Podosome assembly is controlled by the GTPase ARF1 and its nucleotide exchange factor ARNO

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Abstract

Podosomes represent a class of integrin-mediated cell-matrix adhesions formed by migrating and matrix-degrading cells. Here, we demonstrate that in macrophage-like THP1 cells and in fibroblasts stimulated to produce podosomes, down-regulation of G protein ARF1 or the ARF1-GEF ARNO by siRNAs or by pharmacological inhibitors led to striking podosome elimination. Concomitantly, treatments inducing podosome formation increased the level of GTP-bound ARF1. ARNO was found to co-localize with the adhesive rings of podosomes while ARF1 was localized to vesicular structures transiently contacting podosome rings. Inhibition of ARF1 led to an increase in RhoA-GTP levels and triggered assembly of myosin-IIA filaments in THP1 cells, whilst the suppression of myosin-IIA rescued podosome formation regardless of ARF1 inhibition. Finally, expression of constitutively active ARF1 in fibroblasts induced formation of putative podosome precursors: actin-rich puncta coinciding with matrix degradation sites and containing proteins of the podosome core but not of the adhesive ring. Thus, ARNO-ARF1 regulates formation of podosomes by inhibition of RhoA/myosin-II and promotion of actin core assembly.
Introduction

Podosomes are a distinctive form of integrin-mediated cell-matrix adhesion typical of monocyte-derived cells but under some circumstances are produced by cells of other lineages. They usually appear as micron-sized radially symmetrical protrusions containing central actin cores (height about \( \sim 2 \mu m \)) rooted in the cytoplasm surrounded by matrix-associated “adhesive rings” (\( \sim 1 \mu m \) diameter) enriched in integrins and plaque proteins such as talin, paxillin, vinculin and Tks5 (Calle et al., 2006; Cox and Jones, 2013; Labernadie et al., 2014; Meddens et al., 2014; Murphy and Courtneidge, 2011; Seano et al., 2014; Wiesner et al., 2010). In the majority of cell types, podosomes form arrays consisting of numerous individual podosomes connected to each other via a mesh of F-actin-containing links containing myosin-II (Cox et al., 2012; Dries et al., 2013; Panzer et al., 2016). Individual podosome-like structures formed by invasive cancer cells are more stable, protrusive and larger in size than normal podosomes and are often termed invadopodia (Gimona et al., 2008; Murphy and Courtneidge, 2011). Podosomes participate in the processes of cell migration and invasion as well as degradation of extracellular matrix via secretion of matrix metalloproteinases (El Azzouzi et al., 2016; Gawden-Bone et al., 2010; Linder and Wiesner, 2015; Wiesner et al., 2010).

Cells of monocytic origin (for example cultured macrophage-like THP1 cells) form numerous podosomes upon stimulation with transforming growth factor beta (TGF\( \beta \)) or increasing protein kinase C (PKC) activity by phorbol esters (e.g. PMA). Moreover, upon appropriate stimulation, even podosome-lacking cells can be forced to form podosome-like structures. In particular, expression of constitutively active Src in fibroblasts triggers formation of high-order adhesion structures termed podosome “rosettes”, which are capable of degrading the ECM (Tarone et al., 1985). More recently, we have shown that non-transformed fibroblasts that typically do not form podosomes develop podosome-like adhesions under conditions where a cell cannot apply strong traction force to nascent integrin clusters, such as
spreading on fluid RGD-functionalized lipid bilayers, where stress fibres fail to assemble (Yu et al., 2013).

A key process in podosome formation is a local polymerization of actin cores primarily mediated by Arp2/3 complex activated by Wiskott–Aldrich Syndrome protein (WASP) (Burns et al., 2001; Linder et al., 1999; Machesky and Insall, 1998). In turn, WASP activation depends largely upon the activity of the small G protein Cdc42 and can be regulated by WASP-interacting protein (WIP) (Abdul-Manan et al., 1999; Calle et al., 2004; Monypenny et al., 2011; Schachtner et al., 2013; Vijayakumar et al., 2015). Indeed, microinjection of dominant negative Cdc42 has been shown to significantly impair podosome formation in human dendritic cells (Burns et al., 2001). Similarly, podosome formation is impaired in cells microinjected with dominant negative Rac1 (Burns et al., 2001), as well as in Rac1- and especially Rac2-depleted cells (Wheeler et al., 2006) though the downstream pathways are not yet elucidated. Conversely, active RhoA, which typically promotes assembly of stress fibres and focal adhesions, has been generally described to be low in podosome-forming cells (Pan et al., 2011; Yu et al., 2013) and microinjection of active RhoA impairs podosome formation (Burns et al., 2001).

While the role of Rho family GTPases in podosome formation is relatively well-documented, the function of ARF family of G proteins is essentially unknown. Even though these proteins are considered mainly as regulators of membrane traffic, some evidence exists that they also participate in a variety of processes related to regulation of the actin cytoskeleton and involved in a crosstalk with the G proteins of the Rho family. In particular, ARF1, the most abundant ARF family member, known to recruit the coatamer complexes for vesicle budding in the Golgi (Donaldson and Jackson 2011), was shown to be required for clathrin-independent endocytosis (CLIC-GEEC) (Kumari and Mayor, 2008), as well as for formation of “ventral actin structures” in some cell types (Caviston et al., 2014). Thus ARF1 is a potentially interesting candidate for function as a podosome regulator since it could
control fundamental systems involved in podosome formation, actin cytoskeleton and the plasma membrane.

In this study, we demonstrate that regardless of particular stimuli, ARF1 is required for inducing podosome formation in different cell types. Moreover, these stimuli, via an ARF exchange factor ARNO, increase the fraction of GTP-bound ARF1 in cells. ARNO localizes to the adhesive ring of podosomes, and its inhibition interferes with podosome assembly. We demonstrate that the ARNO-ARF1 pathway regulates podosomes by inhibition of RhoA- and ROCK-dependent formation of myosin-II filaments, which antagonizes podosome integrity. In addition, constitutively active ARF1 induces formation of actin-rich puncta co-localizing with matrix degradation sites and containing podosome core markers. Our data strongly suggest a direct role for ARF1 in podosome-type adhesions, and further extends the increasing number of roles for ARF1 at the plasma membrane.
Results

Depletion of endogenous ARF1 interferes with podosome formation

Stimulation by either transforming growth factor-beta 1 (TGFβ1) or the protein kinase C activator, phorbol ester 12-myristate 13-acetate (PMA), has been previously used as a model system to study podosome formation and dynamics in several cell types (Burger et al., 2011; Monypenny et al., 2011; Tatin et al., 2006; Varon et al., 2006). Consistent with numerous previous studies, we define podosomes as F-actin-rich spots with a diameter of about 0.5 μm surrounded by an approximately ring-shaped vinculin-rich zone. We consider a cell as “podosome-forming” if it had more than 10 morphologically identifiable podosomes.

In this study, 85 ± 4.7% (mean ± SD, n =3 independent experiments) of cells of the human monocytic cell line, THP1, plated on fibronectin-coated substrata in the presence of TGFβ1, formed podosomes after 24 hours, with 55 ± 3.2 (mean ± SEM, n=212 cells) podosomes per cell. The corresponding numbers for PMA-stimulated cells were 88 ± 5.5% and 140 ± 19.5 (n=80 cells), respectively. For cells plated on fibronectin in the absence of any additional stimuli, the percentage of podosome-forming cells was only 15 ± 4.7% (n =3 independent experiments) and even among these cells the average number of podosomes did not exceed 22± 8.7 (n= 58 cells).

To investigate the role of ARF1 in podosome dynamics, we depleted ARF1 in TGFβ1-treated THP1 cells by siRNA. Immediately prior to plating, cells were electroporated with the ARF1 or control siRNAs and then seeded onto fibronectin in the presence of TGFβ1. We observed that maximum silencing (>95%) was achieved by 48 hours (Figure 1A).

Depletion of ARF1 led to dramatic decrease of podosome number in TGFβ1-treated cells (Figure 1B,C). Both the number of podosomes per cell and percentage of podosome-forming cells significantly dropped upon ARF1 depletion (Figure 1D-F).
Whilst the mean number of podosomes per cell and percentage of podosome-forming cells in cells transfected with control siRNA did not differ from aforementioned control numbers, the cells transfected with ARF1 siRNA had on average only $8 \pm 1.7$ (n= 96 cells) podosomes per cell. Total intensity of F-actin-containing non-podosomal structures at the ventral surface of the cells became somewhat higher in ARF1-depleted cells (Figure 1B and C, left panels). At the same time, the vinculin-containing non-podosomal structures at the cell periphery that can be classified as small focal adhesions were not apparently affected (Figure 1B and C, right panels). Depletion of ARF1 by siRNA did not affect the integrity of the Golgi apparatus as visualized by cis-Golgi markers GM130/GRASP65 (Figure 1B and C) in agreement with previous publications (Nakai et al., 2013; Szul et al., 2007; Volpicelli-Daley et al., 2005).

The effect of ARF1 siRNA on podosome number was specific since it could be fully reversed by expression of exogenous bovine HA-ARF1 insensitive to human ARF1 siRNA (Figure 1D-F). Interestingly, exogenous HA-ARF1 was often localized in the spots adjacent to the podosomes of transfected cells (Figure 1D'). See below for a detailed analysis of localization dynamics. Significantly, we found that loss of podosome induction due to ARF1 depletion was not exclusive to TGFβ1 stimulation, since after PMA stimulation (Supplementary Figure S1A-I), ARF1 depleted cells demonstrated a significant decrease in both the number of podosomes and the percentage of podosome-forming cells as compared to control cells (Supplementary Figure S1A, D, F, G).

In contrast to ARF1, depletion of ARF6 by siRNA with a silencing efficiency of >95% (Supplementary Figure S1J) did not affect podosome induction in TGFβ1-treated THP1 cells (Supplementary Figure S2K). Both the average number of podosomes per cell and the percentage of cells forming more than 10 podosomes were not significantly different from control siRNA-treated cells (Figure 1E and 1F). Taken
together, these data indicate a specific role for ARF1 in podosome induction in stimulated THP1 cells.

We also examined the effect of expression of dominant negative and constitutively active mutants of ARF1 on adhesion of THP1 cells in the presence of TGFβ1. The dominant negative mutant, CFP-ARF1 (T31N), led to a significant decrease of cell adhesion to fibronectin under these conditions (control: 71 ± 5.5%, ARF1 T31N: 4 ± 0.3%) and the few adherent cells observed did not form podosomes (data not shown). This behaviour resembles a “non-adhesive phenotype”, described previously in the culture of normal human dendritic cells (Burns et al., 2004). Unexpectedly, the expression of a constitutively active mutant of ARF1, CFP-ARF1 (Q71L), also interfered with cell adhesion and completely prevented podosome formation. Thus, sustained high activity of ARF1 is also damaging for cell adhesion and podosome formation in THP1 cells. Overexpression of wild-type ARF1 or constitutively active ARF1 in unstimulated THP1 cells did not induce any apparent phenotypic changes. These cells remain poorly attached to the fibronectin and do not form podosomes.

**ARF1-containing vesicles transiently contact podosomes**

We used fluorescently-tagged ARF1 to further elucidate the localization and dynamics of ARF1 in TGFβ1-stimulated THP-1 cells. Expression of GFP-ARF1 showed a predominant Golgi localization (Figure 1D) in agreement with previous publications (Lippincott-Schwartz et al., 1989; Sciaky et al., 1997). However, in addition to the Golgi localization, we found numerous irregular puncta throughout the cell, some of which were apparently associated with podosomes (Figure 1D’ and Figure 2A).

We employed total internal reflection fluorescence (TIRF) microscopy to explore the spatiotemporal dynamics of ARF1 puncta at the plasma membrane. GFP-ARF1
puncta displayed temporal localization at regions of podosome assemblies and made transient periodic contacts with the adhesive rings of podosomes, as marked by mCherry-vinculin in THP1 cells (Figure 2B, 2B’ and Supplementary movie 1). Up to 80% of podosomes appeared to be in contact with ARF1-containing puncta during 5-minute periods of observation (Figure 2C) with an average dwell time of 10 ± 1.6 seconds (mean ± SEM, Figure 2D). In contrast, CFP-ARF6 does not form puncta-like structures in THP1 cells and no preferential localization of CFP-ARF6 to regions of podosome assembly was found (Supplementary Figure S1L). We further characterized the GFP-ARF1 associated puncta by determining if their mobility was dependent on a cytoskeletal network. We found that GFP-ARF1 patches appeared to be travelling on microtubule tracks identified by labeling with mCherry-ensconsin (Figure 2E,F and Supplementary movie 2). To elucidate the nature of the ARF1 puncta, we co-express GFP-ARF1 with several markers of vesicular traffic carriers, Rab6, Rab7, Rab8 and Rab11 (Supplementary Figure S2A-D). Amongst those, Rab11 (Welz et al., 2014) demonstrated significant co-localization (Supplementary Figure S2E) with ARF1 suggesting that ARF1-containing puncta have a vesicular nature.

**Inhibition of ARF1 activity interferes with formation of podosomes induced by diverse stimuli**

To study the immediate effect of ARF1 inhibition on podosome formation, we used two inhibitors known to suppress ARF1 activity. Brefeldin A (BFA) promotes formation of complexes between GDP-bound ARF1 and Sec7 domains of ARF1 nucleotide exchange factors GBF1, BIG1 and BIG2, and prevents completion of the nucleotide exchange reaction (D'Souza-Schorey and Chavrier, 2006), while sechinH3 inhibits activity of another group of ARF exchange factors, cytohesins (1-4), by binding to their Sec7 domain, without formation of a complex with ARF1 (Casanova, 2007).
Using a G-LISA assay for the measurement of ARF1-GTP levels, we demonstrate that TGFβ1 or PMA treatment of THP1 cells enhances the fraction of active, GTP-bound ARF1, while both secinH3 and BFA significantly reduced it (Figure 3A, Supplementary Figure S1C). Both secinH3 and BFA treatment induced rapid disassembly of all podosomes in about 30-40 minutes (Figure 3B-D, graphs 3E, F and Supplementary movie 3). In the case of secinH3, this process was accompanied by a burst of lamellipodial activity (Figure 3B), the integrity of the Golgi apparatus, as well as localization of ARF1 to Golgi and to vesicular structures in the cytoplasm was not affected (Figure 3B, 3C). Unlike secinH3 treatment, disruption of podosomes with BFA was accompanied by loss of ARF1 localization at the Golgi and at cytoplasmic vesicular structures (Figure 3D) as well as structural disintegration of the Golgi apparatus, in agreement with numerous previous studies (Donaldson et al., 2005; Lippincott-Schwartz et al., 1989). Thus, comparison between the SecinH3 and BFA effects confirmed that active ARF1 is required for podosome integrity and this function of ARF1 does not depend on its role in Golgi stabilization. A second confirmation of independence of podosomes from Golgi traffic can be inferred from experiments with knockdown of COPB1, a subunit of the COPI coatomer protein complex required for retrograde transport from trans-Golgi to cis-Golgi and endoplasmic reticulum (Beck et al., 2009). We found that COPB1 knockdown or its inhibition generated only minor effects on podosome integrity (Supplementary Figure S3A-E). Finally, disruption of podosomes with secinH3 or BFA still proceeded (albeit in a slower rate) in TGFβ1-stimulated THP1 cells expressing constitutively active Cdc42 (GFP-Cdc42 Q61L), a potent podosome-inducing signalling protein (Supplementary Figure S3H). Moreover, treatment of cells with BFA or SecinH3 as well as knockdown of ARNO or ARF1 did not change the level of Cdc42-GTP in TGFβ-stimulated THP1 cells (Supplementary Figure S3I and J).

Visualization of podosomes using structured-illumination microscopy (SIM) revealed a central F-actin core surrounded by patches enriched in adhesion proteins (talin and vinculin) as well as the thin F-actin-rich links connecting neighboring
podosomes (Supplementary Figure S4A, boxed image S4A’ and Supplementary movie 4) in agreement with previous studies (Cox et al., 2012; Dries et al., 2013). Treatment with secinH3 led to the rapid disappearance of the connecting links and gradual concurrent disassembly of both the actin cores and surrounding adhesive rings (Supplementary Figure S4B, C and Supplementary movie 5).

In view of the high podosome turnover rate (Dries et al., 2013), gradual disruption of podosomes upon addition of secinH3 suggests that inactivation of ARF1 changes the balance between podosome assembly and disassembly rather than completely block the assembly processes. The process of disassembly often proceeds through podosome fission and is accompanied by an apparent increase of podosome mobility in the plane of the plasma membrane (Supplementary Figure S4D-F).

We next studied the effect of inhibition of ARF1 on podosome-like structures formed by fibroblast-type cells. Under standard culture conditions, mouse embryonic fibroblasts (MEFs) generally form focal adhesions, which appeared to be resistant to treatment with either BFA (Bershadsky and Futerman, 1994) or SecinH3 (Supplementary Figure S5A). It was recently shown that fibroblasts plated on a fluid substratum (supported RGD-functionalized lipid bilayer), under conditions where they cannot exert traction forces, by default formed podosome-like adhesion structures (Yu et al., 2013). We showed that the level of GTP-ARF1 increased in MEF plated on supported lipid bilayers (Figure 3G). Similarly to “classic” podosomes, podosome-like structures formed by MEFs plated on the lipid bilayer underwent rapid disassembly upon treatment with either BFA or SecinH3 (Figure 3H, I).

A well-known method of induction of podosome-like structures in fibroblast-like cells is ectopic expression of constitutively active Src. In agreement with published results (Tarone et al., 1985), expression of Src Y527F in MEFs led to the formation of prominent rosettes formed as a result of fusion of numerous podosome-like structures (Supplementary Figure S5B and C). Treatment of such cells with either
BFA or SecinH3 resulted in the gradual disassembly of these rosettes and a decrease in the number of rosette-positive cells (Supplementary Figure S5B and C).

Altogether, these data demonstrate that ARF1 activity is required for formation/maintenance of podosome-like structure irrespective of upstream stimuli (TGFβ1, PMA, active Src or fluid substratum). We conclude that a role for ARF1 in podosome dynamics is independent of early signalling pathways that lead to initiation of cellular differentiation to a podosome-generating phenotype.

**ARNO GEF activates ARF1 to drive podosome formation**

The mammalian ARF GTPases are activated by 15 different GEFs categorized in five classes. Among these, only 7 GEFs can activate ARF1: 3 BFA-sensitive (GBF1, BIG1, BIG2) and 4 SecinH3-sensitive (cytohesins 1-4) (D'Souza-Schorey and Chavrier, 2006; Donaldson and Jackson, 2011). Both BFA- and SecinH3-sensitive GEFs share a common conserved SEC7 domain that promotes GDP release and subsequent GTP binding to ARF1. SecinH3-sensitive GEFs have in addition a pleckstrin homology (PH) domain that enables them to interact with phosphoinositides at the plasma membrane (DiNitto et al., 2007; Santy et al., 1999).

We examined the effect of inhibition of a number of ARF1 GEFs on the process of podosome formation (Supplementary Figure S3B, D-G). We found that expression of dominant negative mutants of two BFA-sensitive GEFs, HA-BIG1 (E793K) and HA-BIG2 (E738K) did not prevent formation of podosomes in THP1 cells treated with TGFβ1 (Supplementary Figure S3F and G, lower panel). Conversely podosomes formation was not affected by overexpression of wild-type HA-BIG1 or HA-BIG2 (Supplementary Figure S3F and G, upper panel). Furthermore, both the wild-type HA-BIG1 and HA-BIG2 showed predominant Golgi localization (Supplementary Figure S3F and G) and no podosome localization, consistent with previous reports (Citterio et al., 2006; Ishizaki et al., 2008). To inhibit the activity of the third BFA-sensitive GEF, GBF1, we used a small-molecule inhibitor, Golgicide A (Saenz et al.,
We found only partial dissolution of podosomes in THP1 cells treated with TGFβ1 after application of Golgicide A in a concentration that induced visible fragmentation of the Golgi complex (Supplementary Figure S3B, D and E). Thus, in spite of profound inhibitory effect of BFA on podosome formation, selective inhibition of the BFA-sensitive GEFs produced only minor effect on podosomes.

In contrast, knockdown of one of the SecinH3-sensitive GEFs, ARNO (cytohesin-2), significantly affected podosome formation in TGFβ1- (Figure 4A-D) or PMA-activated THP1 cells (Supplementary Figure S1B, E, H, I). Knockdown of ARNO led to a significant decrease in the number of podosomes per cell as well as the percentage of cells having more than 10 podosomes (Figure 4C and 4D). Additionally we found that ARNO knockdown reduced ARF1 activity in THP1 cells stimulated by TGFβ1 (Figure 4E). Moreover, a dominant negative mutant of ARNO (E156K) also suppressed podosome formation/maintenance (Figure 4F and 4G). The effect of ARNO knockdown on podosomes was specific since knockdown of another secinH3-sensitive GEF, cytohesin-1 did not inhibit podosome formation in stimulated THP1 cells (Figure 4B). Neither ARNO nor cytohesin-1 knockdown produced any significant effect on Golgi integrity (Figure 4B).

Next, we investigated the localization of ARNO and cytohesin-1 in TGFβ1-treated THP1 cells by expressing GFP-fusion construct of these GEFs. ARNO was found to localize to the rings surrounding the actin cores of podosomes (Figure 5A). Similarly, GFP-ARNO localized to the rim around the actin core of podosome rosettes (Figure 5B). Moreover, podosome-like structures formed by normal fibroblasts plated on RGD-functionalized lipid bilayers also contain ARNO in the rings surrounding the actin cores (Figure 5C). Live imaging of GFP-ARNO in all these situations revealed that ARNO localization at the podosome ring was stable and spanned the entire lifetime of a podosome (Figure 5D,D′ and Supplementary movie 6). Unlike ARNO, Cytohesin-1 showed diffuse localization over the plasma membrane and was not enriched at podosomes (Figure 5E).
**Inhibition of ARF1 triggers podosome disassembly via activation of Rho and myosin-IIA**

In search of downstream factors that mediate podosome disruption upon ARF1 inhibition we checked the activity of three major Rho family G proteins in TGFβ1-treated THP1 cells. We found that the fraction of RhoA-GTP significantly increased upon inhibition of ARF1 by SecinH3 (Figure 6A), while activities of both Rac1 and Cdc42 did not change (Figure 6B and C). RhoA and Rho-associated kinase (ROCK) are master regulators of myosin-IIA-driven cell contractility, since ROCK-mediated activation of myosin regulatory light chain (MRLC) phosphorylation promotes assembly of myosin-II filaments as well as myosin-II motor activity (Vicente-Manzanares et al., 2009). Indeed, we have demonstrated that inhibition of ARF1 by SecinH3 promoted assembly of the myosin-II filaments visualized by live imaging of GFP-MRLC using SIM (Figure 6D and Supplementary movie 7). Simultaneous visualization of podosomes and myosin-II filaments revealed that podosome disappearance occurred in those cell regions enriched in myosin-II filaments (Figure 6D), suggesting that podosome disassembly is triggered by local activation of myosin-II-driven contractility. Indeed, treatment of ARF1-inhibited cells lacking a majority of podosomes with an inhibitor of ROCK, Y-27632, led to a burst of podosome formation concurrent with the disappearance of myosin-II filaments (Figure 6E and Supplementary movie 8). To confirm that inhibition of ARF1 led to podosome disruption via activation of myosin-II filament assembly, we performed siRNA-mediated myosin-IIA heavy chain (MYH9) knockdown, which completely blocked formation of myosin-IIA filaments as visualized by antibody to NM-MHCIIA (Figure 6F-H). Myosin-II knockdown by itself did not affect podosome integrity (Figure 6F, I, K and L). While treatment of THP1 cells transfected with control siRNA by SecinH3 led to pronounced disassembly of podosomes (Figure 6G, K and L), the same treatment on myosin-IIA knockdown cells did not disrupt podosomes (Figure 6J, K and L).
Constitutively active ARF1 induces actin-rich puncta in fibroblasts

To test if constitutively active ARF1 could induce formation of podosome-like adhesions in a more general context, we expressed constitutively active ARF1, CFP-ARF1 (Q71L), in cells that normally do not form podosomes, such as mouse embryonic fibroblasts (MEFs). Overexpression of constitutively active but not wild type ARF1 induced formation of numerous actin-rich puncta localized to the ventral surface of these cells, in the same focal plane as focal adhesions (Figure 7A and B). Similar to mature podosomes, the actin-rich puncta induced by constitutively active ARF1 (CFP-ARF1 Q71L) were transiently associated with CFP-ARF1 (Q71L)-containing vesicles (Figure 7B'). Formation of these puncta was accompanied by some reduction in the number of stress fibres and focal adhesions, but even total disassembly of these structures upon expression of dominant negative RhoA (GFP-RhoA T19N) was not sufficient to induce actin-containing puncta (Figure 7C). At the same time, constitutively active ARF1 efficiently triggered formation of such puncta in cells also expressing dominant negative RhoA (Figure 7D). Similarly, inhibition of Rho activity by cell-permeable C3 transferase (2 μg/ml) did not by itself induce formation of the actin puncta and did not interfere with the induction of these puncta by constitutively active ARF1 (Supplementary Figure S5D and E).

Proteins typically associated with podosome cores in different cell types (WIP, N-WASP, cortactin, Arp3, dynamin-2) were found in the actin-rich puncta (Supplementary Figure S5F-J). At the same time, protein components of the podosome “ring”, such as vinculin (not shown) and paxillin (Supplementary Figure S5K), were not found to be associated with active ARF1-induced actin puncta suggesting incomplete podosome formation. ARF1-induced actin puncta were not related to clathrin-depended endocytic activity since they did not co-localize with clathrin-coated pits (Supplementary Figure S5L).
Unlike native podosomes, the puncta induced by constitutively active ARF1 were motile. While podosomes of THP1 cells (Supplementary Figure S4D, right insets), as well as podosome-like structures in fibroblasts plated on fluid substrate (Yu et al., 2013), are essentially stationary with respect to the substratum, the positions of puncta induced by active ARF1 are oscillating with an average velocity of 0.88 ± 0.28 μm/s (± SD). Thus constitutively active ARF1 induced formation of actin-rich puncta in proximity to the ventral cell membrane that can be considered as incompletely anchored podosome-like structures and possibly podosome precursors.

In spite of the difference between authentic podosomes and the actin-rich puncta induced by constitutively active ARF1, the puncta mimic one important podosome function namely, matrix metalloproteinase (MMP)-dependent ability to degrade the matrix. Indeed, the positions of actin puncta induced in the fibroblasts by constitutively active ARF1 (Q71L) coincided with the sites of matrix degradation - dark areas on the substratum covered with fluorescently-labeled gelatin (Figure 7G, G'). Formation of such dark areas could be prevented by treatment with 25 μM of MMP inhibitor GM6001 (Figure 7H,H'), and therefore depended on the exocytosis of MMPs by cells. Thus, our experiments showed that actin puncta induced in fibroblasts by constitutively active ARF1 trigger local matrix degradation by facilitating exocytosis of MMPs independent of podosome ring assembly.
Discussion

In this paper we demonstrate that an ARNO-ARF1 signalling axis is required for the maintenance of podosome integrity (See Figure 8 for flow diagram). First, knockdown of ARF1 but not ARF6 prevents podosome formation by TGFβ1- or PMA-treated THP1 cells. In addition to these “classic” podosomes, we explored podosome-like structures induced in fibroblasts by either expression of constitutively active Src (Tarone et al., 1985) or by plating cells on a fluid substratum (Yu et al., 2013). We checked that specific drugs inhibiting ARF1-activating GEFs, BFA (Niu et al., 2005; Yamaji et al., 2000) and secinH3 (Hafner et al., 2006), led to rapid dissolution of podosomes in THP1 cells and the podosome-like structures in fibroblasts. In addition, we have shown that in both THP1 cells and fibroblasts, treatments inducing podosome formation augmented the fraction of active, GTP-bound ARF1.

BFA and secinH3 inhibit different classes of ARF1-activating GEFs (Donaldson and Jackson, 2011). In our experiments, the BFA-sensitive GEFs (GBF1, BIG1, BIG2) appeared to be functionally unrelated to podosome regulation. The inhibitory effect of BFA could thus be explained by sequestration of ARF1 within the BFA-induced ternary complexes consisting of inhibited GEFs, ARF1-GDP and BFA (Mossessova et al., 2003; Peyroche et al., 1999; Zeghouf et al., 2005). At the same time, we found that one of the secinH3-sensitive GEFs, ARNO (cytohesin-2) but not cytohesin-1, is indispensable for podosome integrity in THP1 cells. Possible functions of several other ubiquitous ARF family proteins (ARF3, ARF4, ARF5) as well as other secinH3-sensitive GEFs, cytohesin-3 and -4, remained to be studied in the context of podosome formation.

Localization studies revealed that ARNO (but not cytohesin-1) is stably co-localized with adhesion proteins in the ring domain of podosomes in THP1 cells as well as with podosome-like structures in fibroblasts. This is consistent with biochemical
data showing direct association of ARNO with paxillin (Torii et al., 2010). Furthermore, live cell imaging showed transient contacts of vesicles containing ARF1 with the periphery and ring domain of podosomes and podosome-like structures in THP1 cells. We demonstrated that ARF1-containing vesicles are moving along microtubules; so one of the functions of microtubules important for the podosome maintenance (Linder et al., 2000) could be delivery of ARF1. It is worth noting that a negative regulator of ARF1 activity, ARF1 GTPase-activating protein ASAP1, was also shown to localize to podosomes (Curtis et al., 2015; Shiba and Randazzo, 2011).

How could active ARF1 affect podosome assembly and stability? The first possibility is based on well-documented functions of ARF1 in the Golgi complex and vesicular traffic (Donaldson et al., 2005). It can be conjectured that some of the ARF1-dependent functions of the Golgi are required for podosome integrity. This possibility cannot be entirely excluded; however, it is worth noting that in our experiments integrity of podosomes can be dissected from the integrity of the Golgi. In particular, inhibition of the ARF1 exchange factor GBF1, responsible for ARF1-dependent COPI recruitment to the Golgi, as well as knockdown of the COPI subunit, βCOP, only marginally affected podosome integrity. ARF1 in principle could be involved in integrin turnover and affect podosome formation via regulation of available integrin adhesion receptors. However, in our experiment, experimental manipulations with ARF1 did not affect the integrity or dynamics of another class of integrin-dependent adhesions, focal adhesions. This suggests that other mechanisms should be considered to explain the specific effect of ARF1 depletion/inhibition on podosome integrity.

Podosomes are part of the actin cytoskeleton and as such likely to be regulated by small G proteins of Rho family. We have shown that inhibition of ARF1 triggered significant activation of RhoA but not Rac or Cdc42. Activation of RhoA in turn triggers the assembly of numerous myosin-IIA filaments, which as we have
demonstrated led to considerable disruption of podosomes. We have shown that suppression of myosin-IIA filament formation by either inhibition of ROCK or knockdown of myosin-IIA, prevented the disruptive effect of ARF1 inhibition on podosome formation. Thus, our experiments suggest that ARF1 functions in podosome formation as an inhibitor of RhoA activity and subsequent myosin-IIA filament formation. This conclusion is consistent with our observation that ARF1-dependent activation of podosome formation by plating of cells on fluid bilayer led to inhibition of RhoA (Yu et al., 2013).

Interestingly, apparently the same mechanism based on suppression of Rho and myosin-II by the cytohesin family exchange factor, Steppke, and a Drosophila ARF was found in a completely different system, during cellularization of Drosophila embryos (Lee and Harris 2013). However, the pathway connecting ARF1 and RhoA remains unknown. It is perhaps worth noting that ARF1 can bind the RhoGAP ARHGAP10/21 and deliver it to the Golgi or plasma membrane (Dubois et al., 2005; Kumari and Mayor, 2008; Menetrey et al., 2007). ARHGAP10/21 is known to inhibit Cdc42 but also shows some RhoA inhibitory activity in vitro (Dubois et al., 2005).

It is also not clear why an excess of myosin-II filaments antagonizes podosome integrity. Myosin-II has been shown to localize to actin links radiating from the podosomes (Dries et al., 2013) but its functional role in podosomes is yet to be established.

In addition to its function as a myosin-II regulator, ARF1 could affect podosomes via regulation of actin polymerization. There are several lines of evidence suggesting involvement of ARF1 in the regulation of Arp2/3 complex-driven actin polymerization: via recruitment of actin nucleation promoting WAVE complex (Humphreys et al., 2012a; Humphreys et al., 2012b), via sequestration and inactivation of Arp2/3 inhibitor, PICK1 (Rocca et al., 2013) and via activation of Cdc42 (Dubois et al., 2005; Heuvingh et al., 2007). We were not able to find evidence of any of these mechanisms in the context of podosome regulation. Neither data
from the literature nor our own observations indicate that WAVE or PICK1 are localized to podosomes. Moreover, ARF1 inhibition did not induce any changes in GTP-bound Cdc42 level, and constitutively active Cdc42 did not prevent or overcome the disruption of podosomes seen upon ARF1 inhibition.

Nevertheless, the effect of ARF1 on the polymerization of actin in the context of podosome formation is seen in our experiments with expression of constitutively active ARF1 in fibroblast-type cells, which normally do not produce podosomes. Active ARF1 not only suppresses stress fibre formation but also induces formation of numerous actin and Arp3-containing patches in such cells. The induction of actin polymerization at the plasma membrane by active ARF1 and ARF6 was previously demonstrated (Caviston et al., 2014). Many actin-associated proteins typical of podosomes (N-WASP, WIP, cortactin, dynamin-II) were also found in these puncta. Moreover, a hallmark of podosome function, the local gelatin matrix degradation by MMPs appeared to be associated with these puncta. At the same time, the puncta were not surrounded by any podosome adhesive ring components. The recruitment of certain adhesion components such as paxillin was shown to require dynamic GTP/GDP turnover of ARF1 (Liu et al., 2002; Liu et al., 2005). This may explain the lack of adhesive ring surrounding podosome core-like structures induced by constitutively active ARF1. Of note, our data show that matrix degrading and adhesion functions could be dissected under conditions of induction of podosome precursors by constitutively active ARF1.

The pathways downstream of ARF1 underlying formation of these podosome precursors are not the same as Rho and myosin-II inhibitory activity of ARNO-ARF1 characterized above, since expression of dominant negative RhoA did not by itself induce formation of the actin-rich puncta in fibroblasts. We cannot exclude that local changes in Cdc42 activity may still play a role in this process (Heuvingh et al., 2007) even though ARF1 did not affect the total level of Cdc42 activity in our experiments.
In summary, we demonstrate that the signalling axis ARNO-ARF1 plays a critical role in the control of podosome integrity and find that this pathway in macrophage-like cells operates via inhibition of RhoA and myosin-II activity. Other pathway(s) found in fibroblasts downstream of active ARF1 induce formation of F-actin-rich puncta resembling podosome actin cores that are not associated with the matrix adhesion components but involved in matrix degradation. These findings open new features of the processes of podosome formation and matrix degradation. Investigation of ARNO-ARF1 upstream and downstream pathways provides a rich source of future studies.
Materials and Method

Cell culture and transfection procedures

THP1 human monocytic leukemia cell line was obtained from Health Protection Agency Culture Collections (Porton Down, Salisbury, UK) and cultured using Roswell Park Memorial Institute (RPMI-1640) supplemented with 10% HI-FBS and 50 µg/ml 2-Mercaptoethanol (Sigma-Aldrich) at 37°C and 5% CO₂.

The suspended THP-1 cells were differentiated into adherent macrophage-like cells either with 1 ng/ml human recombinant cytokine TGFβ1 (R&D Systems) or 50 nM Phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 24 or 48 hours on fibronectin-coated glass substrates. No apparent difference between the phenotype of cells stimulated for 24 or 48 hours were detected. 35-mm ibidi (Cat. 81158) glass-bottomed dishes were coated with 1 µg/ml of fibronectin (Calbiochem, Merck Millipore) in phosphate buffered saline (PBS) for 1-2 hours at 37°C, washed with PBS twice, and incubated in complete medium prior to seeding of cells.

Cells were transiently transfected prior to stimulation with DNA plasmids using electroporation (Neon Transfection System, Life Technologies) in accordance to manufacturer's instructions. Specifically, two pulses of 1400V for 20 milliseconds were used.

For siRNA transfection, THP1 cells were treated with 100nM of ARF1 siRNA (Dharmacon, ON-TARGETplus SMARTpool siRNA, catalogue no. L-011580-00-0005), 150nM of ARF6 siRNA (Dharmacon, ON-TARGETplus SMARTpool siRNA, catalogue no. L-004008-00-0005), 100nM of MYH9 siRNA (Dharmacon, ON-TARGETplus SMARTpool siRNA catalogue no. L-007668-00-0005) or 100nM of COPB1 siRNA (Dharmacon, ON-TARGETplus SMARTpool siRNA, catalogue no. L-017940-00-0005). For control experiments, cells were transfected with (Dharmacon, ON-TARGETplus
Non-targeting pool siRNA, catalogue no. D-001810-10) at a concentration similar to individual gene-targeted siRNAs.

For knockdown of ARF GEFs in THP1 cells, siRNA duplex ‘GCAAUGGGCAGGAAGAAGU’ (Oh et al 2010) against human ARNO sequence and ‘AUGGAGGAGGACGACAGCUAC’ (Sendide et al 2005) against human cytohesin-1 with dT-dT overhangs were purchased from Sigma-Aldrich. For rescue experiments in Figure 1D, ARF1 siRNA transfected THP1 cells were co-transfected with HA-ARF1 (bovine origin, non-sensitive to aforementioned ARF1 siRNA) and fixed 48 hours after plating on fibronectin.

Immortalized rtpαα(+/+) mouse embryonic fibroblasts (Su et al 1999) that was termed MEFs, were obtained from the Sheetz laboratory (Mechanobiology Institute, Singapore). MEFs were cultured in Dulbecco’s modified Eagle’s Medium high glucose (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS, Gibco), 1% L-Glutamine, and 100 IU/mg penicillin-streptomycin (Invitrogen) at 37°C and 5% CO2. MEFs were transiently electroporated with a single pulse of 1400V for 20 milliseconds. MEFs were either seeded on fibronectin-coated 35-mm ibidi or 27-mm IWAKI (Japan) glass-bottomed dishes for 24 hours post-transfection. For plating on supported lipid bilayer membrane, transfected MEFs were seeded on 6-well Nunc (Thermo Fisher Scientific) plastic dishes for 24 hours post-transfection. These MEFs were then treated with trypsin solution, TrypLE™ (ThermoFisher Scientific), for 5 mins and kept in suspension for 15 minutes in complete medium to recover from trypsinization prior to seeding on supported lipid bilayer membrane.

**Plasmids**

mCherry-WIP and GFP-WASP were described in (Vijayakumar et al 2015); GFP-β-actin and mCherry-Talin – in (Cox et al 2012). The following plasmids described in
corresponding references were kindly provided by the following researchers.

EGFP-ARNO (Santy et al 1999) and EGFP-ARNO E156K (Hernández-Deviez et al 2003) – by Dr James Casanova (University of Virginia, VA, USA); EGFP-Cytohesin-1 (Bourgoin et al 2002) – by Dr Sylvain Bourgoin (CHU de Québec Research Center, University of Laval, Quebec, Canada); ARF1-RFP (Hsu et al 2010) – by Dr Nihal Altan-Bonnet (NIH, Bethesda, MD, USA); GFP-Vinculin (Zamir et al 1999) and mCherry-Vinculin – by Dr Michael Davidson (Florida State University, FL, USA); GFP-Paxillin and mApple-Paxillin (Kanchanawong et al 2010) – by Dr Pakorn Kanchanawong (Mechanobiology Institute, Singapore); constitutively active Src Y527F – by Dr Keiko Kawauchi (Mechanobiology Institute, Singapore); EGFP-Rab6A (Miserey-Lenkei S et al 2010) – by Dr Stéphanie Miserey Lenkei (Institute of Curie, Paris, France); GFP-Mannosidase II (Galen J et al 2014) – by Dr Vivek Malhotra (Center of Genomic Regulation, Barcelona, Spain); mApple-Rab11A (verified in-house) – by Dr Vicki Allan (University of Manchester, UK); GFP-Dynamin II (Ochoa et al 2000) – by Dr Pietro De Camilli (Yale University, USA); Myosin regulatory light chain-GFP – by Dr Mark Dodding (King’s College London, UK). All BIG constructs (HA-BIG1, HA-BIG2, HA-BIG1 E793K, HA-BIG2 E738K (Ishizaki et al 2008) – by Dr Hye-won Shin (Kyoto University, Kyoto, Japan).

The following plasmids described in corresponding references were purchased from Addgene (Cambridge, MA, USA): ARF1-GFP (Chun et al 2008, Addgene #39554), ARF1-ECFP (Beemiller et al 2006, Addgene #11381), ARF1(T31N)-ECFP (Beemiller et al 2006, Addgene #11384), ARF1(Q71L)-ECFP (Beemiller et al 2006, Addgene #11385), HA-ARF1 (Furman et al 2002, Addgene #10830), GFP-Rab11 (Choudhury et al 2002, Addgene #12674), mCherry UtrCH (Burkel et al 2007, Addgene #26740), EMTB-mCherry (Miller et al 2009, Addgene #26742), GFP-RhoA (T19N) (Subauste et al 2000, Addgene #12967), ARF6-CFP (Beemiller et al 2006, Addgene #11382), mCherry-Arp3 (Taylor et al 2011, Addgene #27682), mCherry-Cortactin (Taylor et al 2011, Addgene #27676), mCherry-clathrin light chain (Taylor et al 2011, Addgene #27680), dsRed Rab7 (Choudhury et al 2002,
# Addgene 12661), GFP-Rab8A (Guizetti et al 2011, Addgene #31803), EMTB(ensconsin)-mCherry (Miller et al 2009, Addgene #26742).

**Supported lipid bilayer membrane**

Methodologies of supported lipid bilayer preparation and membrane functionalization have been described in Yu *et al* 2011 and Yu *et al* 2013. Briefly, 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dipalmitoyl-sn- glycerol-3-phosphoethanolamine-N-(cap biotinyl) (16:0 biotinyl-Cap-PE) were purchased from Avanti Polar Lipids. The lipids (0.2 mol% of biotinyl-Cap-PE and 99.8 mol% of DOPC) were mixed with an equal volume of PBS and then pipetted onto cleaned glass substratum where a 25-mm coverslip was placed over it for self-assembly of lipid vesicles. The lipid-coated coverslips were immersed into a deionized water bath and then placed and sealed in an Attofluor cell chamber (Life Technologies). The supported lipid bilayer membrane ensemble was kept under aqueous environment at all times. For membrane functionalization, the supported lipid membrane was blocked with 50 µg/ml of Casein. A total of 0.1 µg/ml of Cascade blue neutravidin (Life Technologies) was added onto supported lipid membranes, followed by 1 µg/ml of biotinylated RGD, cyclo [Arg-Gly-Asp-D-Phe-Lys [Biotin-PEG-PEG]; Peptides International). Cells were then added onto the RGD-functionalized lipid bilayer membrane and imaged or fixed within 2-3 hours of preparation.

**Drug treatment**

For drug inhibition studies, cells were treated with 30 µM SecinH3 (Tocris), 10 µM Golgicide A (Santa Cruz Biotechnology), 5 µg/ml Brefeldin A (Sigma-Aldrich), 30 µM Y-27632 (Sigma-Aldrich), 25 µM GM6001 (Enzo Life Sciences), 2 µg/ml C3 transferase (Cytoskeleton) in complete medium for 1-2 hours or 4 hours for GM6001 at 37°C with 5% CO₂ and subsequently fixed with 4% PFA. For live cell
imaging, cells were imaged immediately after addition of appropriate inhibitors, which remained in the medium during the entire period of image acquisition. To study effect of inhibitors on podosomes formed by MEFs plated on RGD lipid bilayer, the cells were treated with appropriate inhibitors 30-45 minutes following cell seeding on the bilayer.

**Immunoblotting**

For verification of knockdown experiments, cells were lysed in RIPA buffer 48 hours after transfection and extracted proteins were separated by 4-20% SDS-polyacrylamide gel (Thermo Fisher Scientific) and transferred to PVDF membranes (Bio-Rad) before incubation at 75V for 2 hours and blocked for 1 hour with 5% non-fat milk (Bio-Rad) or bovine serum albumin (BSA, Sigma-Aldrich). The PVDF membranes were incubated overnight at 4°C with appropriate antibodies: anti-ARF1 (Abcam, catalogue no. ab108347, dilution 1:1000); anti-ARF6 (Abcam, catalogue no. ab77581, dilution 1:1000); anti-ARNO (Abcam, catalogue no. ab56510, dilution 1:1000); anti-cytohesin-1 (Merck Millipore, catalogue no. MABT14, dilution 1:500); anti-α-tubulin (Sigma-Aldrich, catalogue no. T6199, dilution 1:3000); Anti-βCOP (Abcam, catalogue no. ab2899, dilution 1:1000); anti-HA (Cell Signaling Technology, catalogue no. 2367, dilution 1:1000); anti-Cdc42 (Cell Signaling Technology, catalogue no. 2462, dilution 1:1000); anti-RhoA (Santa Cruz Biotechnology, Inc., catalogue no sc-418, dilution 1:1000); anti-Rac1 (BD Biosciences, catalogue no. 610650, dilution 1:1000); Anti-non muscle myosin-IIA (Sigma-Aldrich, catalogue no. M8064, dilution 1:1000).

After three washes (10 minutes each), appropriate secondary antibodies conjugated with horseradish peroxidase (Bio-Rad) were incubated with the membrane for 1 hour, washed three times (15 minutes at room temperature), and detected by Pierce™ ECL western blotting substratum (Thermo Fisher Scientific) using CL-Xposure film (Thermo Fisher Scientific).
Small G protein activity assay

Total cell lysates were collected and immediately quantified by the G-LISA ARF1 or Cdc42 Activation Assay Biochem Kit (colorimetric-based) and performed as per manufacturer's protocol (Cytoskeleton, Inc.). Samples were run in duplicates per sample, averaged and then normalized to the total ARF1 or Cdc42 levels detected by immunoblotting. For each set of experiment, data were normalized to TGFβ1-treated THP1 cells giving a fold-change value from zero (minimum) to one (maximum). Pull-down assay using GST-tagged RhoA-binding domain of Rhotekin was used to precipitate GTP-bound RhoA while GST-tagged Rac1/Cdc42-binding domain of PAK1 (PBD) beads were used to precipitate GTP-bound Rac1 or Cdc42 in THP1 cells. Pulled-down RhoA, Rac1 and Cdc42 were immunoblotted using respective antibodies as described above.

Immunofluorescence

Cells were fixed with 3.7% paraformaldehyde in PBS, washed twice and permeabilized with 0.5% triton X-100 (Sigma-Aldrich) in PBS for 10 minutes, and then washed twice. Fixed cells were blocked with 5% BSA or 5% FBS for 1 hour at room temperature or overnight at 4°C prior to incubation with appropriate primary antibodies: anti-GM130 (BD Biosciences, catalogue no. 610822, dilution 1:400); anti-GRASP65 (Abcam, catalogue no. ab30315, dilution 1:500); anti-HA (Cell Signaling Technology, catalogue no. 2367, dilution 1:400), anti-vinculin (Sigma-Aldrich, catalogue no. V9131, dilution 1:400); anti-ARF6 (Abcam, catalogue no. ab77581, dilution 1:200); Anti-βCOP (Abcam, catalogue no. ab2899, dilution 1:200); Anti-non muscle myosin-IIA (Sigma-Aldrich, catalogue no. M8064, dilution 1:800). Cells were washed thrice with PBS and incubated with Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 hour at room temperature followed by three washes in PBS. Actin staining was carried out using either Alexa
Fluor 488 Phalloidin (Thermo Fisher Scientific), Phalloidin-TRITC (Sigma-Aldrich) or Alexa Fluor 647 Phalloidin (Thermo Fisher Scientific).

**Matrix degradation assay**

50% sulphuric-acid-washed coverslips were coated with 50 mg/ml poly-D-Lysine for 30 minutes at room temperature, and then fixed with 0.5% glutaraldehyde for 15 minutes. 0.2% gelatin warmed at 37°C was mixed with Oregon Green 488 conjugated-pig gelatin at 6:1 ratio. Coverslips were coated with gelatin mix for 10 minutes, washed with 1xPBS and then quenched with 5mg/ml sodium borohydride for 15 minutes followed by numerous washes. For matrix degradation assay, MEFs were seeded on these coated coverslips for 4 hours and then fixed for immunofluorescence imaging as described above. Dark spots corresponding to areas of cells indicate degradation of the matrix.

**Live cell imaging and microscopy**

Cells were imaged in complete medium (unless stated otherwise) at an acquisition rate from 5 seconds to 1 minute intervals using a spinning-disc confocal microscope (PerkinElmer Ultraview VoX) attached to an Olympus IX81 inverted microscope, equipped with a 100x oil immersion objective (1.40 NA, UPlanSApo), an EMCCD camera (C9100-13, Hamamatsu Photonics) for image acquisition, and Volocity software (PerkinElmer) to control the acquisition protocol. Fixed samples and live imaging were also imaged with a Nikon confocal A1R system and Nikon structured illumination microscopy (N-SIM) attached to a Nikon Ti-E inverted microscope with Perfect Focus System (PFS) using a 100x oil immersion objective (1.40 NA, CFI Plan-ApochromatVC). The cameras, from Andor technology Neo sCMOS and DU-897 were used to acquire images for confocal A1R and N-SIM systems respectively, with the Nikon NIS-Elements AR software to control the acquisition protocol. For z stack
images, cells were imaged at a step-size of 0.2-0.5 μm with a total height of 15-20 μm.

**Image processing and data analysis**

Image processing and analysis were performed with ImageJ or Volocity Software. The number of podosomes (marked by core or ring marker) was quantified automatically using an ImageJ-based tool for counting Nuclei (ImageJ plugin), which was manually verified for the first ten cells in the specimen to account for undetected podosomes (less than 10%). Line intensity measurements (arbitrary unit, a.u.) of GFP-ARNO, GFP-Cytohesin-1 and mCherry-UtrCH were quantified by measuring the mean intensity of GFP or mCherry fluorescence per area (μm²), background subtracted and normalized with values ranging from 0 (lowest) to 1 (highest).

**Statistical analyses**

Prism version 6 (GraphPad Software) was used to plot, analyze and represent the data. Significance of the differences was determined using two-tailed unpaired Student's t-test or one-way ANOVA for more than two groups. The methods for statistical analysis and sizes of the samples (n) are specified in the results section or figure legends for all of the quantitative data. Differences were accepted as significant for \( P < 0.05 \).
Online supplementary material

Fig. S1 shows that knockdowns of either ARF1 or ARNO prevent formation of podosomes in THP1 cells stimulated by PMA, while ARF6 is not involved in podosome formation. Fig. S2 shows that ARF1-containing puncta are positive for Rab11 but not for Rab6, Rab7 or Rab8. Fig. S3 shows that inhibition of β-COP as well as ARF exchange factors GBF1, BIG1 and BIG2 do not lead to podosome disruption in TGFβ1-stimulated THP1 cells. In addition, ARF1-mediated pathway of podosome formation does not involve Cdc42. Fig. S4 shows dynamics of podosome disassembly by SecinH3 visualized using structured-illumination microscopy (SIM). Fig. S5 shows that cell treatment with the drugs suppressing ARF1 activity results in disruption of Src-induced podosome rosettes in fibroblasts, but does not disrupt focal adhesions. In addition, this figure shows actin-rich puncta in fibroblasts transfected with constitutively active ARF1 and localization of podosome core proteins to these puncta.

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Author contributions
A.D.B. conceived and designed the project together with G.E.J. N.B.M.R designed and performed all experiments and prepared the manuscript; L.Z.Z. and C.H.Y. provided assistance in carrying out experiments and discussed results. A.D.B., G.E.J. and P.T.M. discussed results and prepared the manuscript.

Conflict of interest statement
The authors declare no competing financial interest.
Figure Legends

Figure 1

Depletion of endogenous ARF1 disrupts podosomes. (A) Western blot showing ARF1 levels in cells treated with scramble (control) or ARF1 siRNA; α-tubulin was used as a loading control. (B, C) ARF1 knockdown leads to disruption of podosomes but not the Golgi apparatus. Actin labeled with phalloidin (left panel) and vinculin visualized by antibody staining (right panel) in control (B) and ARF1 siRNA-transfected (C) THP1 cells 48 h after TGFβ1 stimulation. The Golgi apparatus in the same cells was visualized by staining with antibody against cis-Golgi proteins, GM130 (left panel, green) and GRASP65 (right panel, red). Scale bars, 5 µm. (D) Expression of HA-tagged bovine ARF1 in ARF1-depleted human THP1 cells rescues podosome formation. Podosomes are visualized by phalloidin staining (left and right panels) and HA-ARF1 by immunostaining with anti-HA antibody (central and right panels). Scale bar, 5 µm. HA-ARF1 was localized to Golgi and to punctate structures shown with high magnification in (D’) representing the enlarged area boxed in (D). Scale bar, 1 µm. Labeling in (D’) shows actin (upper image), HA-ARF1 (middle image), and merged image of both (lower panel). Width of the images 7 µm. (E,F) Quantification of the effect of ARF1 and ARF6 knockdown on podosomes integrity. Both number of podosomes per cell (E) and percentage of cells having more than 10 podosomes (F) decreased upon ARF1 but not ARF6 knockdown. This effect was rescued by expression of exogenous HA-ARF1. The graphs represent results of 3 independent experiments with 100-200 cells used for each group. The numbers of podosomes per cell are presented as box-and-whiskers plot while the percentage of cells with more than 10 podosomes as mean ± SD. The significance of the difference between groups was estimated by two-tailed Student’s t-test, the range of P-values >0.05 (non-significant), ≤ 0.05, ≤0.01, ≤0.001, ≤ 0.0001 are denoted by “ns”, one, two, three and four asterisks (⋆), respectively.
**Figure 2**

Localization and dynamics of ARF1 puncta in TGFβ1-stimulated THP1 cells. (A) TIRF image of the ventral surface of cell with podosomes labeled by mCherry-vinculin (left panel) and ARF1 puncta labeled by GFP-ARF1 (central panel). Merged image (right panel) shows non-random distribution of ARF1 puncta with a tendency to co-localize to podosome periphery. Scale bar, 5 µm. Boxed area (2.5x2.5 µm²) contains a podosome, where co-localization dynamics with ARF1 puncta is presented in (B). (B) Kymograph representing fluorescent intensities in a line scan through the podosome boxed in (A). While mCherry-vinculin is stably labeled in the podosome ring (upper panel), GFP-ARF1 was transiently concentrated at one side of the ring (central panel and merged image at the bottom). See also Supplementary movie 1. The time course of fluorescence intensity of GFP-ARF1 at the podosome ring is shown in (B’). (C) Each dot corresponds to a single cell and represents percentage of podosome rings (labeled by vinculin) contacted by either ARF1-containing puncta or Rab6-containing vesicles within 5 minutes of image acquisition. (D) Frequency distribution of the durations of podosome contacts (in seconds) with ARF1-containing puncta (35 podosomes from 10 cells were filmed as shown in kymograph B). (E,F) GFP-ARF1 puncta are moving along microtubules. (E) Left panel: microtubule labeling with 125 kDa microtubule-associated protein, ensconsin (mCherry-ensconsin); central panel: GFP-ARF1 puncta in the same cell; right panel: merged imaged. The dynamics of microtubules and ARF1 puncta in the boxed area (8 x 7.5 µm²) of C is shown in (F). Scale bar, 5 µm. Movement of puncta along the microtubule is indicated by arrowhead. See also Supplementary movie 2.

**Figure 3**

ARF1-GTP levels and podosome formation. (A) Quantification of ARF1-GTP levels by G-LISA assay in control, stimulated and inhibitor-treated THP1 cells. Both TGFβ1
and PMA increased the fraction of GTP-bound ARF1 compared to control, while treatment with SecinH3 or BFA dramatically reduced it. Pooled results of 3 independent experiments are shown. (B) Disruption of podosomes labeled with mCherry-Utrophin (UtrCH) upon treatment with SecinH3 (upper panel). Note that integrity of the Golgi apparatus labeled with GFP-mannosidase II was preserved in the same SecinH3-treated cell (lower panel). See also Supplementary movie 3. (C,D) Disruption of podosomes labeled with mCherry-vinculin by SecinH3 (upper panel of C) and BFA (upper panel of D). Whilst the effect of SecinH3 in these cells is not accompanied by changes in localization of ARF1 to the Golgi and cytoplasmic puncta (lower panel of C), BFA disrupted both Golgi and ARF1 puncta (lower panel in D); Scale bars, 5 \( \mu \)m. Insets (1 x 1 \( \mu \)m\(^2\)) show evolution of individual ARF1 puncta in each case. (E,F) Quantification of the effect of SecinH3 and BFA on average number of podosomes per cell (E) and percentage of cells with more than 10 podosomes (F). (G) ARF1-GTP levels increase in fibroblasts plated on a RGD-functionalized fluid lipid bilayer as compared to fibroblasts plated on glass coverslip. (H) Effect of SecinH3 on the integrity of podosome-like structures formed by fibroblasts plated on fluid lipid bilayer. (I) Quantification of the disruptive effect of SecinH3 and BFA on podosome-like structures formed by fibroblasts on lipid bilayer. The percentage of podosome-forming cells significantly decreased upon treatment by each of the inhibitors. The data were presented and the significances of the difference were assessed as indicated in the legend to Figure 1. Pooled data of three independent experiments are presented for each group.

**Figure 4**

Knockdown of ARF1 exchange factor ARNO (cytohesin-2), but not cytohesin-1 leads to podosome disruption. (A) Western blot showing ARNO and cytohesin-1 levels in cells treated with control (scrambled) siRNA, ARNO or cytohesin-1 siRNAs; \( \alpha \)-tubulin was used as a loading control. (B) Upper panel: TGF\( \beta \)1-stimulated THP1 cells; podosomes labeled with phalloidin and Golgi apparatus labeled by antibody
against GM130. Middle panel: siRNA-mediated knockdown of ARNO disrupted podosomes leaving the Golgi undisturbed. Lower panel: Cytohesin-1 knockdown disrupt neither podosomes nor Golgi. Scale bars, 5 µm. (C,D) Quantification of the effect of ARNO and cytohesin-1 knockdowns on average number of podosomes per cell (C) and percentage of cells with more than 10 podosomes (D). (E) G-LISA quantification of ARF1-GTP level in non-transfected control cells, scrambled siRNA-transfected cells, and cells transfected with ARNO siRNA. (F,G) Quantification of the effect of expression of wild-type GFP-ARNO and of dominant negative ARNO mutant (GFP-ARNO E156K) on average number of podosomes per cell (F) and percentage of cells with more than 10 podosomes (G). The data were presented and the significances of the difference were assessed as indicated in the legend to Figure 1. Pooled data of three independent experiments are presented for each group.

**Figure 5**

ARNO but not cytohesin-1 is localized to podosomes and podosome-like structures in different cell types. (A,B,C) Localization of F-actin marker, mCherry-UtrCH and GFP-ARNO in TGFβ1-stimulated THP1 cell (A), active Src-transformed fibroblast (B) and fibroblast on a RGD-functionalized fluid lipid bilayer (C). Left panels: F-actin cores of podosomes (A), podosome rosettes (B), and podosome-like structures formed on fluid bilayer (C). Central panels: GFP-ARNO localized to periphery of F-actin cores (A,B,C). Right panels: merged images. The boxed areas (A and B: 2.5 x 2.5 µm², Scale bar: 1 µm, C: 14 x 14 µm², Scale bar, 5 µm) of merged images are enlarged and line scanned as shown in inset. The graphs on the right demonstrate intensity profiles of F-actin and ARNO in individual podosome (A), podosome “rosette” (B), and podosome-like structure on bilayer (C). (D, D’) Time course of ARNO localization to the podosome periphery. Dynamics of F-actin (labeled by mCherry-UtrCH) and GFP-ARNO fluorescence intensities in the podosome shown in the boxed area (3 x 3 µm²) in D are presented in the sequences in D’. Time interval between frames is 30 seconds. See also Supplementary movie 6. (E) Cytohesin-1 is not
localized to podosomes. Left panel: F-actin cores of podosomes in TGFβ1-stimulated THP1 cell. Central panel: GFP-cytohesin-1 localization in the same cell. Right panel: merged image. Line scanning through the individual podosome in the boxed area (4 x 1.5 µm²) of the merged image shown in inset is quantified in the graph on the right. No enrichment of GFP-cytohesin-1 at podosome core or periphery was detected. Scale bars, 5 µm.

Figure 6

Inhibition of ARF1 activity induces RhoA activation. (A-C) 1 hour incubation of TGFβ1-stimulated THP1 cells with 30 µM SecinH3 led to increase in RhoA-GTP (A) but not Rac1-GTP (B) or Cdc42-GTP (C) fractions as indicated by western blots after pull-down assay. (D-J) Structured-illumination microscopy (SIM) visualization of podosome dynamics in TGFβ1-stimulated THP1. (D and E) Live imaging of cell stably transfected with GFP-MRLC to visualize myosin-II filaments and RFP-lifeact to visualize podosome cores. (D) Cell treated with 30 µM SecinH3 show an increase in myosin-IIA filament assembly (green) and disruption of podosomes (red). Enlarged image of white-boxed area (5 x 4.5 µm²) of D shows co-localization between appearance of myosin-IIA filaments and podosome disruption (See supplementary movie 7). (E) Time course of podosome re-appearance after addition of 30 µM ROCK inhibitor Y-27632 to cell incubated in secinH3 containing medium. Note that podosomes (red) appeared after disassembly of myosin-II filaments (green). (F-J) TGFβ1-stimulated THP1 cells were transfected with either control scrambled siRNA (F and G) or with siRNA to NM-myosin-IIA heavy chain, MYH9 (I and J) and, after 48 hours, treated with either 0.1% DMSO (F and I) or 30 µM SecinH3 (G and J) for 1 hour. (F, G, I, J) After fixation, the cells were stained with phalloidin (left panels) and antibodies to NM-myosin-IIA heavy chain (middle panels) and vinculin (right panels). (H) Western blot showing protein levels of NM-myosin-IIA heavy chain in control cells (transfected with scrambled siRNA) or in NM-myosin-IIA knockdown cells (transfected with MYH9 siRNA); α-tubulin was used as a loading control. (K
and L) Effect of secinH3 treatment of control and NM-myosin-IIA knockdown cells on the average number of podosomes per cell (K) and percentage of cells with more than 10 podosomes (L). The data are presented as indicated in the legend to Figure 1. Pooled data of at least two independent experiments are presented for each group.

**Figure 7**

Constitutively active ARF1 induces F-actin-rich puncta (labeled by mCherry-UtrCH) in mouse fibroblasts. (A) Transfection with wild-type CFP-ARF1 did not change actin cytoskeleton of fibroblast. (B) Numerous F-actin-rich puncta in the fibroblast transfected with constitutively active ARF1 mutant, CFP-ARF1 Q71L. Scale bars, 5 μm. (B’) Images of the boxed area (6 x 4.5 μm²) in (B) showing F-actin (red, upper row), CFP-ARF1 Q71L (green, middle row), and their superimposition (lower row) at 3 time points taken with a 3 second time interval are presented. Transient contacts (yellow) of CFP-ARF1 Q71L-containing puncta with the F-actin-rich puncta are seen. Scale bar, 1 μm. (C) Cell transfected with dominant negative RhoA (GFP-RhoA T19N) do not contain stress fibres. (D) Cell co-transfected with dominant negative RhoA (GFP-RhoA T19N) and constitutively active ARF1 (CFP-ARF1 Q71L) form numerous F-actin-rich puncta. (E) Control fibroblast forming stress fibres (left) does not demonstrate localization of mCherry-WIP (right). (F) F-actin-rich puncta in fibroblast expressing CFP-ARF1 Q71L are enriched with mCherry-WIP. (G and H) Fibroblasts transfected with constitutively active ARF1 (CFP-ARF1 Q71L) were plated on fluorescent gelatin-coated coverslips in the control medium containing 0.2% DMSO (G) or in the medium containing 25 μM MMP inhibitor GM6001 (H) and incubated for 4 hours. The matrix degradation sites in the boxed areas are seen at high magnification in the left panel of G’ but not H’. Actin puncta were visualized by phalloidin staining; high magnifications of boxed areas in G and H as well as merged images of actin and fluorescent gelatin are shown in central and right panels of G’ and H’ respectively. Note that actin puncta co-localize with dark
areas corresponding to degraded fluorescent gelatin in control fibroblasts (white arrowheads in G'), while gelatin degradation is completely prevented in cells treated with GM6001 (H'). Scale bars, 5 μm.

**Figure 8**

A flow diagram illustrating the role of ARNO-ARF1 signaling axis in the podosome formation. A variety of external factors known to switch cells towards podosome formation activate the ARF GEF ARNO. The ARNO activates ARF1, which in turn induce two major pathways regulating the podosomes. First, ARF1 inhibits Rho, which negatively regulate podosomes via ROCK-dependent formation of myosin-II filaments. Second, ARF1 promotes formation of Arp2/3- and actin-enriched podosome core-like structures associated with matrix degradation sites. The solid arrows represent the direct pathways while the dashed arrows indicate to the existence of unknown intermediate steps in the depicted pathways.
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