N-WASP Involvement in Dorsal Ruffle Formation in Mouse Embryonic Fibroblasts

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The Wiskott–Aldrich syndrome protein (WASP) family activates the Arp2/3 complex leading to the formation of new actin filaments. Here, we study the involvement of Scar1, Scar2, N-WASP, and Arp2/3 complex in dorsal ruffle formation in mouse embryonic fibroblasts (MEFs). Using platelet-derived growth factor to stimulate circular dorsal ruffle assembly in primary E13 and immortalized E9 Scar1^{+/+} and Scar1 null MEFs, we establish that Scar1 loss does not impair the formation of dorsal ruffles. Reduction of Scar2 protein levels via small interfering RNA (siRNA) also did not affect dorsal ruffle production. In contrast, wiskostatin, a chemical inhibitor of N-WASP, potently suppressed dorsal ruffle formation in a dose-dependent manner. Furthermore, N-WASP and Arp2 siRNA treatment significantly decreased the formation of dorsal ruffles in MEFs. In addition, the expression of an N-WASP truncation mutant that cannot bind Arp2/3 complex blocked the formation of these structures. Finally, N-WASP^{-/-} fibroblast-like cells generated aberrant dorsal ruffles. These ruffles were highly unstable, severely depleted of Arp2/3 complex, and diminished in size. We hypothesize that N-WASP and Arp2/3 complex are part of a multiprotein assembly important for the generation of dorsal ruffles and that Scar1 and Scar2 are dispensable for this process.

INTRODUCTION

The actin cytoskeleton is crucial for numerous cellular processes, including cell motility, vesicle trafficking, and cell division. The generation of specialized F-actin structures, such as lamellipodia, dorsal ruffles, and filopodia enable actin to function in these diverse cellular events. One pathway that regulates the formation of these structures involves the Wiskott–Aldrich Syndrome protein (WASP) family proteins and the Arp2/3 complex (Machesky *et al.*, 1999; Suetsugu *et al.*, 2003).

Dorsal ruffles are short-lived, dynamic, F-actin-enriched “waves” that occur across the top surface of the cell (for review, see Buccione *et al.*, 2004). Also known as actin ribbons, these structures are generated by fibroblasts and differentiated epithelial cell types in response to agonists such as platelet-derived growth factor (PDGF) and epidermal growth factor. Dorsal ruffles are transient in nature, typically occurring after 2 min of agonist stimulation and disappearing within 30 min. Numerous cellular roles have been attributed to dorsal ruffles. These roles include preparing a static cell for subsequent movement (Krueger *et al.*, 2003), macropinocytosis (Dowrick *et al.*, 1993; Araki *et al.*, 2000),

and the internalization of cell surface receptors (Orth *et al.*, 2006).

In mammalian cells, there are five members of the WASP family, called WASP, N-WASP, and Scar/WAVE1-3 (for review, see Millard *et al.*, 2004). These proteins are important activators of Arp2/3 complex-induced actin nucleation (Machesky and Insall, 1998; Miki *et al.*, 1998; Machesky *et al.*, 1999). At their C termini, all the WASP proteins contain G-actin and Arp2/3 complex binding motifs that enable them to form a trimer nucleus with the Arp2/3 complex and to induce de novo actin polymerization. At the extreme N termini, the Scar proteins have a unique Scar homology domain (SHD) (Bear *et al.*, 1998). Instead of an SHD, WASP and N-WASP have Enabled/VASP homology 1/WASP homology 1 and Cdc42 and Rac interactive binding (CRIB) domains. The differences in domain structures at the N termini are important in defining the regulation of these proteins. Both WASP and N-WASP interact via their CRIB motifs with GTP-Cdc42, relieving an autoinhibited state and allowing them to bind and activate the Arp2/3 complex (Rohatgi *et al.*, 1999; Higgs and Pollard, 2000). However, the Scar proteins do not possess a CRIB domain and their activity is regulated by the Scar complex proteins (reviewed in Bompard and Caron, 2004).

Scar1 and Scar2 were proposed to have differential roles in lamellipodia and dorsal ruffle formation. Loss of Scar1 resulted in loss of dorsal ruffles but not lamellipodia, whereas Scar2 deficiency led to decreased lamellipodia but not dorsal ruffles (Suetsugu *et al.*, 2003). In addition to Scar1 and Scar2, the WASP family member N-WASP has been localized to dorsal ruffles along with WIP, dynamin 2, and cortactin after PDGF stimulation (Anton *et al.*, 2003; Krueger *et al.*, 2003).

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Inhibition of dynamin-cortactin binding by the injection of anti-dynamin 2 antibodies or overexpression of truncation mutants abrogated dorsal ruffle formation, indicating that an interaction between these proteins is required for wave formation (Krueger *et al.*, 2003). WIP presence is also important for dorsal ruffle formation (Anton *et al.*, 2003). Microinjection of anti-WIP antibodies or the absence of WIP in fibroblasts resulted in decreased and delayed PDGF-induced dorsal ruffle production. Anton *et al.* (2003) proposed that WIP, in conjunction with its binding partner cortactin, could both function in dorsal ruffle formation through activation of the Arp2/3 complex and stabilization of actin filaments. After PDGF stimulation, N-WASP-deficient fibroblasts still formed lamellipodia; however, the effects on dorsal ruffle formation have not been studied in detail (Snapper *et al.*, 2001).

In this study, we investigated the roles of the WASP family proteins in dorsal ruffle formation after PDGF treatment. Using two separate sources of Scar1 null mouse embryonic fibroblasts (MEFs), Scar1 deficiency did not impair the generation or kinetics of dorsal ruffles. In addition, siRNA knockdown of Scar1 and Scar2 protein levels did not result in the decreased formation of dorsal ruffles, indicating either functional redundancy between Scar1 and Scar2 or a lack of requirement in dorsal ruffles. In contrast, treatment of MEFs with wiskostatin, a chemical inhibitor of N-WASP, potentially inhibited dorsal ruffle formation in a dose-dependent manner. Depletion of N-WASP and Arp2 levels with siRNA oligonucleotides (oligos) resulted in a statistically significant reduction in the production of dorsal ruffles. In addition, ectopic expression of an N-WASP truncation mutant deficient in Arp2/3 complex binding inhibited dorsal ruffle formation. Therefore, a complex of N-WASP and Arp2/3 complex is required for dorsal ruffle formation in MEFs. Finally, N-WASP null fibroblast-like cells (FLCs) generated actin protrusions in response to PDGF, but these structures were highly unstable, reduced in frequency and size, and depleted of Arp2/3 complex. In conclusion, it seems that N-WASP and not Scar1 or Scar2, is the WASP family member required for robust dorsal ruffle formation in MEFs.

MATERIALS AND METHODS

Antibodies and Reagents

All chemicals were purchased from Sigma Chemical (Poole, Dorset, United Kingdom) unless stated otherwise. Antibodies from the following sources: anti-Scar1 (Launay *et al.*, 2003), anti-Scar2, anti-Scar3, and anti-p34Arc (Upstate Biotechnology, Lake Placid, NY), anti-N-WASP (kind gift of Pontus Aspenstrom, Uppsala, Sweden), horseradish peroxidase-conjugated secondary antibody anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA), goat anti-rabbit fluorescein isothiocyanate (FITC) and Alexa-488 conjugates (Invitrogen, Paisley, United Kingdom).

DNA Constructs

N-WASP constructs were a kind gift of Adrian Thrasher (Hospital for Sick Children, London, United Kingdom). Scar Δ A constructs were cloned into pRK5-myc and have been described previously (Machesky and Insall, 1998; Machesky *et al.*, 1999). Human WASP full-length and bovine N-WASP full-length and N-WASP Δ WWCA (residues 1-404) constructs were cloned into pEGFPN1 (Clontech, Mountain View, CA).

Cell Culture and Lysis

The immortalized wild-type and Scar1 knockout cells were generated from E9 embryos and were a kind gift from Yoshifumi Itoh (Imperial College London, London, United Kingdom). The E9.5 N-WASP FLCs were a kind gift from Scott Snapper (Massachusetts General Hospital, Boston, MA). These cells were cultured in DMEM containing 10% fetal bovine serum (FBS). Cell lysates were generated from a dish of confluent cells by incubating for 15 min in ice-cold lysis buffer (1% Triton-X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 1

μ g/ml each of chymostatin, aprotinin, leupeptin, and pepstatin). Lysates were clarified by centrifugation at $15,000 \times g$ for 10 min at 4°C and the protein concentration was determined by Bio-Rad protein assay.

Preparation of Primary Wild-Type and Scar1 Null MEFs

The Scar1 null mice were a kind gift from Seung Kwak (Wyeth-Ayerst, Princeton, NJ). Heterozygote Scar1^{+/−} mice were mated, and the embryos were extracted from embryo day 13 (E13) pregnant mice. The embryo heads were removed and kept for genotyping (see methods below). All cell lines used subsequently for this report were probed with an anti-Scar1 antibody to confirm the absence of Scar1 in the knockout MEFs. Dissected embryos were trypsinized and resuspended in DMEM containing 10% FBS and plated out into 10-cm dishes in an incubator at 37°C, 5% CO₂. After 24 h, the medium was changed, and the cells then left to reach confluence.

Scar1, Scar2, Arp2, and N-WASP siRNA Treatment

All siRNA oligos were purchased as siGENOME SMARTpool mix of four oligos targeted specifically against mouse Scar1, Scar2, N-WASP, and Arp2 (Dharmacon, RNA Technologies, Lafayette, CO). We transfected 75 nmol siGENOME duplex in MEFs by using Lipofectamine 2000 transfection reagent (Invitrogen) for 48 h per manufacturer's instructions.

Polymerase Chain Reaction (PCR) Protocol for Genotyping

The DNA preparation from tail tips and embryos was done following the protocol in Laird *et al.* (1991). Primers were designed so that the wild-type mice will only produce a 400-bp band, the heterozygotes will have 400- and 150-bp bands, and the Scar1 knockouts will have a 150-bp band (see Supplemental Figure 1 for details). The primers were diluted to a final concentration working stock of 6.6 μ M and used in the following PCR reaction: WAVE1_1F: 5'-CCT-TAGCTCATCATCAGGAC-3'; WAVE1_2R: 5'-AGTAATTAGCACAACCA-TGG-3'; LTR2-2F: 5'-TGGCGTTACTTAAGCTAGCT-3'; and PCR program: 1) 94°C for 3 min, 2) 94°C for 30 s, 3) 60°C for 45 s, 4) 72°C for 1 min, 5) go to 2 and repeat for 35 cycles, and 6) 72°C for 8 min.

Immunofluorescence Microscopy

Cells were fixed in 4% formaldehyde for 10 min, neutralized in 50 mM NH₄Cl in phosphate-buffered saline (PBS) for 10 min, and permeabilized for 4 min in 0.1% Triton X-100/PBS. Cells were stained with 1:25 anti-Scar1, 1:25 anti-Scar2, 1:50 anti-N-WASP, and 1:500 anti-cortactin antibodies. Secondary antibodies used were goat anti-rabbit FITC and Alexa-488 conjugates (Invitrogen). To visualize F-actin, tetramethylrhodamine B isothiocyanate (TRITC) phalloidin or Alexa-350 phalloidin (Invitrogen) diluted 1:40 in PBS were used. Coverslips were incubated in primary and secondary antibodies for 30 min with three washes in PBS between every step. The coverslips were mounted in Mowiol containing *p*-phenyldiamine antifade. Slides were viewed using an MRC100 confocal laser scanning microscope (Bio-Rad, Hercules, CA) or a using a Zeiss Axioskop2 microscope equipped with a digital camera C4742-95 (Hamamatsu, Bridgewater, NJ) and 63 $\times$ oil immersion objective and processed using Openlab 4.0.3 (Improvision, Coventry, United Kingdom) and Adobe Photoshop 7.0 software (Adobe Systems, Mountain View, CA) for Macintosh.

PDGF and Wiskostatin Assays

The MEFs growing on glass coverslips were washed twice in PBS and incubated overnight in serum-free media (DMEM alone). The media were removed and PDGF-BB (Calbiochem, San Diego, CA) (10 μ g/ml stock) was diluted in serum-free media to desired concentration (10 ng/ml in all experiments except Supplemental Figure 3 where 2, 1, and 0.5 ng/ml were used) and added to the coverslips. In the wiskostatin experiments, the MEFs were preincubated with wiskostatin (Calbiochem) or DMSO carrier in serum-free media for 5 min before incubation in media containing $\pm$ wiskostatin and 10 ng/ml PDGF. After fixed incubation lengths at 37°C, 5% CO₂, the coverslips were washed in PBS, fixed, and stained.

Time-Lapse Microscopy

The MEFs were allowed to adhere to WillCo-35-mm glass bottom dishes (WPI, Sarasota, FL) and were serum starved overnight. The media were removed and replaced with 2 ml of prewarmed DMEM containing 10 ng/ml PDGF. The cells were then observed with phase-contrast microscopy at 5 s/frame by using a Zeiss Axiovert 100 microscope equipped with a QICAM-FAST 32-0088B-172 12 bit camera and 32 $\times$ objective.

Measurements of Dorsal Ruffle and Cell Areas

After 5 min PDGF stimulation, the N-WASP FLCs were washed twice in PBS, fixed, and stained for F-actin. To determine the average area of the dorsal ruffles, 30 images were taken from random fields of view on the coverslips by using a Zeiss Axioskop2 microscope equipped with a Hamamatsu digital camera C4742-95 and 63 $\times$ oil immersion objective. These images were opened

Figure 1. Dorsal ruffle production in E13 primary MEFs. (A) DNA isolated from embryos and mouse tail tips were used in a PCR genotyping reaction. Products were run on a 2% agarose gel. Lane 1, 100-bp ladder; lane 2, heterozygote; lane 3, wild-type; and lane 4, Scar1 null. (B) We analyzed 50 μ g of total protein from wild-type and Scar1 null MEFs by immunoblot with anti-Scar1, Scar2, and actin antibodies. (C) Wild-type and Scar1 null MEFs were stimulated with PDGF for 0, 2, 5, and 7 min. The cells were fixed and stained for F-actin and the percentage of cells generating at least one dorsal ruffle was determined. (D) The MEFs were PDGF stimulated for 2, 5, and 7 min, and the average number of dorsal ruffles (white arrows) per cell containing at least one dorsal ruffle was calculated. For C and D, >200 cells were counted per time point, and each point represents the average value from three independent experiments (error bars + SD). Student's *t* test p values are shown on graphs.

in ImageJ, and each dorsal ruffle circumference was outlined (McCarty *et al.*, 2004). A Java plugin for ImageJ was then used to calculate the number of pixels contained within the outline of the dorsal ruffles. This was then repeated to measure the total areas of the cells. Imaging of a graticule then enabled the areas to be converted from pixels to micrometers. Each assay was repeated three times, measuring 30 dorsal ruffles per experiment.

RESULTS

Dorsal Ruffle Formation in Scar1 Null E13 Primary MEFs

To investigate the role of Scar1 in dorsal ruffle formation, we generated wild-type and Scar1 null primary MEFs. The Scar1 knockout mice were generated using a retroviral gene-trap insertion (Dahl *et al.*, 2003). Scar1 null mice displayed numerous neuroanatomical abnormalities and postnatal lethality and so could not be used for breeding (Dahl *et al.*, 2003). Therefore, heterozygote mice were bred to generate Scar1 null embryos. Embryonic day 13 (E13) embryos were used to generate primary MEFs. The embryo heads were used in genotyping PCR reactions (Figure 1A). For explanation of the genotyping protocol, refer to Supplemental Figure 1. Wild-type embryos produced a 400-bp band, and

Scar1 null embryos had a 150-bp PCR product. Protein lysates from wild-type and Scar1 null MEFs also were probed with anti-Scar1, anti-Scar2, and anti-actin antibodies (Figure 1B). The anti-Scar1 antibody showed a strong band at approximately 66 kDa in the wild-type lysate that was absent in the Scar1 null lane. This confirmed the loss of Scar1 protein expression in the knockout MEFs. Anti-Scar2 immunoblots showed the presence of a 70-kDa band in both wild-type and Scar1 null cells. Scar2 levels remained comparable between the two cell types, indicating there was no increase in Scar2 expression to compensate for Scar1 loss. Consistent with previous reports, an anti-Scar3 blot showed no detectable expression of Scar3 in either wild-type or Scar1 knockout MEFs but did detect a strong band in mouse brain lysates and COS-7 cell lysates transfected with full-length Scar3-myc (data not shown; Suetsugu *et al.*, 2003).

Endogenous Scar1 and Scar2 localized to dorsal ruffles, and Scar2 (but not Scar1) consistently localized to peripheral lamellipodia (Supplemental Figure 2). To investigate the role of Scar1 in dorsal ruffle production, serum-starved MEFs were treated with 10 ng/ml PDGF $\beta\beta$ for 0, 2, 5, and 7 min.

At these time points, the cells were fixed and stained for F-actin and endogenous Arp2/3 complex (anti-p34). The percentage of cells with dorsal ruffles at each time point is shown in Figure 1C. At 0 min, the serum-starved cells had no dorsal ruffles, consistent with cells requiring agonist stimulation to generate dorsal ruffles. By 2 min, the cells started to generate dorsal ruffles, with 5% of the wild-type and the Scar1 null cells containing at least one dorsal ruffle. At 5 min, dorsal ruffle numbers have reached an optimum, with ~30% of cells containing dorsal ruffles. The percentage of cells with dorsal ruffles then starts to decrease at 7 min as some ruffles close and the number tends to zero after 20 min (data not shown). Staining with anti-p34 showed a strong enrichment of endogenous Arp2/3 complex within the dorsal ruffles and at peripheral ruffles (data not shown). Importantly, at all time points, the Scar1 null cells produced the same percentage of dorsal ruffles as the wild-type cells, as shown by Student's *t* tests (see Figure 1C for *p* values).

Figure 1D shows an example of a wild-type MEF producing three dorsal ruffles after 5 min of PDGF treatment. Therefore, because MEFs can produce multiple dorsal ruffles per cell, we calculated the average total number of dorsal ruffles per cell in a time course. Cells without dorsal ruffles were not included in this assay, therefore, no value was recorded at 0 min. Figure 1D shows the average number of dorsal ruffles per cell after 2, 5, and 7 min PDGF treatment. Interestingly, no optimal value was recorded at 5 min, with the response staying constant at two dorsal ruffles per cell at all time points. This is consistent with the dorsal ruffles forming, expanding, and contracting over a time scale of minutes and rarely splitting into multiple separate ruffles at later time points. Both the wild-type and Scar1 null cells showed the same constant response to the PDGF. Therefore, Scar1 is not essential for the generation of dorsal ruffles by the E13 primary MEFs in response to PDGF.

Dorsal ruffles are highly dynamic structures, expanding and contracting over time. PDGF treatment of MEFs was therefore followed using real-time phase microscopy to determine if Scar1 loss affected dorsal ruffle dynamics. Representative movies are shown in Figure 2 (A1–A5 wild-type, B1–B5 Scar1 null MEFs; Supplemental Movies 1 and 2, respectively). After ~1 min of PDGF treatment, dark punctate structures start occurring on the dorsal surface of the MEFs (A2 and B2 arrows). By 5 min, the dorsal ruffles have fully formed, consistent with the optimum peak seen in Figure 1C. These waves pulse, expand, and contract over time before finally closing (A5 and B5). Interestingly, phase bright macropinosomes formed upon closure of the dorsal ruffles in both wild-type and Scar1 null MEFs (A5 and B5 arrowheads). A magnified example of macropinosome formation is shown in images C1–C4 (Supplemental Movie 3). As the dorsal ruffle zips closed from right to left, significant numbers of phase-bright, 1- to 2- μ m-wide macropinosomes are formed (C2–C4, arrowheads). The visualization of these structures is consistent with reports that dorsal ruffles are involved in macropinocytosis (Dowrick *et al.*, 1993; Araki *et al.*, 2000). Imaging multiple dorsal ruffles from wild-type and Scar1 null cells did not reveal any qualitative differences in dorsal ruffle dynamics. Both cell types generated waves through dark punctate precursors, which then expanded into large dorsal ruffles. These structures constricted over time and closed normally to generate macropinosomes. Therefore, Scar1 deficiency does not detectably affect the dynamics of dorsal ruffle formation or closure.

Figure 2. Time-lapse phase microscopy of the primary E13 wild-type and Scar1 null cells producing dorsal ruffles in response to 10 ng/ml PDGF treatment. A1–A6, wild-type cell. B1–B6, Scar1 null cell. C1–C4, enlarged movie of a dorsal ruffle in a Scar1 null cell. Arrows follow the formation of the dorsal ruffles. Arrowheads indicate phase bright macropinosome formation during dorsal ruffle closure. Time in seconds is indicated in top right corner. Bar, 20 μ m (see Movies 1, 2, and 3).

Dorsal Ruffle Formation in E9 Scar1 Null Immortalized MEFs

The results obtained in Figures 1 and 2 were particularly significant because they contradict previous findings that Scar1 is important for dorsal ruffle formation in MEFs (Suetsugu *et al.*, 2003). This could be due to differences in the genetic background or preparation of the MEFs used for these studies, although the background C57BL/6 was reported to be the same in both cases. Because Suetsugu *et al.* (2003) used E9 MEFs, it is possible that the E13 primary MEFs have undergone further differentiation/gene expression changes to compensate for the loss of Scar1. Therefore, it was necessary to confirm the results with the same MEFs used in the previous study. We obtained immortalized E9 wild-type and Scar1 null MEFs (a gift from Y. Itoh and T. Takenawa, University of Tokyo, Tokyo, Japan) and repeated the PDGF assays to investigate dorsal ruffle production (Figure 3). Lysates from the E13 primary and E9 immortalized wild-type and Scar1 null MEFs were probed with anti-Scar1, Scar2, and actin antibodies (Figure 3A). These immunoblots confirmed the loss of Scar1 in the immortalized knockout cells with Scar2 levels staying constant (Figure 3A). The overall intensity of the Scar1 and Scar2 staining between the wild-type and immortalized cells was also comparable, indicating that both MEF sources expressed similar levels of

Figure 3. Dorsal ruffle production in E9 immortalized MEFs. (A) We analyzed 50 μ g of total protein from E13 primary and E9 immortalized wild-type and Scar1 null MEFs by immunoblot with anti-Scar1, Scar2, and actin antibodies. (B) Representative picture showing phalloidin staining of Scar1 null E9 MEFs after 5 min of PDGF treatment. Scale bar, 20 μ m. (C) Wild-type and Scar1 null E9 MEFs were stimulated with PDGF for 0, 2, 5, 7, 10, 12, and 15 min. For C, >200 cells were counted per time point, and each point represents the average value from three independent experiments. The cells were fixed and stained for F-actin, and the percentage of cells generating at least one dorsal ruffle was determined (error bars + SD). Student's *t* test *p* values are shown on the graph.

Scar1 and Scar2. None of the MEFs had any detectable Scar3 expression (data not shown).

The immortalized MEFs were stimulated with 10 ng/ml PDGF, and the percentage of cells producing dorsal ruffles was determined (Figure 3, B and C). Phalloidin staining of the MEFs revealed that nearly 100% of the wild-type and Scar1 null cells produced dorsal ruffles after 5-min PDGF treatment (Figure 3C). This response was much stronger than with the primary MEFs, where only 30% of the cells produced dorsal ruffles after 5 min (Figure 1C). The dorsal ruffles in the immortalized MEFs were also much more persistent, with a significant percentage of cells retaining dorsal ruffles even after 15-min stimulation (Figure 3C). This difference between primary and immortalized MEFs in dorsal ruffle formation could result from additional genetic/gene expression changes induced during cell transformation, which potentially increase the sensitivity to growth factor stimulation. Regardless of these differences, the wild-type and Scar1 null-immortalized MEFs responded identically to PDGF stimulation, with equal number of cells generating dorsal ruffles at all time points considered as shown by Student's *t* tests (Figure 3C).

As mentioned, treatment of the immortalized MEFs with 10 ng/ml PDGF induced a robust, 100% response in dorsal ruffle formation. Taking into account a potential increase in sensitivity to PDGF in those cells, the lack of an obvious phenotype could therefore be due to saturating amount of growth factor. To test this hypothesis, decreasing amounts of PDGF were used to stimulate the immortalized MEFs, and the percentage of wild-type and Scar1 null cells produc-

ing dorsal ruffles was determined (Supplemental Figure 3). With 2 ng/ml PDGF (graph A), the response was skewed, with the response at 2 min dropping from 30 to 2% and the optimum moving from 5 to 7 min. At 1 and 0.5 ng/ml, the response was greatly reduced, with peak responses at 7 min of only 20 and 10%, respectively (graphs B and C). Independent of PDGF concentration or strength of response, the wild-type and Scar1 null immortalized MEFs produced a statistically similar response at all time points considered (Student's *t* test *p* values shown on graphs). Therefore, neither the E13 primary nor the E9 immortalized Scar null MEFs showed any defect in the production of dorsal ruffles, indicating that Scar1 is not essential for dorsal ruffle formation.

Wiskostatin Inhibition of Dorsal Ruffle Formation in MEFs

Studies on Scar1 did not reveal any obvious dorsal ruffle phenotype. Therefore, it was interesting to investigate the role of its related family member N-WASP. Endogenous N-WASP, along with its binding partners dynamin, cortactin, and WIP have all been localized to dorsal ruffles (Anton *et al.*, 2003; Krueger *et al.*, 2003). To investigate the endogenous localization of N-WASP relative to Arp2/3 complex and cortactin, PDGF-stimulated wild-type MEFs were stained using anti-Arp2 or anti-cortactin and anti-N-WASP antibodies (Supplemental Figure 4). Staining of N-WASP and Arp2 showed a strong colocalization with F-actin within dorsal ruffles (Supplemental Figure 4, A–D). N-WASP also colocalized with cortactin within dorsal ruffles (Supplemental Figure 4, E–H), consistent with previous reports (Krueger *et al.*, 2003). Therefore, N-WASP, Arp2/3 complex, and cortactin all colocalize in dorsal ruffles after PDGF stimulation.

The activity of N-WASP is tightly regulated by the binding of numerous proteins and lipids (Higgs and Pollard, 2000). In its native form, N-WASP is autoinhibited due to intramolecular folding (Rohatgi *et al.*, 1999). Wiskostatin is a chemical inhibitor of N-WASP. It functions by binding a cleft in the regulatory GTPase binding domain of N-WASP and stabilizing its native, autoinhibited conformation (Peterson *et al.*, 2004). WASP expression is limited to hematopoietic cell lines, and it is not expressed in MEFs (Parolini *et al.*, 1997). Therefore, it is possible to study N-WASP function in dorsal ruffle formation by inhibiting it with wiskostatin.

When treated with wiskostatin alone, serum-starved immortalized wild-type and Scar1 null MEFs did not generate any dorsal ruffles (Figure 4A). Pretreatment of the MEFs with DMSO for 5 min, followed by 5 min 10 ng/ml PDGF treatment resulted in ~100% of cells producing dorsal ruffles, independently of the Scar1 status. Finally, the MEFs were preincubated with 10 μ M wiskostatin to inhibit N-WASP and then treated for 5 min with PDGF and wiskostatin. Inhibition of N-WASP with wiskostatin abolished the formation of dorsal ruffles but not peripheral ruffles in both wild-type and Scar1 null cells. Figure 4C shows an example of a wild-type MEF treated with 10 μ M wiskostatin. No dorsal ruffles are present, but endogenous Arp2/3 complex can still localize into peripheral ruffles (arrows), consistent with reports that N-WASP null cells can still generate lamellipodia (Snapper *et al.*, 2001). These data indicate that N-WASP, unlike Scar1 or Scar2, is important for dorsal ruffle formation.

To further investigate the effects of wiskostatin, decreasing concentrations were used on wild-type MEFs to determine how potently it inhibited dorsal ruffle formation. Wiskostatin at 5 μ M still strongly inhibited dorsal ruffle formation (Figure 4B, <10%). As the concentration reached

Figure 4. Wiskostatin treatment of E9 immortalized MEFs. (A) Serum-starved wild-type and Scar1 null MEFs were treated for 5 min with 10 μ M wiskostatin alone (Wisk), 10 ng/ml PDGF (+ DMSO carrier) alone (PDGF), or PDGF and wiskostatin (PDGF + Wisk). The cells were then fixed and stained with TRITC phalloidin, and the percentage of cells producing dorsal ruffles was determined. (B) Serum-starved wild-type E9 MEFs were pretreated with decreasing concentrations of wiskostatin for 5 min and then incubated with 10 ng/ml PDGF and wiskostatin. The same concentration of wiskostatin was used in the pretreatment as during the PDGF stimulation. After PDGF treatment, the average percentage of cells generating dorsal ruffles was determined. For A and B, >200 cells were counted per time point, and each point represents the average value from three independent experiments (error bars + SD). (C) Representative image of a wild-type MEF treated with 10 μ M wiskostatin and 10 ng/ml PDGF. Cells were fixed and stained with TRITC phalloidin (A) and anti-p34 (B). Arrows indicate localization of Arp2/3 complex in peripheral ruffles. Bar, 20 μ m.

2.5, 1.25, and 0.625 μ M, dorsal ruffle production returned rapidly to 100% levels. Therefore, 3–4 μ M wiskostatin is sufficient to inhibit 50% of dorsal ruffle production in these MEFs. This indicates that inhibition of N-WASP with wiskostatin has a potent inhibitory effect on dorsal ruffle formation.

Scar1, Scar2, N-WASP, and Arp2 siRNA Studies in MEFs

To confirm the Scar1 data and examine the role of Scar2 in dorsal ruffle formation, siRNA oligos specific to mouse Scar1 and Scar2 were used to specifically downregulate their protein levels. Wild-type immortalized MEFs transfected for 48 h with Scar1 and Scar2 siRNA oligos showed a 70 and

Figure 5. siRNA studies on dorsal ruffle formation in E9 immortalized MEFs. (A) Wild-type MEFs were transfected for 48 h with siRNA specific to mouse Scar1, Scar2, N-WASP, and Arp2. Knockdown of protein levels was shown by immunoblot with polyclonal antibodies specific to each protein. Anti-tubulin blots were included as protein loading controls. (B) The siRNA-treated MEFs were serum starved and stimulated with 10 ng/ml PDGF for 5 min. The cells were fixed and stained with TRITC phalloidin, and the percentage of cells generating at least one dorsal ruffle was determined. N-WASP and Arp2 siRNA treated cells were also cotransfected with human WASP-GFP and human Arp2-myc constructs. More than 200 were counted per time point, and each point represents the average value from three independent experiments (error bars + SD). Student's *t* test *p* values are shown on graphs.

75% knockdown of protein levels, respectively, as determined by the intensity of bands produced after immunoblots with anti-Scar1 and anti-Scar2 antibodies (Figure 5A). Anti-tubulin loading controls confirmed roughly equal loadings between the control and siRNA lanes. The siRNA-treated cells were serum starved, treated for 5 min with PDGF, and fixed and stained for F-actin and endogenous Arp2/3 complex. The percentage of cells generating dorsal ruffles was then determined and plotted in Figure 5B.

As a control, wild-type MEFs were treated with transfection reagent alone before PDGF treatment and staining. On average, ~55% of wild-type cells treated with Lipofectamine 2000 alone generated dorsal ruffles. The 70 and 75% knockdown of Scar1 and Scar2, respectively, had no detectable effect on the percentage of cells producing dorsal ruffles (see Figure 5B for Student's *t* test *p* values). These experiments were repeated six times, and on each occasion neither Scar1 nor Scar2 siRNA treatment resulted in a statistically significant decrease in dorsal ruffle production. These data confirm the previous Scar1 results and indicate that Scar2 is also not essential for dorsal ruffle formation.

To confirm the wiskostatin data, wild-type MEFs were treated with mouse N-WASP siRNA oligos to knock down the endogenous protein levels of N-WASP. In addition, the Arp2/3 complex was targeted using Arp2 siRNA (Figure 5, A and B). Knockdown of N-WASP and Arp2 protein levels was confirmed by immunoblot (Figure 5A). Depletion of N-WASP protein levels (85% reduction) resulted in a dramatic loss of dorsal ruffles. Control cells produced on average 55% dorsal ruffles; however, this number was reduced to <10% in N-WASP siRNA-treated cells ($p < 0.0005$). This N-WASP siRNA inhibition was not as potent as wiskostatin treatment, which completely blocked dorsal ruffle production. This block is probably for two main reasons. First, the siRNA did not completely deplete N-WASP protein levels, meaning the remaining 10% could be sufficient to retain dorsal ruffle production in a subset of cells. Second, it is possible that wiskostatin has other protein targets or cytotoxic effects, resulting in indirect inhibition of dorsal ruffle production (Guerriero and Weisz, 2006). However, we used 10 μ M or less wiskostatin in our assays, which gave only a weak off-target effect in the study of Guerriero and Weisz (2006). Interestingly, it was possible to partially retrieve the N-WASP siRNA phenotype by cotransfection of human WASP-GFP (Figure 5B). In N-WASP siRNA-treated cells retrieved by human WASP-GFP expression, WASP-GFP colocalizes with endogenous Arp2/3 complex and F-actin in dorsal ruffles (Supplemental Figure 5, A–D, arrows). However, fewer than 10% of cells not transfected with WASP-GFP had dorsal ruffles. This is consistent with WASP-GFP being recruited to the dorsal ruffles and compensating for N-WASP loss. Therefore, inhibition of N-WASP function through wiskostatin or siRNA treatment results in dorsal ruffle inhibition, and there is a degree of functional redundancy between the N-WASP and WASP in this structure.

In addition to N-WASP, Arp2/3 complex was shown to be important in dorsal ruffle formation. Reduction of endogenous Arp2 levels (70% reduction; Figure 5A) resulted in a statistically significant inhibition in the percentage of cells generating dorsal ruffles ($p < 0.001$; Figure 5B). The phenotype was not as severe as with N-WASP siRNA treatment (20% compared with <10%). This was probably due to differences in the efficiency of the siRNA treatments. Cotransfection of human Arp2-myc retrieved this phenotype back to control levels (Figure 5B). Similar to WASP-GFP, triple staining showed Arp2-myc colocalization with endogenous Arp2/3 complex and F-actin in dorsal ruffles (Supplemental Figure 5, E–H, arrows). Therefore, like N-WASP, Arp2/3 complex function is necessary for dorsal ruffle formation in MEFs.

Numerous roles have been attributed to dorsal ruffles, including the dissolution of stress fibers required to prepare a static cell for subsequent movement (Krueger *et al.*, 2003). The consequence of N-WASP and Arp2 siRNA treatment was evident in the stress fiber organization of the PDGF treated cells. Fifteen minutes into PDGF treatment, the wild-type, Scar1 null, and Scar2 siRNA-treated immortalized MEFs contained large dorsal ruffles. Within the waves, there was a dramatic disassembly of the stress fibers (Supplemental Figure 6, arrows). However, PDGF treated N-WASP and Arp2 siRNA MEFs retained large numbers of stress fibers in the main body of the cells (Supplemental Figure 6A, arrowheads). Therefore, this result confirms previous reports that dorsal ruffle formation is associated with stress fiber disassembly and demonstrates that N-WASP and the Arp2/3 complex are required for this process (Krueger *et al.*, 2003).

The importance of Scar1, Scar2, Scar3, and N-WASP in dorsal ruffle formation was finally investigated using the

Figure 6. Effects of expressing truncation mutants of N-WASP and Scar proteins in E9 immortalized MEFs. Wild-type (white bars) and Scar1 null (gray bars) E9-immortalized MEFs were treated with transfection reagent alone (U) or transfected with Scar1 Δ A (S1dA), Scar2 Δ A (S2dA), Scar3 Δ A (S3dA), wild-type N-WASP (Wt NW), or N-WASP Δ WWCA (NWdWWCA) mutants as indicated. The cells were serum starved, stimulated with PDGF for 5 min, and the percentage of cells generating dorsal ruffles was determined. More than 200 cells were counted per time point, and each point represents the average value from three independent experiments (error bars + SD). Student's *t* test *p* values are shown on graphs.

ectopic expression of truncation mutants in wild-type and Scar1 null MEFs stimulated for 5 min with PDGF. To investigate Scar function, delta-A Scar constructs were used. These constructs lack the C-terminal amino acids essential for Arp2/3 complex binding (Machesky and Insall, 1998). Approximately 50% of control cells treated with transfection reagent alone generated dorsal ruffles (Figure 6). Transfection of Scar1 Δ A, Scar2 Δ A, or Scar3 Δ A into wild-type MEFs (white bars) did not invoke a statistically significant decrease in dorsal ruffle production, consistent with none of the Scar proteins being essential for dorsal ruffle production. Wild-type N-WASP expression did not inhibit dorsal ruffle formation. However, transfection of an N-WASP truncation mutant deficient in Arp2/3 complex binding (N-WASP Δ WWCA) resulted in a decrease in cells with dorsal ruffles ($p < 0.02$; drop from 50 to 20%). This is probably due to the sequestration of endogenous SH3 domain proteins, such as dynamin and WIP, away from the Arp2/3 complex. Finally, Scar1 Δ A and Scar2 Δ A were expressed in Scar1 null MEFs (Figure 6, gray bars) to determine whether the presence of any Scar isoform is required for dorsal ruffle formation. Neither Scar1 Δ A nor Scar2 Δ A expression in the Scar1 null MEFs inhibited dorsal ruffle formation. Therefore, it seems that an N-WASP–cortactin–Arp2/3 ternary complex is important for generating dorsal ruffles, whereas Scar1, -2, and -3 are dispensable for this process.

Dorsal Ruffle Formation in N-WASP Null FLCs

Previous studies on N-WASP function have shown its activity is dispensable for cell migration, adhesion to fibronectin substrate, and lamellipodia extension (Snapper *et al.*, 2001; Rogers *et al.*, 2003). To further investigate N-WASP function in dorsal ruffle formation, E9.5 N-WASP $^{-/-}$ FLCs were obtained (Snapper *et al.*, 2001). These cells differ from MEFs because they were transformed with simian virus 40 (SV40) large-T-containing retrovirus to enable viability in cell cul-

Figure 7. PDGF treatment of N-WASP FLCs. (A) We analyzed 30 μ g of total protein from wild-type MEFs (positive control) and the N-WASP FLCs by immunoblot with anti-NWASP and tubulin antibodies. Loadings: lane 1, wild-type MEFs; lane 2, +/+ RV-N-WASP; lane 3, N-WASP^{+/+}; and lane 4, N-WASP^{-/-}. (B) The N-WASP^{+/+} (A–C), +/+ RV-N-WASP (D–F) and N-WASP^{-/-} (G–O) FLCs were PDGF stimulated for 5 min and fixed and stained with TRITC phalloidin and anti-p34. Arrows indicate collapsed dorsal ruffles and arrowheads show Arp2/3-enriched peripheral ruffles in the N-WASP^{-/-} FLCs. Bar, 20 μ m.

ture. Immunoblot analysis with anti-N-WASP antibody on protein lysates confirmed the absence of a 66-kDa band in the N-WASP null FLCs (N-WASP^{-/-}; Figure 7A). The wild-type SV40 FLCs (N-WASP^{+/+}) expressed similar levels of N-WASP as the MEF-positive control. Finally, SV40-FLCs infected with an N-WASP-expressing retrovirus (+/+ RV-N-WASP) showed a marked increased in N-WASP protein levels.

The N-WASP FLCs were stimulated for 5 min with PDGF and then fixed and stained for F-actin and endogenous Arp2/3 complex (Figure 7B). The N-WASP^{+/+} (A–C) and +/+ RV-N-WASP (D–F) FLCs generated large, circular dorsal ruffles that were strongly stained for endogenous Arp2/3 complex. To our surprise, the N-WASP^{-/-} cells were able to generate dorsal actin protrusions in response to PDGF (G–O). However, there were numerous obvious deficiencies associated with these structures. They were often collapsed, forming bright, punctate structures under F-actin staining

Figure 8. Analysis of dorsal ruffle formation in N-WASP FLCs. (A and B) The +/+ RV-N-WASP and N-WASP^{-/-} FLCs were PDGF stimulated for 0, 2, 5, and 7 min, and the percentage of cells generating any dorsal actin-rich protrusions (A) or only ring-shaped dorsal actin-rich protrusions (B) was calculated. More than 200 cells were counted per time point, and each point represents the average value from three independent experiments (error bars $\pm$ SD).

(arrows in H and K). In cells that did generate full ring-shaped dorsal ruffles, these were typically smaller than the equivalent structures in the N-WASP^{+/+} and +/+ RV-N-WASP FLCs and displayed greatly reduced staining for endogenous Arp2/3 complex (M–O). However, the N-WASP^{-/-} cells were still able to generate normal Arp2/3 complex-enriched peripheral ruffles, consistent with N-WASP not functioning in lamellipodia formation (arrowheads in N; Snapper *et al.*, 2001). Therefore, although not essential in these cells, N-WASP deficiency has resulted in highly abnormal dorsal ruffles that are unstable and depleted of Arp2/3 complex.

To quantify these deficiencies, after 5 min of PDGF stimulation, the average areas of the dorsal ruffles in +/+ RV-N-WASP and N-WASP^{-/-} cells were quantified using a Java plugin for ImageJ (see *Materials and Methods*). The dorsal ruffles in +/+ RV-N-WASP cells were on average 3 times larger than the N-WASP^{-/-} cells (6500 μ m² compared with 2100 μ m²). Measurement of the average areas of the cells showed that the difference in dorsal ruffle size was not due to N-WASP^{-/-} FLCs being smaller than their +/+ RV-N-WASP counterparts (20,000 μ m² compared with 19,000 μ m²).

Finally, the +/+ RV-N-WASP and N-WASP^{-/-} FLCs were stimulated for 0, 2, 5, and 7 min with PDGF to follow the generation of dorsal ruffles over time (Figure 8, A and B). At each time point, the cells were fixed, and the percentage of cells generating dorsal ruffles was determined. Consistent with Figures 1C and 3C, serum-starved FLCs did not gen-

erate dorsal ruffles. The $+/+$ RV-N-WASP FLCs showed a strong and persistent response to PDGF, with $>70\%$ of cells generating dorsal ruffles at 2, 5, and 7 min (Figure 8A). The N-WASP $^{-/-}$ cells were severely deficient in producing dorsal ruffles, with only 20% generating dorsal ruffles at 2 min and a maximal response of 50% at 5 min. In this count, N-WASP $^{-/-}$ FLCs generating punctate, collapsed dorsal structures and full ring structures were both counted as dorsal ruffles. Therefore, the assay was repeated and the abnormal, collapsed structures generated by both $+/+$ RV-N-WASP and N-WASP $^{-/-}$ FLCs were not included (Figure 8B). The graph for the $+/+$ RV-N-WASP cells was largely unaffected, with $>70\%$ of cells generating large, ring dorsal ruffle structures at 2, 5, and 7 min. The N-WASP $^{-/-}$ response was greatly reduced, with $<20\%$ of cells generating dorsal ruffles at all time points. This demonstrates that a large proportion of N-WASP $^{-/-}$ FLCs generate small, abnormal dorsal ruffles. Movies of PDGF stimulated $+/+$ RV-N-WASP and N-WASP $^{-/-}$ FLCs are included as Supplemental Movies 4 and 5. The $+/+$ RV-N-WASP cells generated large, stable dorsal ruffles that expand and contract steadily over time, similar to the wild-type and Scar1 null MEFs in Figure 2. However, the N-WASP $^{-/-}$ cells displayed dramatic and persistent distortions at the dorsal surface, but these were unable to form into larger dorsal ruffle structures. In conclusion, N-WASP is required for the formation of large and stable dorsal ruffle waves as seen in primary and immortalized MEFs and FLCs.

DISCUSSION

We propose that N-WASP, but not Scar1- or Scar2-mediated recruitment of Arp2/3 complex is important for the formation of dorsal ruffles. Scar1 deficiency, in our experiments, did not affect the frequency, appearance or number of dorsal ruffles per cell or dorsal ruffle kinetics during live cell imaging; 70 and 75% reduction in Scar1 and Scar2 by siRNA also did not alter the percentage of cells generating dorsal ruffles. Finally, Scar1 null MEFs obtained from John Scott's group (The Vollum Institute, Portland, OR) also did not show any qualitative differences in dorsal ruffle formation (data not shown; Soderling *et al.*, 2003). Therefore, it is unlikely that Scar1 is essential for dorsal ruffle formation.

These data are particularly interesting in light of a previous report where Scar1 and Scar2 were shown to have differential roles in dorsal ruffle and peripheral ruffle formation, respectively (Suetsugu *et al.*, 2003). Staining for endogenous Scar1 and Scar2 showed localization of both Scar1 and Scar2 to dorsal ruffles (Supplemental Figure 2). However, only Scar2 localized consistently to peripheral lamellipodia, and this is consistent with Scar2 functioning in lamellipodia formation (Suetsugu *et al.*, 2003). The presence of Scar1 and Scar2 in similar localizations in dorsal ruffles presents the possibility that these proteins can functionally compensate for each other. Although we could achieve knockdown of either Scar1 or Scar2 individually, we were unable to observe MEFs with both proteins at undetectable levels. Complete removal of all Scar protein may be detrimental to MEF adhesion or integrity. However, expression of dominant-negative Scar2 ΔA in the Scar1 null MEFs did not inhibit dorsal ruffle formation and indicates that the presence of Scar is not a prerequisite for dorsal ruffle formation.

Numerous roles have been attributed to dorsal ruffles, including the dissolution of stress fibers in preparing a static cell for movement (Krueger *et al.*, 2003), macropinocytosis (Dowrick *et al.*, 1993; Araki *et al.*, 2000), and the internalization of cell surface receptors (Orth *et al.*, 2006). Evidence for

a role in macropinocytosis has come from observing the closure of dorsal ruffles by using real-time microscopy and uptake of fluorescent dextran (Dowrick *et al.*, 1993; Araki *et al.*, 2000). Peripheral ruffles have also been proposed to be the site of macropinocytosis (Suetsugu *et al.*, 2003). Therefore, it seems that macropinocytosis can occur at both peripheral and dorsal surfaces. In addition, wild-type, Scar1 null, and Scar2 siRNA-treated MEFs still made dorsal ruffles, which resulted in almost complete disruption of the stress fibers within the waves. In contrast, N-WASP- and Arp2 siRNA-treated MEFs were unable to generate dorsal ruffles; therefore, the stress fibers remained unaffected. Our study confirms previous reports that dorsal ruffles are involved in the removal of stress fibers in static cells (Krueger *et al.*, 2003).

Invadopodia and podosomes are another class of membrane-deforming F-actin structures, which have striking similarities to dorsal ruffles (for review, see Buccione *et al.*, 2004). Invadopodia and podosomes depend on a dynamin-cortactin-Arp2/3 complex-N-WASP multiprotein assembly. WASP/N-WASP localize to all these structures, and targeted disruption of this complex using dominant-negative N-WASP constructs or depletion of N-WASP protein levels by using null cells or siRNA inhibits invadopodia and podosome formation (Mizutani *et al.*, 2002; Calle *et al.*, 2004; Lorenz *et al.*, 2004; Yamaguchi *et al.*, 2005). Recent evidence supports a similar model for dorsal ruffle formation. Inhibition of dynamin-cortactin binding by the injection of anti-dynamin 2 antibodies or overexpression of truncation mutants abrogated dorsal ruffle formation, indicating that an interaction between these proteins is required for wave formation (Krueger *et al.*, 2003). Recently, overexpression of the dynamin-binding, multidomain scaffolding protein Tuba has been shown to induce dorsal ruffles, and this was inhibited by wiskostatin treatment (Kovacs *et al.*, 2006), further supporting the idea of an N-WASP-dynamin-containing actin assembly in dorsal ruffles.

Analysis of N-WASP null fibroblast-like cells showed that N-WASP deficiency was not terminal for dorsal ruffle production in these cells. However, the dorsal ruffles of N-WASP null cells were severalfold smaller, depleted of Arp2/3 complex and highly unstable than the N-WASP overexpressing cells. We were unfortunately unable to quantify whether the wild-type fibroblast-like cell line produced the same number of dorsal ruffles as the overexpressing cells, because in our hands, the wild-type cell line did not grow well and formed clumps in tissue culture. Comparison between wild type and knockout would have obviously been the best control for the knockout cells, but we think that the significant differences observed between the knockout cells and overexpressing cells still strongly supports the role of N-WASP in dorsal ruffles. The ability of the fibroblast-like cells to generate dorsal ruffles is also complicated by their transformation with SV40 virus. Tyrosine phosphorylation and hyperactivation of Src targets, such as dynamin and cortactin, could potentially compensate for N-WASP loss and enable highly abnormal dorsal ruffles to form. The similarities between dorsal ruffles and podosomes support this hypothesis, because podosomes spontaneously form in Src-transformed cells (Shriver and Rohrschneider, 1981). Interestingly, the wiskostatin treated MEFs and N-WASP null FLCs were still able to recruit Arp2/3 complex and form normal peripheral ruffles. In contrast, Scar2 null MEFs reportedly cannot generate peripheral ruffles under some conditions (Suetsugu *et al.*, 2003; Yan *et al.*, 2003). Therefore, although morphologically similar structures, the

mechanisms underlying dorsal and peripheral ruffle formation may be fundamentally different.

In conclusion, the data presented here support a model where N-WASP and Arp2/3 complex function is critical for robust dorsal ruffle formation. Scar1 and Scar2 also colocalize to dorsal ruffles but are not essential for their formation. It is possible that Scar function is required to reinforce N-WASP-mediated Arp2/3 complex activation or for metalloproteinase-mediated degradation of extracellular matrix during three-dimensional migration mediated by dorsal ruffles. Secretion of the metalloproteinase MMP-2 has been linked to the expression of Scar1 with Scar1 null or Scar1 RNAi treated MEFs showing decreased secretion of MMP-2 (Suetsugu *et al.*, 2003). In addition, Scar3 (WAVE3) siRNA treatment in MDA-MB-231 cells led to the inhibition of cell migration and a decrease in MMP-1, MMP-9, and PDGF-induced MMP-3 mRNA levels (Sossey-Alaoui *et al.*, 2005). Therefore, we propose that N-WASP is an important activator of the Arp2/3 complex in dorsal ruffles, whereas Scar1 and Scar2 are perhaps more important in controlling subsequent matrix degradation and migration.

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