably.
cells but did induce membrane ruffling. The IRSp53 phenotypes in both N-WASP and Mena/VASP KO cells were similar. Thus both N-WASP and Mena are important for IRSp53-mediated filopodia formation but not for membrane ruffling/lamellipodia formation (Fig. 6, A and B). We also determined the effect of IRSp53 KD on the N-WASP-driven filopodia formation. As shown in Fig. 6, D and E, IRSp53 KD significantly inhibited N-WASP driven filopodia formation.

**Reconstitution of N-WASP in N-WASP KO Cells**—In the next set of experiments we titrated in N-WASP cDNA to the KO cells...
with IRSp53 cDNA. Low levels of N-WASP cDNA were tolerated by cells and allowed us to carry out reconstitution experiments with IRSp53. N-WASP KO cells microinjected with N-WASP cDNA responded to IRSp53 by filopodia formation (Fig. 7A, compare panels a and b; see supplemental Movies 3 and 4), and neurite-like processes were also formed (data not shown). IRSp53FP/AA was not competent to generate filopodia in N-WASP reconstitution experiments (Fig. 7A, panels d and e) suggesting that IRSp53 interaction with N-WASP is essential for filopodia formation in these reconstitution experiments (controls determining the morphological activity of IRSp53FP/AA alone in N-WASP WT and KO cells are shown in supplemental Fig. S2B). Finally, we were able to demonstrate positive FRET between mRFP-IRSp53 and GFP-N-WASP in KO cells (data not shown) similar to that seen in N1E115 and CHO-1 cells (Fig. 3C).

N-WASPΔWA, a mutant unable to interact with the Arp2/3 complex, was used next in reconstitution experiments. The IRSp53-N-WASPΔWA combination was able to reconstitute filopodia formation in KO cells, but neurite-like processes were not observed. Reconstitution with the N-WASPΔWA mutant induced filopodia on the dorsal surface of the KO cells as well as on the periphery (Fig. 7A, panel c; see supplemental Movie 5).

Using the N-WASPH208D mutant in the reconstitution system, we examined the role of Cdc42-N-WASP interaction. The
IRSp53 Couples Membrane Protrusion and Actin Dynamics

FIGURE 5—continued

B

Cdc42 V12

Cdc42 V12 and RacN17

N-WASP WT

N-WASP KO

N-WASP KO and N-WASP

N-WASP WT

N-WASP KO

N-WASP KO and N-WASP

N-WASP WT

N-WASP KO

N-WASP KO and N-WASP

FIGURE 5—continued
FIGURE 6. IRSp53 phenotype in Mena/VASP WT and KO cells. A, Mena/VASP WT and KO cells were microinjected with IRSp53 and GFP-actin cDNA, and cells were allowed to express for over 6 h. Time-lapse analysis was then carried out as described under “Materials and Methods.” Panels a, c, and c’, WT cells; panels b, d, and d’, KO cells. Panels a and b, GFP-actin; panels c and d’, DIC. Panels c’ and d’ are duplicate images of panels c and d with tracings of morphological structures induced; red, filopodia; black, lamellipodia/membrane ruffling. Bar = 10 μm. B and C, statistical analysis of experiments illustrated in A. N-WASP WT and KO cell data are presented for comparison. Cells were scored for filopodia per cell and % lamellipodia/membrane ruffle per cell as described under “Materials and Methods.” All measurements are presented as an average ± S.D., n = 7 per experiment, from three experiments. D and E, comparison of the effect of IRSp53 on N-WASP KO cells, Mena KO cells, and N-WASP on IRSp53 KD cells. IRSp53 RNAi as described in F and under “Materials and Methods.” Morphological structures were analyzed as described above. D, % filopodia was set at 100% for IRSp53 phenotype in N-WASP WT cells or neuroblastoma N1E115 cells. E, % lamellipodia/membrane ruffles was set at 100% for IRSp53 phenotype in N-WASP KO cells or neuroblastoma N1E115 cells. Values for 100% can be obtained from Fig. 4, B and C. All measurements are presented as an average ± S.D., n = 7 per experiment, from three experiments. F, effect of RNAi on IRSp53 protein expression. Cells were transfected with N-WASP cDNA with either no addition or RNAi, scramble RNAi or IRSp53-specific RNAi.
FIGURE 7. Reconstitution and characteristics of IRSp53-induced filopodia formation by N-WASP, N-WASPΔWA and NWASP H208D. A, N-WASP KO cells were microinjected with GFP-actin and IRSp53 cDNA (panel a), IRSp53 and N-WASP cDNA (panel b), IRSp53 and N-WASPΔWA cDNA (panel c), or IRSp53 and N-WASPH208D cDNA (panel d), and cells were incubated for up to 6 h for expression. GFP-actin-positive cells were imaged using DIC time-lapse microscopy. Panels a’–d’ are duplicates of images from a to d with tracings of morphological structures induced; red, filopodia; black, lamellipodia/membrane ruffling. Panels e and f, statistical analysis of cells illustrated in panels a–d. Cells were scored for filopodia per cell and % lamellipodia/membrane ruffling per cell as described under “Materials and Methods.” Bar = 10 μm. All measurements are presented as an average ± S.D., n = 7 per experiment, from three experiments.
N-WASP-H208D mutant was able to reconstitute filopodia formation driven by IRSp53 (Fig. 7A, panels d and e).

**Function of the IRSp53-N-WASP Interaction**—Next, we compared filopodia induced by IRSp53/N-WASP with IRSp53/N-WASPΔWA in reconstitution experiments for length and lifetime (a control determining the morphological activity of N-WASPΔWA alone in N-WASP KO cells is shown in supplemental Fig. S2C). The filopodia induced in the two situations differed significantly only in lifetime. The effect of the WA deletion was to increase the lifetime of the filopodia from 60 to 160 s (Fig. 7B), which was caused by an increase in time taken for disassembly.

**IRSp53 Interacts with Mena and Eps8 in Filopodia**—Unlike Cdc42, IRSp53, or N-WASP (Fig. 1A), overexpression of Mena or Eps8 fails to induce filopodia (data not shown). Both GFP-Mena and GFP-Eps8 were located primarily in distinct vesicular structures, and filopodial localization was not observed (data not shown). When GFP-Mena and GFP-Eps8 were transfected with mRFP-IRSp53, their distribution was changed, and they could be observed in filopodia. To determine whether IRSp53 interacted directly with Mena and Eps8 and thereby recruit these proteins to filopodia, we used the acceptor photobleaching FRET approach as described above. Fig. 8 shows examples of individual filopodia where positive FRET signals between GFP-Mena or GFP-Eps8 and mRFP-IRSp53 were found. In these experiments Eps8 was found throughout the filopodia, whereas Mena had a preference for the tip complex (Fig. 8).

We also examined the role of the IRSp53-mDia2 interaction in filopodia formation. Expression of mRFP-IRSp53 with either myc-mDia2 or YFP-mDia2 led to inhibition of the IRSp53-mediated filopodia formation (data not shown). In most cells there were no filopodia. IRSp53 and mDia2 colocalized in the perinuclear area.

**Role of the IRSp53 I-BAR Domain in Filopodia Formation**—Recent studies have suggested that the I-BAR domain has F-actin bundling activity, and the role of IRSp53 in filopodia is to bundle F-actin (14, 15, 36). We compared the F-actin bundling activity of IRSp53 with that of Fascin using low speed sedimentation assays as first described for IRSp53 in Ref. 15. Fascin was able to bundle F-actin effectively at 60 nM. With the IRSp53 I-BAR domain we obtained variable results, but at least 5–10 μM was required to see any F-actin bundling (supplemental Fig. S3). We then assayed the cellular concentration at which GFP-IRSp53 was able to generate filopodia formation using FCS (see under “Materials and Methods”). Cellular concentrations of the IRSp53 ranged from 29.7 to 453 nM (supplemental Fig. S4), with 29.7 nM being sufficient to generate filopodia.

Next we overexpressed the GFP-I-BAR domain with mRFP-actin (Flag-tagged-I-BAR and GFP-actin were also used in

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N-WASP KO cells were microinjected with GFP-actin, IRSp53, and N-WASP cDNA (panel a) or N-WASPΔWA cDNA (panel b), and cells were incubated for up to 6 h for expression. GFP-actin-positive cells were imaged using DIC time-lapse microscopy as described under “Materials and Methods.” Filopodia generated by either treatment were followed during formation and disassembly, and the time taken for both processes was monitored. The time-lapse series shown gives an example of one such measurement. Filopodia were measured for length (panel c), lifetime (panel d), and duration (panel e). Blue bars represent assembly, and green bars represent disassembly (panel e). Bar = 5 μm. Lifetime, determined by following individual filopodia from appearance to the cell membrane to disappearance. Duration, filopodia assembly is defined as the time taken from appearance to maximum length. Filopodia, disassembly is defined as the time taken from maximum length to disappearance. All measurements are presented as an average ± S.D., n = 7 per experiment, from three experiments.

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IRSp53 Couples Membrane Protrusion and Actin Dynamics

IRSp53 Binds Actin—To further characterize the differences between IRSp53 and its I-BAR domain, we attempted to measure FRET between GFP-actin and mRFP-IRSp53 as well as between mRFP-actin and GFP-I-BAR and GFP-I-BAR-4K. There was positive FRET between GFP-actin and mRFP-IRSp53 in filopodia-like structures, ribs, neurite-like processes, and areas of ruffling (Fig. 10). However, both I-BAR and I-BAR-4K failed to show positive FRET with actin. These data suggest that the I-BAR domain does not interact with actin in the same way as IRSp53 (Fig. 10).

DISCUSSION

Definition of Filopodia—Filopodia are F-actin-based morphological structures at the periphery of cells. In particular, neurons have prominent filopodia in their growth cones, and these structures are thought to help axons find their targets (39). Filopodia are constructed from parallel bundles of F-actin that lie perpendicular to the cell periphery. The length of endogenous filopodia in the mammalian cells examined in this study varied between 8 and 15 μm. These filopodia are highly dynamic with lifetimes between 79 and 142 s. Mammalian cells form a number of structures that resemble filopodia. For example, retraction fibers are F-actin-based structures present at the cell periphery and have similar overall dimensions to filopodia. However, retraction fibers are static structures that arise from the cell membrane withdrawing from the leading edge. Thus, it is essential to use time-lapse analysis with GFP-actin to distinguish filopodia from other structures at the cell periphery.

Cdc42 Pathways to Filopodia Formation—N-WASP was the first Cdc42-interacting protein implicated in filopodia formation (37). Expression of dominant negative N-WASP constructs or microinjection of N-WASP antibodies into bradykinin-treated cells inhibited filopodia formation. Subsequently,
FIGURE 9. Characterization of I-BAR domain-driven protrusive structures. A, N-WASP WT were microinjected with GFP-BAR and mRFP-actin (panels a–c), and cells were left between 1 and 6 h for cDNA expression. Positive cells were imaged using time-lapse microscopy as described under "Materials and Methods." Panel a, static protrusions with actin. Panel b, static protrusions without actin. Panel c, dynamic protrusions without actin. Panel d, N-WASP WT cells microinjected with IRSp53 and GFP-actin. Bar = 5 μm. B, I-BAR-induced protrusions can be classified into three groups as follows: (i) static protrusions with actin; (ii) static protrusions without actin; and (iii) dynamic protrusions without actin. Dynamic protrusions with actin (filopodia) were not produced by I-BAR or I-BAR-4K. Filopodia produced by full-length IRSp53 is used for comparison as group iv. Protrusions were scored for lifetime, length, and width. Protrusions were

| Protrusions                  | I-BAR | I-BAR-4K | IRSp53 |
|-----------------------------|-------|----------|--------|
| Lifetime (sec)              |       |          |        |
| Length (μm)                 |       |          |        |
| Width (μm)                  |       |          |        |
| Lifetime (sec)              |       |          |        |
| Length (μm)                 |       |          |        |
| Width (μm)                  |       |          |        |

1. Static protrusions with actin
   >10 mins 10.3±0.8 0.98±0.06 ND ND ND ND ND
2. Static protrusions without actin
   >10 mins 4.5±0.06 0.59±0.11 ND ND ND ND ND
3. Dynamic protrusions without actin
   17±4 10.3±0.62 0.74±0.06 17±6 32 5.7±0.9 1.9±0.15 ND ND ND
4. Dynamic protrusions with actin
   ND ND ND ND ND 197±38 6.3±1.8 1.25±0.12

ND = Not Detected
two studies using N-WASP KO cells came to different conclusions about the role of N-WASP in Cdc42-mediated filopodia formation. Snapper et al. (38) were unable to discriminate between retraction fibers and filopodia, and thus it is difficult to comment on the basal filopodial activity observed in N-WASP KO cells in this study. However, Snapper et al. (38) did find substantial reduction in “filopodia” numbers in N-WASP KO cells, which led them to conclude that their data “support earlier studies (37) implicating a crucial role for N-WASP in the induction of filopodia by Cdc42.” In contrast, Lommel et al. (30) found the ability of Cdc42 to generate filopodia was unaffected by N-WASP KO. The data presented in our study taken together with previous work (37, 38) suggest an important role for N-WASP in filopodia formation. The use by Lommel et al. (30) of the mixture of Cdc42L61/RacN17/C3 toxin to investigate filopodia formation represents one major difference in the studies. C3 toxin may induce the dissociation of a RhoA-mDia2 complex allowing mDia2 to work with Cdc42 (40) in the absence of N-WASP. Another potential discrepancy between the studies is the definition of filopodia. We believe it is crucial that bona fide filopodia have the characteristics described in Table 1.

IRSp53 Couples Membrane Protrusion and Actin Dynamics

FIGURE 10. IRSp53 and I-BAR interaction with actin. GFP-actin and mRFP-IRSp53 or mRFP-actin and GFP-I-BAR were expressed in CHO-1 cells, and FRET analysis was carried out as described in Fig. 3C and under “Materials and Methods.” A, panels a and b show examples of cells transfected with I-BAR or I-BAR-4K, and then acceptor photobleaching was carried out. The traces shown in panels a′ and b′ represent GFP and mRFP intensities during the experiment. B, statistical analysis of experiments carried out in A. %FE and CC data are presented. Positive FRET is defined as FE > 3% and CC values between -0.7 and 1.0. Data presented are averages ± S.D. from three experiments, with n = 7–10. FRET was measured on fixed samples and therefore structures are designated filopodia-like. Bar = 10 μm.
IRSp53 SH3 domain binding partners and identified N-WASP as a novel interactor. Yeast two-hybrid and pulldown experiments in vitro demonstrated that IRSp53 can interact directly with N-WASP. Furthermore, FRET experiments show that IRSp53 and N-WASP interact in vivo and that, using the IRSp53-FP/AA mutant, the IRSp53 SH3 domain is essential for this interaction.

IRSp53 Phenotypes in N-WASP and Mena KO Cells—To investigate the role of the IRSp53-N-WASP and IRSp53-Mena interactions, we employed the use of KO fibroblasts and RNAi mediated KD. Overexpression of IRSp53 in N-WASP and Mena WT fibroblasts induced strong filopodia formation. However, in N-WASP KO and Mena/VASP KO cells, overexpression of IRSp53 induced membrane ruffling but not filopodia. Furthermore, N-WASP-induced filopodia formation was significantly reduced by IRSp53 KD. We conclude that both N-WASP and Mena are essential for IRSp53-mediated filopodia formation.

We also noticed that IRSp53 induced significant stimulation of lamellipodia/membrane ruffling in N-WASP and Mena KO. We suspect that IRSp53-SH3-WAVE2 and IRSp53-Tiam1 (Tiam1 is a Rac1 exchange factor) interactions are responsible for this phenotype. In support of this, both the FP/AA mutation and Rac1N17 reduced the ability of IRSp53 to stimulate lamellipodia/membrane ruffling. Thus the IRSp53 phenotype and function may be determined by competition between target-interacting proteins.

Because the IRSp53-N-WASP interaction has not been described before we investigated the role of N-WASP further, IRSp53 in combination with Rac1N17 revealed even stronger filopodia formation in N-WASP WT cells but still none in N-WASP KO cells. If N-WASP cDNA is included with IRSp53 in the injection/transfection of N-WASP KO cells, reconstitution of filopodia formation is observed. The IRSp53 SH3 domain is essential for these effects as the FP/AA mutation did not generate filopodia in N-WASP WT cells and N-WASP could not reconstitute filopodia formation with this mutant in N-WASP KO cells.

To extend this analysis we carried out the reconstitution with the mutant, N-WASPΔWA, which is unable to bind the Arp2/3 complex. N-WASPΔWA was fully competent to reconstitute filopodia formation. However, the filopodia observed in the N-WASPΔWA reconstitution experiments had a longer lifetime because of a slower disassembly phase. These results clearly show that the role of N-WASP in IRSp53-mediated filopodia formation is not to activate actin nucleation via the Arp2/3 complex. The N-WASPΔH208D mutant was able to reconstitute filopodia formation, and thus it is unlikely that the Cdc42-N-WASP interaction is important for IRSp53-mediated filopodia formation. So what could be the role of IRSp53-N-WASP interaction? Three possible roles for IRSp53-N-WASP interaction in filopodia formation could be determined as follows. (i) N-WASP induces a conformational change in IRSp53 allowing activation of the membrane deformation activity of protein. (ii) IRSp53 could sequester N-WASP, reducing actin filament elongation. (iii) N-WASP is localized to vesicles and is implicated in promoting their movement. The IRSp53-N-WASP interaction may promote vesicle recycling, which could

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**FIGURE 11. Model of IRSp53 mediated filopodia formation.**

**A,** IRSp53 I-BAR domain generates three distinct membrane protrusions (type 1–3) but not filopodia. Only full-length IRSp53 generates filopodia with uniform actin and with similar characteristics (length, width, and lifetime) to filopodia generated by Cdc42 and N-WASP. The use of GFP-actin and time-lapse microscopy is essential to determine whether bona fide filopodia are being induced.

**B,** step 1: Cdc42-GTP recruits IRSp53 to the plasma membrane. We have shown previously that a mutant IRSp53 that is unable to bind Cdc42 fails to localize with actin and does not generate filopodia (11). Disanza et al. (36) have shown biochemically that Cdc42 can recruit IRSp53 to the plasma membrane. Step 2: IRSp53 normally exists in a closed conformation where the SH3 domain is masked. Krugmann et al. (12) have shown that C-terminal of IRSp53 binds N-terminal. IRSp53 needs to adopt an open conformation, but the mechanism for this is unclear. One possibility is that N-WASP could promote an open IRSp53 conformation. Further work is necessary to address this issue. Step 3: recruitment by the IRSp53 SH3 domain of N-WASP, Mena and Eps8 allows coupling of I-BAR mediated membrane protrusion with actin dynamics and subsequent generation of filopodia formation.
be important for delivering proteins to the membrane for filopodia formation. Further work will be necessary to understand the contribution played N-WASP in IRSp53- and Cdc42-mediated filopodia formation.

**BAR Domain Function**—The role of BAR domain proteins in membrane trafficking has been recognized for some time (41). In these proteins the BAR domain induces membrane curvature, and this is coupled to actin dynamics via proteins such as dynamin to facilitate membrane vesicle formation and endocytosis. Members of the BAR domain family also include proteins Toca-1, CIP4, and FBPI7 that have a similar overall structure to IRSp53, namely a BAR domain linked to a Cdc42-binding site and an SH3 domain. In the case of Toca-1 it is clear that its SH3 domain binds N-WASP, and Toca-1 can regulate N-WASP actin polymerization activity *in vitro*. Thus the domain structure of these proteins (Toca-1, IRSp53, CIP4, and FBPI7) could allow the coupling of the membrane curvature with actin dynamics under the control of Cdc42. Interestingly, structural and functional studies of the IRSp53 I-BAR domain suggest it would induce membrane curvature opposite that of proteins such as amphiphysin leading to membrane protrusion rather than membrane invagination (16, 42) and hence the designation I (Inverse)-BAR domain. Here, we show that the IRSp53 I-BAR domain induces dynamic membrane protrusions that lack actin in live mammalian cells (see Fig. 11A).

**Function of the I-BAR Domain in Filopodia Formation—** Yamagishi et al. (15) and Millard et al. (14) have suggested that the I-BAR domain of IRSp53 is sufficient for filopodia formation. Our experiments fail to detect *bona fide* filopodia formation with the I-BAR domain. At high levels of I-BAR, we detect static actin-based protrusions that do not possess typical filopodial morphology. At lower expression levels, the I-BAR domain induces membrane protrusions, some of which are dynamic, but which do not contain actin. These membrane protrusions are smaller and thinner than endogenous or IRSp53-induced filopodia. We term these I-BAR domain induced structures as partial-filopodia. The 4K mutation in the I-BAR domain eliminates the induction of static actin-based structures but does not affect the membrane protrusions lacking actin. Thus, the 4K mutation allows us to dissociate the I-BAR-domain-induced membrane protrusions from changes in actin. The I-BAR-domain-induced structures were similar in both N-WASP WT and N-WASP KO cells suggesting that N-WASP does not play a role in the induction of these structures. Further work is necessary to clarify the physiological link between actin and membrane protrusion for the I-BAR domain.

**Mechanism of IRSp53-mediated Filopodia Formation—** IRSp53 KD has recently been reported to block Cdc42-induced filopodia formation (36) validating the importance of IRSp53 in this pathway. We have shown here that N-WASP-driven filopodia formation requires IRSp53. A number of studies (14, 15, 36) suggest that the apparent F-actin bundling activity of IRSp53 (possibly with Eps8) is sufficient to induce filopodia formation. In parallel assays we found the IRSp53 I-BAR domain has F-actin bundling activity ∼83-fold weaker than Fascin. *In vitro* assays require at least 5 μM I-BAR domain to see any F-actin bundling compared with 60 nM for Fascin. We estimate by FCS that cellular IRSp53 concentrations of 30 nM are sufficient to generate filopodia. Furthermore, our FRET analysis suggests that the I-BAR domain does not bind actin in the static actin-based protrusions, whereas full-length IRSp53 does bind actin in filopodia–like structures. In line with our findings, two recent reports (16, 17) fail to see F-actin bundling by the IRSp53 I-BAR domain (named IMD in these studies) at physiological salt concentrations. Thus the I-BAR-domain-mediated F-actin bundling is unlikely to be the main mechanism used by IRSp53 to generate filopodia.

The I-BAR domain was unable to induce *bona fide* filopodia as determined by time-lapse microscopy with GFP-actin or mRFP-actin. However, the I-BAR domain and the I-BAR-4K mutant did generate dynamic membrane protrusions that lacked actin and were of a smaller size than filopodia. Recent studies using lipid vesicles *in vitro* demonstrate that the I-BAR domain of IRSp53 can generate membrane deformation in the form of buds independent of F-actin (18). Taken together, these results suggest that the prime function of the I-BAR domain is to induce membrane protrusion.

In conclusion, our data suggest a mechanism for filopodia formation; Cdc42 helps to localize IRSp53 through the partial-CRIB domain (see Fig. 11). Subsequently, IRSp53 mediates the coupling of I-BAR-mediated membrane protrusion with actin dynamics via its SH3 domain binding partners N-WASP and Mena. Other SH3 domain binding proteins such Eps8 (36) are also likely to be involved in filopodia formation. Work is currently underway to define the exact molecular roles played by N-WASP, Mena, and Eps8 in actin dynamics (F-actin microfilament assembly and disassembly) during IRSp53-mediated filopodia formation.

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