

The *Drosophila* Arf1 homologue Arf79F is essential for lamellipodium formation

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Summary

The WAVE regulatory complex (WRC) drives the polymerisation of actin filaments located beneath the plasma membrane to generate lamellipodia that are pivotal to cell architecture and movement. By reconstituting WRC-dependent actin assembly at the membrane, we recently discovered that several classes of Arf family GTPases directly recruit and activate WRC in cell extracts, and that Arf cooperates with Rac1 to trigger actin polymerisation. Here, we demonstrate that the Class 1 Arf1 homologue Arf79F colocalises with the WRC at dynamic lamellipodia. We report that Arf79F is required for lamellipodium formation in *Drosophila* S2R+ cells, which only express one Arf isoform for each class. Impeding Arf function either by dominant-negative Arf expression or by Arf double-stranded RNA interference (dsRNAi)-mediated knockdown uncovered that Arf-dependent lamellipodium formation was specific to Arf79F, establishing that Class 1 Arfs, but not Class 2 or Class 3 Arfs, are crucial for lamellipodia. Lamellipodium formation in Arf79F-silenced cells was restored by expressing mammalian Arf1, but not by constitutively active Rac1, showing that Arf79F does not act via Rac1. Abolition of lamellipodium formation in Arf79F-silenced cells was not due to Golgi disruption. Blocking Arf79F activation with guanine nucleotide exchange factor inhibitors impaired WRC localisation to the plasma membrane and concomitant generation of lamellipodia. Our data indicate that the Class I Arf GTPase is a central component in WRC-driven lamellipodium formation.

Key words: Abi, Cyfip, Nap1, PIR121, Scar, Sra1

Introduction

Lamellipodia are sheet-like protrusions generated at the leading edge of a cell which play crucial roles in cell morphology and motility (Ridley, 2011; Weijer, 2009). The protrusive force is supplied by polymerisation of actin filaments initiated via the ubiquitous Arp2/3 complex which is activated by nucleation promoting factors (NPFs) (Campellone and Welch, 2010). In mammalian and *Drosophila* cells, the critical NPF underlying lamellipodium formation is the WRC {WAVE [WASP (Wiskott–Aldrich syndrome protein)-family verprolin-homologous protein] regulatory complex}, which is thought to be activated at the plasma membrane by the Rho GTPase Rac1 (Kunda et al., 2003; Miki et al., 1998). Purified mammalian WRC is inactive, but can be activated in buffer by high concentrations of Rac1 (Ismail et al., 2009). The low affinity of Rac1 for WRC (Chen et al., 2010) suggested that additional unknown factors are required to facilitate WRC-dependent actin polymerisation (Davidson and Insall, 2011). We showed that active Rac1 was indeed insufficient for recruitment and activation of the WRC at the membrane in mammalian cell extract (Koronakis et al., 2011). This led to our discovery that Arf family GTPases directly recruit and activate the WRC, which is enhanced when Arf and Rac1 work together to trigger actin polymerisation. Here, we sought to demonstrate that Arf family GTPases play a role in WRC-dependent generation of lamellipodia.

Results and Discussion

WRC is composed of five subunits, namely SCAR/WAVE, Sra1/PIR121, Nap1, Abi and HSPC300 (Gautreau et al., 2004). In human cells, paralogous genes give rise to SCAR/WAVE1, WAVE2 and WAVE3, Sra1 and PIR121, and Abi1 and Abi2 to generate divergent WRC combinations. The mammalian Arf GTPase family is also divergent and can be divided into three subfamilies: Class 1 (Arf1, Arf3), Class 2 (Arf4, Arf5) and Class 3 (Arf6) (Donaldson and Jackson, 2011), which display significant functional redundancy (Volpicelli-Daley et al., 2005). In *Drosophila* cells however, each Arf class is represented by a single isoform, namely Arf79F (Class 1), Arf102F (Class 2) and Arf51F (Class 3). Therefore, to bypass this additional layer of complexity, we used *Drosophila* S2R+ cells where only single WRC subunits (SCAR, Sra1, Kette/Nap1, Abi and HSPC300) are encoded, all of which are required for lamellipodium formation, which is very prominent in this cell type (Kunda et al., 2003; Rogers et al., 2003; Zallen et al., 2002). We first examined whether the *Drosophila* Arf1 homologue Arf79F localised at lamellipodia of S2R+ cells by immunofluorescence using antibodies against human Arf1 and *Drosophila* Sra1 (Fig. 1A). While Arf79F was predominantly found at the perinuclear Golgi apparatus, Arf79F was also observed enriched at the actin-rich cell edge where it colocalised with the WRC component Sra1 (inset). Anti-Arf1 specifically

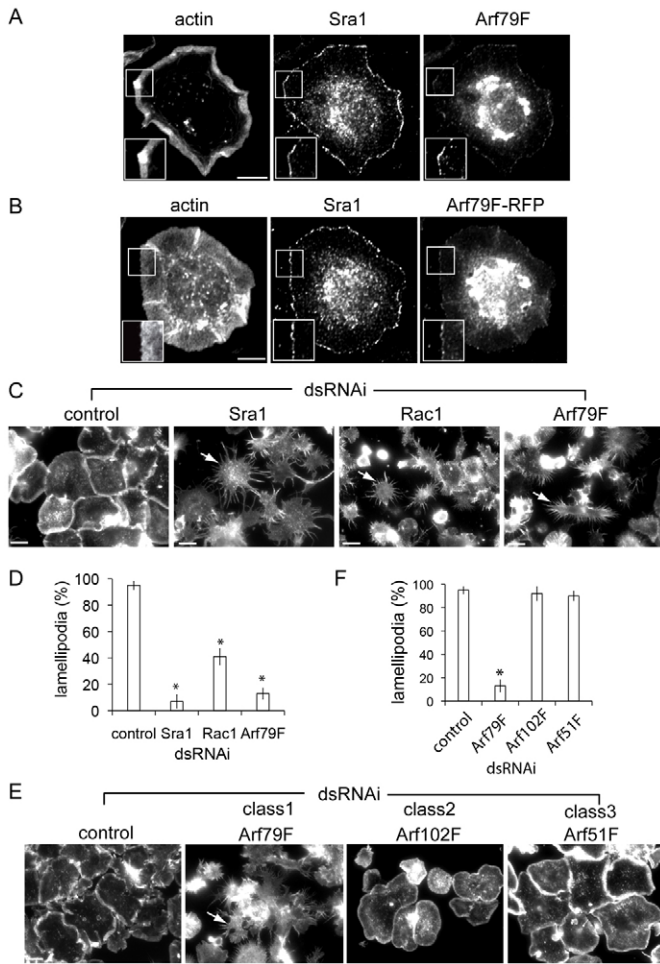


Fig. 1. Arf79F is required for lamellipodium formation in S2R+ cells. (A) Arf79F and control Sra1 visualised at lamellipodia (insets) using anti-Sra1 or anti-Arf1 antibodies and fluorescent phalloidin (actin). (B) Cells expressing RFP-tagged Arf79F labelled as in panel A. (C) Lamellipodia in control-, Sra1-, Rac1- and Arf79F-dsRNAi cells visualised by staining actin (arrows indicate starfish phenotype) then quantified microscopically (D). Asterisks indicate a significant difference from the control. Error bars represent \pm s.e.m. (E) Lamellipodia in control-, Arf79F-, Arf102F- and Arf51F-dsRNAi cells visualised as in panel B and quantified in (F), as in panel D. Scale bars: 10 μ m (A,B), 20 μ m (C,E).

recognised Arf79F as detection of Arf at the Golgi and plasma membrane was abolished when Arf79F gene expression was silenced by double-stranded RNA interference (dsRNAi) (supplementary material Fig. S1A; Fig. S2A). Like endogenous Arf79F, recombinant RFP-tagged Arf79F was also detected by immunoblotting with Arf1 antibody (supplementary material Fig. S2B) and was visualised at the cell edge where it colocalised with Sra1 and actin (insets) (Fig. 1B). This was not the case in cells expressing RFP alone which was predominantly cytosolic relative to Arf79F (supplementary material Fig. S2C,D). The dynamics of fluorescent Arf79F was also monitored in real time where it was enriched at the plasma membrane and the Golgi (supplementary material Movie 1) which was further corroborated when Arf79F-RFP cells were stained with anti-Arf1 antibody (supplementary material Fig. S2E).

We next investigated the requirement for Arf79F in lamellipodium formation. Actin-rich lamellipodia were clearly apparent in \sim 95% of control cells (Fig. 1C,D) but Sra1 dsRNAi (supplementary material Fig. S1B) impaired lamellipodia in \sim 93% of cells giving rise to a characteristic “starfish” phenotype (Kunda et al., 2003), typified by multiple thin extensions and disruption of cortical actin filaments (Fig. 1C,D). This was also seen in SCAR or Kette dsRNAi cells (data not shown). Rac1-silencing also impaired lamellipodium formation in \sim 59% of cells (Fig. 1C,D), a much milder phenotype than in WRC-silenced cells as previously reported (D’Ambrosio and Vale, 2010; Kunda et al., 2003; Rohn et al., 2011). In Arf79F-silenced cells, lamellipodia were abolished in \sim 87% of cells showing that Arf79F is critical for lamellipodium formation (Fig. 1C,D).

We next tested the requirement for specific classes of Arf GTPases in lamellipodium formation using dsRNAi (supplementary material Fig. S1C). In contrast to Arf79F dsRNAi, lamellipodia were unaffected by Arf102F and Arf51F dsRNAi (Fig. 1E,F). To confirm a role for Class 1 Arf GTPases in lamellipodium formation, we examined the morphology of cells equivalently expressing a readily available bank of dominant-negative human Arf GTPases (hArf^{TN}) (supplementary material Fig. S3A). These are known to bind and sequester endogenous Arf guanine nucleotide exchange factors (GEF), thereby blocking Arf activation (Dascher and Balch, 1994; Hart et al., 1994). As human and *Drosophila* Arfs retain high amino acid identity, we speculated that hArf^{TN} would also sequester class specific *Drosophila* Arf GEFs. While lamellipodia were not compromised in cells expressing hArf3^{T31N}, hArf4^{T31N}, hArf5^{T31N} or hArf6^{T27N}, their formation was impaired in \sim 84% of hArf1^{T31N} cells (Fig. 2A,B; supplementary material Fig. S3B) further supporting a specific role for class 1 Arf79F.

We next sought to restore lamellipodium formation in Arf79F-silenced cells. Human Arf1 shares 95% amino acid identity with Arf79F, but only possesses 77% mRNA identity making it ideal for Arf79F dsRNAi rescue experiments. Like Arf79F (Fig. 1B), RFP-tagged hArf1 was observed at the Golgi and at actin-rich lamellipodia (Fig. 2C, arrows) which were formed equivalently in non-transfected (-; \sim 91%) and hArf1-transfected cells (hArf1; \sim 93%) (Fig. 2D, control dsRNAi). When hArf1 was expressed in Arf79F-silenced cells, lamellipodium formation was restored from \sim 15% to \sim 62% (Fig. 2C, arrows; Fig. 2D, Arf79F dsRNAi). Importantly, while \sim 93% of control cells expressing constitutively active Rac1^{G12V} formed lamellipodia, Rac1^{G12V} failed to restore lamellipodium formation in Arf79F-silenced cells (Fig. 2E, arrows; Fig. 2F) showing that Arf79F-dependent lamellipodium formation is not mediated via upstream activation of Rac1.

Arf GTPases are predominantly observed at the perinuclear Golgi apparatus (Fig. 1A,B) where they are best characterised as having roles in membrane trafficking and Golgi organisation (Donaldson and Jackson, 2011). We therefore addressed whether it is Arf79F-mediated membrane trafficking from the Golgi that is imperative for lamellipodia by examining the Golgi apparatus in Arf79F-silenced cells (Fig. 3A). Immunofluorescence of the *cis*-Golgi marker GM130 following Arf79F dsRNAi revealed a clearly defined Golgi network (\sim 84% of cells) comparable to that seen in control cells (\sim 93%) with lamellipodia. This was not the case in cells expressing dominant-negative hArf4^{T31N} where the Golgi was impaired in \sim 75% of cells (Fig. 3B). Despite this Golgi disruption, cells expressing hArf4^{T31N} still displayed lamellipodia and Sra1 localisation at the cell cortex in \sim 90%

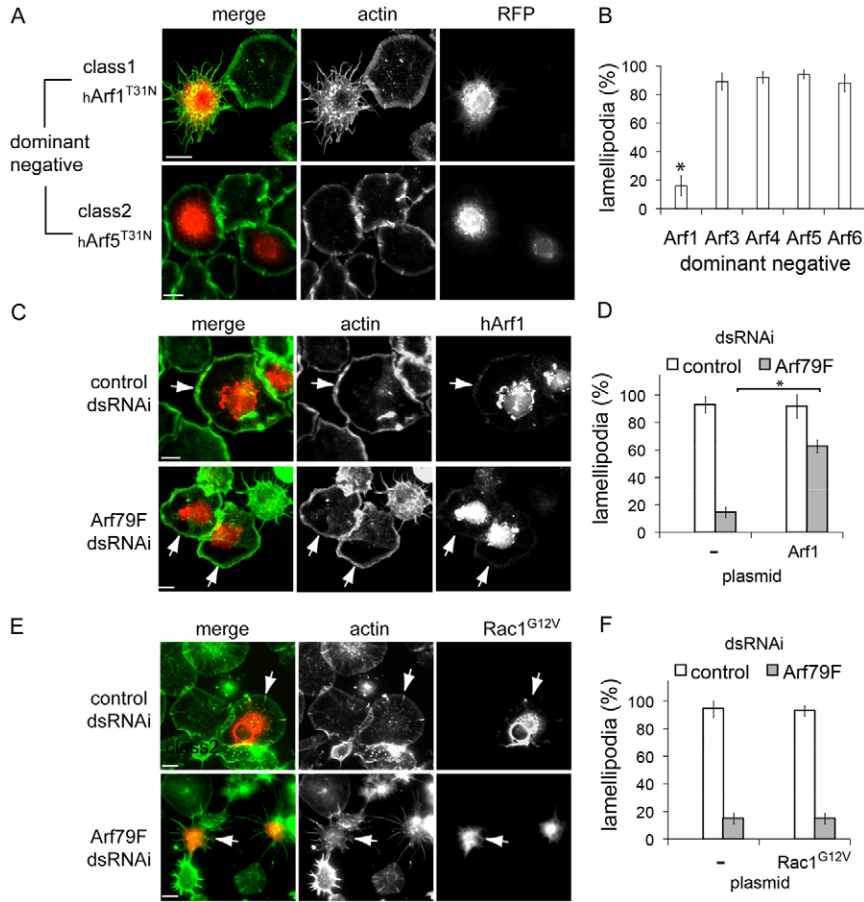


Fig. 2. Rescue of lamellipodium formation in Arf79F-silenced cells by Arf1 and Rac1. (A) Lamellipodia in cells expressing RFP-tagged human dominant-negative Arf GTPases (hArf), hArf1^{T31N} or hArf5^{T31N}, visualised by staining actin then quantified in (B), as Fig. 1D. Lamellipodia in control- or Arf79F-dsRNAi cells expressing either (C) hArf1-RFP or (E) HA-tagged Rac1^{G12V} visualised by staining actin and Rac1 with anti-HA antibody (arrows indicate transfected cells). Lamellipodia quantified in non-expressing (-) and either (D) hArf1-RFP or (F) Rac1^{G12V} cells from experiments in panels C and E were quantified as in panel B. Scale bars: 10 μ m.

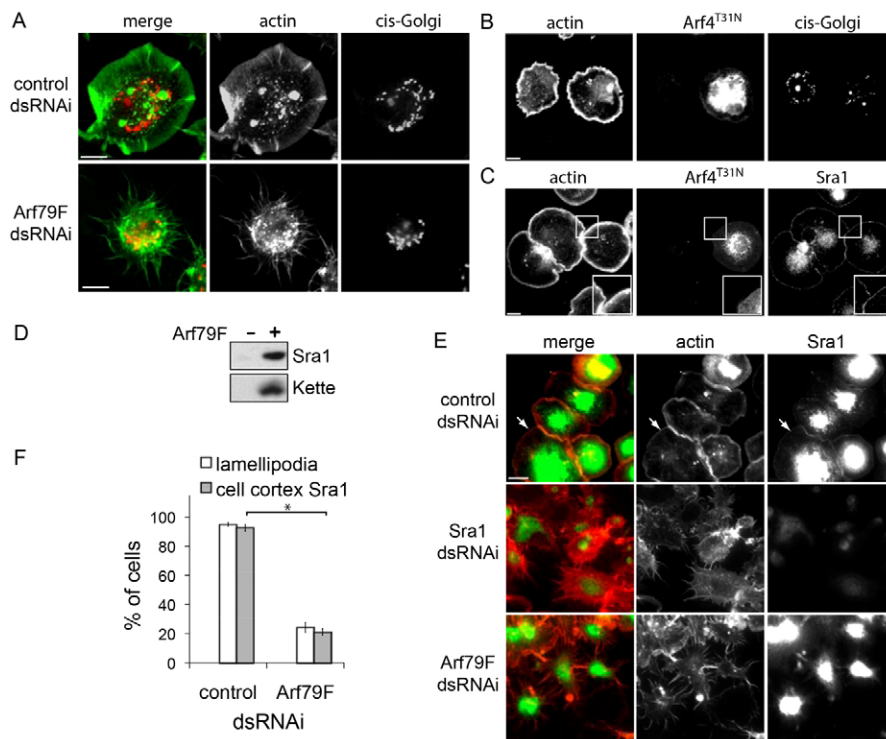


Fig. 3. Arf79F is required for Sra1 localisation to the plasma membrane. (A) *cis*-Golgi in control- or Arf79F-dsRNAi cells visualised with anti-GM130 antibody and actin as a control. Cells expressing RFP-tagged dominant-negative hArf4^{T31N} labelled with antibodies to (B) GM130 for the *cis*-Golgi or (C) Sra1 (magnified in insets) with actin as a control. (D) Arf79F recruitment of WRC components to the membrane. Silica beads coated with phospholipid bilayers alone (-) or with anchored myristoylated Arf79F loaded with GTP γ S (+) were isolated from *Drosophila* cell extract, then the recruited proteins were analysed by SDS-PAGE and immunoblotting with antibodies to Sra1 and Kette. (E) Sra1 in control-, Sra1- or Arf79F-dsRNAi cells were visualised at the cell cortex with anti-Sra1 antibody and actin as a control, then cell cortex Sra1 and lamellipodia were quantified (F) as in Fig. 1D ($P < 0.001$, Student's *t*-test). Scale bars: 10 μ m (A-C); 20 μ m (E).

of cells (Fig. 3C). These results suggest that Arf79F may facilitate lamellipodium formation independently of its role in Golgi organisation and membrane trafficking.

We recently demonstrated that mammalian Arf1 directly binds the structural homologues Sra1 and Nap1 (Koronakis et al., 2011) and therefore wondered whether Arf79F drives lamellipodium formation by recruiting WRC to the plasma membrane. First, purified *in vitro* myristoylated Arf79F was anchored to silica beads coated in a phospholipid bilayer consisting of equal amounts of phosphatidylcholine and phosphatidylinositol (PC:PI). Anchored Arf79F was locked in an active state by loading with non-hydrolysable GTP γ S before addition of the beads to S2R+ cell extract. The beads were isolated from the extract, washed and the recruited proteins extracted from the membrane before analysis by SDS-PAGE and immunoblotting to detect WRC components Sra1 and Kette (Nap1 homologue) (Fig. 3D). In contrast to control beads with no Arf79F (-), active Arf79F (+) recruited Sra1 and Kette demonstrating active Arf79F acts like mammalian Arf GTPases by recruiting the WRC to the membrane. We next tested whether Arf79F could also recruit the WRC to the cell plasma membrane by examining Sra1 localisation in Arf79F-silenced cells by immunofluorescence. In control cells, Sra1 was observed enriched at the perinuclear region and at the plasma membrane (arrows) in ~92% of cells (Fig. 3E,F). Sra1 was virtually non-detectable in control Sra1-silenced cells confirming the specificity of the Sra1 antibodies (Fig. 3E). In Arf79F-silenced cells, Sra1

was still found at the perinuclear area but cell cortex Sra1 was abolished in ~79% of cells.

WRC recruitment from cell extract by GTP bound Arf79F (Fig. 3D) indicated that Arf79F activation would be key to Sra1 plasma membrane localisation and therefore lamellipodium formation. To test this hypothesis, we employed Arf GEF inhibitors SecinH3 and BrefeldinA (BFA) (Fig. 4A,B). Lamellipodia were generated by ~95% of control cells which was reflected in Sra1 plasma membrane localisation in ~92% of cells. SecinH3 treatment abrogated lamellipodia and cell cortex Sra1 by ~35% (insets), while ~50% of remaining SecinH3-treated cells displayed an abnormal cell edge resulting in disjointed lamellipodia (white arrows), likely representing incomplete pathway inhibition as indicated by Sra1 plasma localisation in these cells (white arrows). BFA eliminated lamellipodia and Sra1 at the cell cortex in ~84% of cells. Differences in effectiveness between inhibitors is probably due to their mechanism where SecinH3 targets the cytohesin-family of Arf GTPase activators, Steppke in *Drosophila* (Donaldson and Jackson, 2011; Fuss et al., 2006; Hafner et al., 2006), while BFA forms a ternary complex with Arf1-GDP and Arf GEFs that would sequester class 1 Arf GTPase(s) (Mossessova et al., 2003). To dissect whether the reduction in Sra1 localisation ensues directly from inhibiting Arf or indirectly as a consequence of inhibiting lamellipodia, we uncoupled the two with the actin polymerisation inhibitors latrunculin B (LatB) and cytochalasin

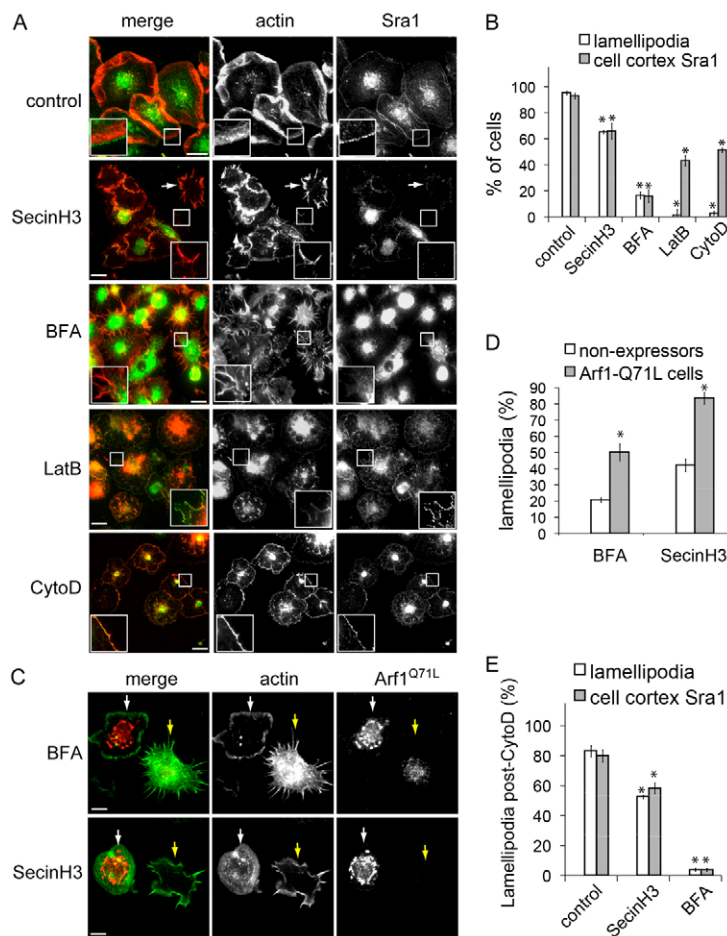


Fig. 4. Active Arf79F recruits Sra1 to the cell membrane. (A) Sra1 in control-, SecinH3-, BFA-, LatB- and CytoD-treated cells visualised at the cell cortex (magnified in insets) with anti-Sra1 antibody and actin as a control (arrows indicate disjointed lamellipodia), then cell cortex Sra1 and lamellipodia quantified (B) as in Fig. 1A. (C) Lamellipodia in cells expressing constitutively active Arf1^{Q71L} (white arrows) or adjacent non-expressing cells (yellow arrows) visualised by staining actin then quantified microscopically (D) as in Fig. 1D ($P < 0.05$, Student's *t*-test). (E) Lamellipodia and cell cortex Sra1 in cells treated with CytoD (30 min) that were subsequently incubated with either growth medium (control), SecinH3 or BFA (1 h) were quantified as in panel B. Scale bars: 20 μ m (A); 10 μ m (C).

D (CytoD) (Fig. 4A,B). While both inhibitors abrogated lamellipodium formation, cell cortex Sra1 still remained in ~44% of LatB-treated cells and ~51% of CytoD-treated cells (insets) demonstrating that Sra1 still localised to the cell periphery upon inhibition of lamellipodial actin polymerisation. Consistent with our finding, WRC components have been previously observed at the plasma membrane of mammalian cell treated with LatB (Weiner et al., 2007).

To address whether impaired lamellipodia following BFA- and SecinH3-treatment was due to inhibiting Arf79F, we examined whether expressing constitutively active Arf1^{Q71L} would restore lamellipodia (Fig. 4C,D). Relative to non-expressers (yellow arrows), cells expressing Arf1^{Q71L} generated ~2.5-fold more lamellipodia in BFA-treated cells (white arrows) and ~2-fold more in SecinH3-treated cells demonstrating that the Arf GEF inhibitors target *Drosophila* Arf1 (i.e. Arf79F).

Having established that Arf79F and its activation is required for maintaining lamellipodia, we investigated whether active Arf79F triggers re-generation of lamellipodia following their destruction by CytoD (Fig. 4E). When CytoD-treated cells were washed then subsequently incubated with either growth media (control), SecinH3 or BFA, lamellipodia were produced in ~83% of control cells but only in ~52% SecinH3-treated cells and ~4% of BFA-treated cells demonstrating a role for active Arf79F in establishing new lamellipodia. The impairment in lamellipodium formation caused by Arf GEF inhibition was mirrored in the cell cortex localisation of Sra1 indicating that recruitment of Sra1 to the plasma membrane by Arf79F is key for triggering lamellipodia.

Arf GTPases are best known for their roles in membrane trafficking and organelle organisation (Donaldson and Jackson, 2011) but they have also been implicated in actin-dependent processes at the plasma membrane (Boulay et al., 2008; Cohen et al., 2007; Myers and Casanova, 2008; Stalder et al., 2011). Here, we establish that Arf79F not only localises at the cell edge with the WRC component Sra1 but is also essential for lamellipodium formation. This builds on our recent demonstration that Arf1 directly recruits and activates mammalian WRC *in vitro* (Koronakis et al., 2011).

High-throughput RNAi screening of *Drosophila* S2R+ cells has enabled the discovery of cellular factors driving lamellipodia (D'Ambrosio and Vale, 2010; Kiger et al., 2003; Rogers et al., 2003; Rohn et al., 2011); though as yet, Arf79F has not been implicated. This could be due to a 8% false negative rate in RNAi screens (Booker et al., 2011) and the relatively long time required for complete Arf79F dsRNAi (Fig. 1C; supplementary material Fig. S1A). Our discovery that Arf79F is key to lamellipodia establishes a new platform to dissect the Arf–WRC pathway. This role appears to be specific for Arf79F as Class 1 but not Class 2 or 3 Arfs were required for lamellipodia. This was surprising as WRC can be activated in cell extract by divergent Arf GTPases such as Arf5 (Arf102F homologue) and Arl1 (Koronakis et al., 2011). However, Gautier and colleagues showed that Arf79F, but not related *Drosophila* Arf isoforms, was found bound to immunoprecipitated WRC (Gautier et al., 2011) underlining Arf specificity for the WRC. Since Arf1 alone is insufficient for WRC activation *in vitro* (Koronakis et al., 2011) and Arf must cooperate with Rac1 for WRC-driven actin assembly (Humphreys et al., 2012; Koronakis et al., 2011), it seems that it is only active Arf79F which is temporally and spatially coincident with Rac1 activation in *Drosophila* cells.

Recent structural studies of WRC indicate that its plasma membrane localisation is mediated by Sra1–Rac1 interactions that would release the SCAR/WAVE VCA domain for Arp2/3 activation (Chen et al., 2010). However, WRC–Rac1 interactions are of low affinity suggesting recruitment and activation of WRC requires additional factors (Davidson and Insall, 2011). This is supported by a recent report revealing a role for clathrin in WRC recruitment which is independent of its connection to Arf–AP1/AP2 trafficking (Gautier et al., 2011). However, we demonstrated that Rac1 is insufficient for WRC activation (Koronakis et al., 2011) so while clathrin could promote the recruitment of WRC to the plasma membrane, its activation would only be triggered when both active Arf and Rac1 cooperate. We showed the role for Arf79F in lamellipodium formation is independent of Rac1 activation and trafficking. Consistent with this, we confirmed effects on lamellipodia were not due to altered Golgi function implying direct Arf–WRC interactions and not indirect Arf-mediated membrane trafficking triggers WRC-signalling. A direct mechanism involving Arf79F is further supported by its ability to recruit WRC components *in vitro* which was reflected in cells where active Arf79F was critical for Sra1 localisation and concomitant generation of lamellipodia. The data indicate Arf GTPase is a key component of the WRC signalling pathway in cells.

Materials and Methods

Plasmids and recombinant proteins

The following plasmids were generated by Invitrogen Gateway methodology: pAHW-Rac-V12 (Rac1G12V) and pMT-RFP (Liu et al., 2010) were used to create RFP-tagged Arf79F, human Arf1, Arf1Q71L, Arf1T31N, Arf3T31N, Arf4T31N, Arf5T31N and Arf6T27N. Arf79F was cloned into pET20b, expressed and purified as previously for human Arf1 (Koronakis et al., 2011).

Drosophila cell culture, transfection and dsRNAi

S2R+ cells were cultured at 25°C in M3 insect medium (Sigma) with 10% heat-inactivated fetal bovine serum (JRH Biosciences) and 1% penicillin/streptomycin (Sigma–Aldrich) as described (Liu et al., 2010). When appropriate, cells were incubated with 0.5 µM Latrunculin B or cytochalasin D, 5 µM Brefeldin A (Sigma–Aldrich) or 25 µM SecinH3 (Merck) for 1 h. *Drosophila* cell transfection and RNAi experiments were carried out on concanavalin A-coated coverslips as previously described (Liu et al., 2010). RNAi was performed with double-stranded RNA generated from the following primer pairs: Arf79F (Arf79F_01_f: 5'-TAATACGACTCACTATAGGGTGTGATTGGTTGGCTTTT-3', Arf79F_01_r: 5'-TAATACGACTCACTATAGGGACATCAGAAGGAATTTTCACAAA-3' and Arf79F_02_f: 5'-TAATACGACTCACTATAGGAGGGGTAGGGAGAGGACAA-3', Arf79F_02_r: 5'-TAATACGACTCACTATAGGAACTGGTACATCCAGGCGAC-3'); Arf102 (Arf102F_f: 5'-TAATACGACTCACTATAGGATGCTGTGG-AAAACGACT-3', Arf102F_r: 5'-TAATACGACTCACTATAGGCCATGTTC-TGTAGTTCTCTTTCAGC-3'); Arf51 (Arf51F_f: 5'-TAATACGACTCACTATAGGTAACCCCATGAAATCCAG-3', Arf51F_r: 5'-TAATACGACTCACTATAGGCTTGGCGTTGGTTTTCTTTG-3'); SCAR (Scar_f: 5'-TAATACGACTCACTATAGGTCGATGAGGACGCACTAATTT-3', Scar_r: 5'-TAATACGACTCACTATAGGCACGTCGCGGGCCAGTTC-3'); Sra1 (Sra1_f: 5'-TAATACGACTCACTATAGGAGTTATGGGCTTTGGCCTGT-3', Sra1_r: 5'-TAATACGACTCACTATAGGAAATGCACCATTAGATCCGC-3'); Kette (Kette_f: 5'-TAATACGACTCACTATAGGATGGAATCCAGTAGGCGGG-3', Kette_r: 5'-TAATACGACTCACTATAGGATTCGATGCCACGGATCTTA-3'); Rac1 (Rac1_f: 5'-TAATACGACTCACTATAGGTCGAAACACGGTGGGTATGTA-3', Rac1_r: 5'-TAATACGACTCACTATAGGAGCAACAGTCTCCGCCG-3'). RNAi was quantified in each case by EXPRESS One-Step SYBR® GreenER qRT-PCR according to manufacturer's instructions (Invitrogen) with RP49 as an internal control.

Immunofluorescence

Immunofluorescence microscopy and images assembled as described (Humphreys et al., 2009). RFP intensity (arbitrary units) within regions-of-interest at the cell edge were compared to equivalent regions in the cytoplasm (4 regions per cell) using Velocity 3D Image Analysis Software (PerkinElmer). Antibodies used were Sra1 and Kette (gifts from Buzz Baum), anti-Arf1 (Abcam-ab58578) and anti-GM130 (Abcam-ab30637). Alexa Fluor-labelled secondary antibodies, Alexa Fluor- and TxRed-labelled phalloidin (Invitrogen).

WRC recruitment to Arf79F anchored phospholipid beads

Drosophila S2R+ cell extract was prepared by passage through a 22-gauge needle in 10 mM HEPES (pH 7.4), 100 mM KCL, 1 mM MgCl and Complete EDTA-free protease inhibitor cocktail (Roche) then ultracentrifugation (180,000 g, 20 min) separated membranes from the high-speed supernatant cytoplasm which was used for experiments. Preparation of phospholipid-coated beads and pulldowns have been described in detail (Koronakis et al., 2011).

Statistics

In all experiments ($n \geq 3$), at least 300 cells were analysed for quantification per experiment. Comparisons between two data points were made using Student's *t*-test, between three or more data points using ANOVA combined with post-hoc Bonferroni's multiple comparison test. Asterisks indicate $P < 0.01$ unless stated otherwise. Error bars represent \pm s.e.m.

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Supplementary material available online at

<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.108092/-/DC1>

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