Sodium fluoride induces podosome formation in endothelial cells

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Background information. Fluoride is a well-known G-protein activator. Exposure of cultured cells to its derivatives results in actin cytoskeleton remodelling. Podosomes are actin-based structures endowed with adhesion and matrix-degradation functions. This study investigates actin cytoskeleton reorganization induced by fluoride in endothelial cells.

Results. Treatment of cultured endothelial cells with sodium fluoride (NaF) results in a rapid and potent stimulation of podosome formation. Furthermore, we show that Cdc42 (cell-division cycle 42), Rac1 and RhoA activities are stimulated in NaF-treated cells. However, podosome assembly is dependent on Cdc42 and Rac1, but not RhoA. Although the sole activation of Cdc42 is sufficient to induce individual podosomes, a balance between RhoGTPase activities regulates podosome formation in response to NaF, which in this case are often found in groups or rosettes. As in other models, podosome formation in endothelial cells exposed to NaF also involves Src. Finally, we demonstrate that NaF-induced podosomes are fully competent for matrix protein degradation.

Conclusions. Taken together, our findings establish NaF as a novel inducer of podosomes in endothelial cells in vitro.

Introduction

RhoGTPases regulate the organization of the actin cytoskeleton, a dynamic, structural component of cells that plays a key role in a variety of biological processes, including changes in cell morphology, motility, invasion, intracellular trafficking and cell cycle progression. Indeed, studies performed in fibroblasts have established that upon activation, the three main members of the Rho family remodel the cytoskeleton, each giving rise to a distinct pattern of F-actin structures. Constitutively active forms of Cdc42 (cell-division cycle 42), Rac1 and RhoA induce filopodia, lamellipodia and stress fibres respectively (Nobes and Hall, 1995). Acting as a link between membrane receptor signalling and the cytoskeleton, RhoGTPase activities are tightly regulated. They cycle between an inactive GDP-bound and an active GTP-bound state and the transition is dependent on two main classes of regulators, GEFs (guanine-nucleotide-exchange factors) and GAPs (GTPase-activating proteins). GEFs catalyse the exchange of GDP for GTP and hence activate the GTPases, whereas GAPs enhance the intrinsic GTPase activity, returning the GTPases to their basal inactive GDP-bound state (Jaffe and Hall, 2005).

Fluoride is a well-known G-protein activator. AlF has long been used to activate heterotrimeric G-protein α-subunits. AlF was shown to act as a phosphate analogue, mimicking the γ-phosphate of GTP, thereby promoting formation of the GTPase transition state not only of Gα subunits of heterotrimeric G-proteins but also of several classes of small GTPases in the presence of their GAP. Indeed, the use of AlF, which enhances by 100-fold the binding of GAP to the GDP-bound form of RhoGTPase,
provided conditions suitable for the elucidation of three-dimensional structures of small GTPases (Rittinger et al., 1997; Nassar et al., 1998). Other studies then revealed that formation of GAP–RhoGTPase complexes is independent of aluminium and of the guanine nucleotide, suggesting a role for fluoride distinct from that of a γ-phosphate-mimic, formulated in the initial hypothesis (Vincent et al., 1998). Indeed, NaF, in the absence of aluminium, specifically induces the formation of a high-affinity complex between the GTPase RhoA and p190RhoGAP in vitro (Vincent et al., 1998). In vivo, exposure of cultured fibroblasts to NaF resulted in a stimulation of stress fibre formation due to RhoA activation after p190RhoGAP sequestering in NaF-induced GAP–RhoGTPase complexes (Vincent and Settleman, 1999). This example provides evidence that the inactivation of RhoGAPs activities represents an effective means of promoting GTPase-mediated cellular processes.

Podosomes are uncommon microdomains endowed with adhesion and matrix-degradation functions. Their assembly is strongly dependent on Rho-GTPase activities (Linder, 2009). Podosomes consist of a densely packed actin core and actin-regulatory proteins (gelsolin, cortactin, Arp2/3 (actin-related protein 2/3), WASP (Wiskott–Aldrich syndrome protein)/N-WASP (neuronal WASP)) surrounded by a ring of components commonly found in focal adhesion structures such as vinculin, integrins and signalling proteins. Another divergence between podosomes and focal adhesions relies on the presence of matrix-degrading enzymes in these structures, including the major metalloproteinase MT1-MMP (membrane-type 1 matrix metalloproteinase) (Linder, 2009). Podosomes form spontaneously in monocyte-derived cells such as macrophages, osteoclasts and immature dentritic cells (Linder, 2009). Previously, we discovered that endothelial cells are also capable of assembling podosomes but only in response to specific inducers such as a constitutively active form of Cdc42 (Moreau et al., 2003), an oncogenic form of Src (Tatin et al., 2006), phorbol esters (Tatin et al., 2006) or transforming growth factor-β (Varon et al., 2006).

In the present study, we examined the remodelling of the actin cytoskeleton in response to fluoride treatment in endothelial cells. We found that NaF promotes actin reorganization into podosome rosettes on the basis of podosome markers and functional capacities. We also found that NaF leads not only to the activation of RhoA, but also to that of Rac1 and Cdc42 in this model. We further demonstrated that NaF-induced podosomes are dependent on Cdc42, Rac1 and Src-family kinase activities. We report NaF as a novel inducer of podosomes in endothelial cells.

Results
NaF induces F-actin reorganization in endothelial cells
Since fluoride has been shown to induce actin cytoskeleton remodelling into various actin configurations (Radhakrishna et al., 1996; Vincent and Settleman, 1999; Wang et al., 2001), we examined the cytoskeletal response of cells from the PAE (porcine aortic endothelial) cell line to fluoride treatment. PAE cells were treated with AlF and stained with fluorescently labelled phalloidin to analyse F-actin organization. After 30 min of treatment, F-actin staining showed a profound reorganization of the cytoskeleton (Figure 1A). The most striking change was the loss of thick stress fibres and the formation of thin microfilament bundles and dot-like structures, often clustered into rings. To determine if such cytoskeleton remodelling was dependent on aluminium, cells were treated with NaF alone. After 30 min of treatment, most cells showed an F-actin pattern indistinguishable from that obtained after AlF, indicating that actin remodelling was not dependent on aluminium (Figures 1A and 1B). In a dose-response experiment, the maximum response was reached upon 20 mM NaF for a 1-h treatment period, then intracellular actin-rich dots progressively disappeared (Figure 1C) and stress fibres returned after 4 h. Thus, in these cells, NaF induces peculiar F-actin structures not reported for other NaF-treated cells (Radhakrishna et al., 1996; Vincent and Settleman, 1999; Wang et al., 2001). Similar results were obtained in primary endothelial cells. Indeed, in HAECs (human arterial endothelial cells), stress fibre organization is the main F-actin configuration at the basal state, and NaF caused a phenotype switch from stress fibres to thin F-actin bundles and actin dots (see Supplementary Figure S1 at http://www.biolcell.org/boc/102/boc1020489add.htm). In contrast, in PHAEC (primary HAECs) or HUAECs (human umbilical
Figure 1 | Fluoride induces actin cytoskeleton remodelling in PAE cells

(A) PAE cells were left untreated (top left panel), treated with 30 mM NaF and 50 μM AlCl₃ (AlF), or 50 μM AlCl₃ (AlCl) or 30 mM NaF (NaF) for 30 min at 37°C. Cells were then fixed and F-actin was labelled using rhodamine-phalloidin. Scale bar, 25 μm.

(B) Quantification of the experiment described in (A) after 30 min or 1 h of treatment. Cells showing F-actin-rich dots were counted and data are presented as a percentage. Each bar represents the mean ± S.D. of two independent experiments where approx. 400 cells were counted per coverslip. (C) PAE cells were treated with the indicated concentration of NaF for 1, 2 or 3 h. After F-actin labelling, cells showing actin dots were counted and data are presented as a percentage.
artery endothelial cells), addition of NaF led to the formation of stress fibres (Supplementary Figure S1).

**F-actin structures formed in NaF-treated cells are genuine podosomes**

We noticed that the rings observed in NaF-treated PAE cells were reminiscent of podosome rosettes formed in HUVECs (human umbilical vein endothelial cells) exposed to phorbol esters (Tatin et al., 2006). Double staining for F-actin and vinculin revealed the characteristic podosomal arrangement of these two proteins in NaF-treated cells, with vinculin forming a ring around each F-actin dot (Figure 2A). P85α, the regulatory subunit of phosphoinositide 3-kinase and p190RhoGAP-A, a negative regulator for RhoA, both known to localize to podosome actin cores (Burgstaller and Gimona, 2004; Chellaiah et al., 2001; Guegan et al., 2008) co-localized with F-actin in NaF-treated PAE cells (Figure 2A). Likewise, N-WASP and WIP (WASP-interacting protein), two proteins involved in the actin polymerization machinery, were also recruited to these structures (see Supplementary Figure S2 at http://www.biolcell.org/boc/102/boc1020489add.htm). In this analysis, no difference in protein composition could be noted between sporadic individual actin structures and those organized into rings or rosettes.

A specific feature of podosomes resides in their ability to degrade the extracellular matrix. Since...

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**Figure 2** Identification of podosomes after NaF treatment

(A) Podosomal markers localize at NaF-induced actin structures. PAE cells were treated with 20 mM NaF for 1 h. Cells were labelled for F-actin using rhodamine-phalloidin (red) and co-stained with anti-vinculin, anti-p85α or anti-p190RhoGAP-A (p190A) antibodies revealed with Alexa Fluor®-488-labelled secondary antibodies (green). High magnification images show vinculin arranged in a ring (arrows) that surrounds the actin-rich core of podosomes. Scale bar, 10 μm. (B) Proteolytic activity is associated with NaF-induced podosomes. HAECs were seeded on FITC-gelatin (green) coated coverslips. The cells were treated with NaF for 1 h and stained for F-actin (blue) and MT1-MMP (red). Note areas of degraded gelatin that overlap with podosomes. Scale bar, 10 μm.
cells of the PAE cell line are intrinsically defective for this activity (Violaine Moreau, unpublished work), we tested the proteolytic capacities of NaF-induced podosomes in HAECs. Immunofluorescence studies revealed the presence of the major proteinase MT1-MMP at podosomes (Figure 2B). Moreover, in the gelatin-FITC degradation assay, matrix degradation was visualized as dark areas in the fluorescent coating directly underneath podosomes (Figure 2B). These results establish that NaF-induced podosomes are fully competent for matrix protein degradation. We conclude that NaF-induced actin dots represent genuine podosomes.

**NaF induces activation of RhoA, Rac1 and Cdc42 in endothelial cells**

This observation prompted us to seek out alterations in the status of small GTPases, which could occur as a result of NaF treatment. GST (glutathione transferase)-fusion proteins containing Rho or Rac/Cdc42 effector binding domains were coupled to agarose beads to pull down GTP-bound GTPases from NaF-treated or control PAE cells. Affinity-purified proteins were analysed by electrophoresis, followed by Western blotting using anti-RhoA, Rac1 or Cdc42 antibodies. We found that RhoA activity was dramatically increased upon NaF treatment (Figure 3A). In addition, Rac1 and Cdc42 were also activated under these conditions, although to a lesser extent than RhoA (Figure 3A). From these experiments, we conclude that NaF stimulates RhoA, Rac1 and Cdc42 activation in endothelial cells.

In order to examine the role played by RhoGTPases in podosome induction upon exposure to NaF, we used RNA interference to individually silence RhoA, Rac1 or Cdc42. Interestingly, in PAE cells, Cdc42 and Rac1 were required for podosome formation whereas RhoA was dispensable (Figure 3B). Knockdown of RhoA did not alter the percentage of cells with podosomes or podosome organization in the modified cells. To rule out the possibility that the small amount of RhoA remaining in siRNA (small interfering RNA) knocked down cells could still become activated upon NaF treatment and influence podosome formation under these conditions, we employed the exoenzyme C3 transferase from Clostridium botulinum to inhibit Rho protein activities. Neither highly purified C3 transferase nor its modified cell permeable form (tat-C3) was able to compromise podosome formation (Figure 3C). On the contrary, Rho protein inhibition led to a 2-fold increase in the number of podosome-positive cells (Figure 3C). Thus, our results demonstrate that NaF induces podosome formation via Rac and Cdc42 activation in endothelial cells and suggests that inactivation of Rho facilitates their assembly.

**Src co-ordinates RhoGTPase activities to promote podosome rosette assembly**

Cdc42 and RhoA are known to act downstream of Src kinase in the process of podosome formation (Berdieux et al., 2004; Tatin et al., 2006). Likewise, the pharmacological Src inhibitor PP1 completely ablated podosome formation in PAE cells exposed to NaF (Figure 4A). Examination of RhoGTPase activities in this situation revealed that PP1 altered their regulation by NaF. Whereas PP1 had only a slight effect on Cdc42 activation, it further increased that of RhoA upon NaF exposure (Figure 4B). Thus, NaF regulates RhoGTPase activation and podosome assembly, in a Src-dependent manner. Taken, together, these results suggest that NaF could perturb the activation/deactivation balance of RhoGTPase activities in a Src-dependant manner to promote the formation of the F-actin structures.

**Discussion**

In the present study we describe podosomal structures induced by NaF treatment in endothelial cells. The observations made in the PAE and HAECs are distinct from those obtained in fibroblasts and other cells, where NaF induces the formation of a dense network of F-actin cables (Vincent and Settleman, 1999). However, in endothelial cells from other vascular sources (PHAEC and HUAEC), NaF was also found to promote the formation of stress fibres. As RhoGTPases are major modulators of the actin cytoskeleton, we assume that the integration of the individual contribution of each GTPase may account for the resulting phenotype upon NaF treatment.

In the literature, fluoride derivatives are extensively used for their ability to form ternary complexes, where fluoride stabilizes the association of GDP-bound GTPase with its GAP. NaF promotes stable complexes between RhoA and p190RhoGAP (Vincent and Settleman, 1999). These observations, linked to the massive induction of stress fibres led...
Figure 3 | Small GTPases are involved in podosome formation after NaF treatment
(A) NaF activates RhoA, Rac1 and Cdc42 in PAE cells. PAE cells were treated without (−) or with (+) 20 mM NaF for 1 h. Cells were then lysed and active GTPases were affinity-precipitated with GST–RBD-rhotekin or GST–CRIB-PAK, eluted from the beads and analysed by Western blot using relevant antibodies. For each point, a fraction of the lysate was run to monitor the amount of GTPase before precipitation. The graph shows quantification of bands resulting from three experiments. Each bar represents the mean ± S.E.M. of three independent experiments. *P < 0.01, **P < 0.005, ***P < 0.001 using the t test when compared with control.
(B) Knockdown of Cdc42 or Rac expression inhibited NaF-induced podosome formation. siRNA designed against RhoA, Rac1 and Cdc42 down-regulated their targeted protein. PAE cells were transfected with the indicated siRNA. After 72 h, protein extracts were analysed by Western blot and cells were analysed by immunofluorescence. Quantification of the experiment was performed by counting the number of cells showing podosomes after transfection and the results are presented as fold increase of the control response obtained with cells transfected with control siRNA. Each bar represents the mean ± S.D. of three independent experiments. Ns, non-significant, **P < 0.01, ***P < 0.001 using the t test when compared with control.
(C) PAE cells were treated without or with 1 μg/ml of tat–GFP, 1 μg/ml of tat-C3 or 20 μg/ml of C3 for 7 h and then treated with 20 mM NaF for 1 h. After fixation, cells were analysed by immunofluorescence. Quantification of the experiment was done by counting the number of cells showing podosomes and the results are presented as fold increase of the control response obtained with untreated cells (control for C3) or tat–GFP treated cells (control for tat-C3). *P = 0.041, **P = 0.0056 using the t test when compared with control.
Sodium fluoride induces podosomes

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Figure 4 | NaF regulates RhoGTPase activation and podosome assembly in a Src-dependent manner

(A) Src inhibition modulates NaF-induced events. PAE cells were treated or not with 20 mM NaF and/or 5 μM PP1 for 1 h. PP1 inhibits NaF-induced podosomes. Cells were fixed and stained for F-actin. The percentage of cells presenting podosomes is indicated at the bottom of each image. Podosomal structures are shown by arrows. (B) PP1 alters the NaF-induced GTPase response. Cells were lysed and active GTPases were affinity-precipitated and analysed by Western blot using relevant antibodies. A fraction of each cell lysate was run to monitor the amount of GTPase before precipitation. The graphs show quantification of bands resulting from three experiments. Each bar represents the mean ± S.E.M. Ns, non significant, *P < 0.05, **P < 0.005 using the t test when compared with control without PP1.
the authors to suggest that the sequestration of p190RhoGAP is responsible for the biologically significant activation of RhoA. In our endothelial cell model, RhoA became activated when cells were exposed to NaF. By showing that Cdc42 and Rac1 were also found under their GTP-bound form upon NaF treatment, we now extend this observation to two other members of the RhoGTPase family. Future studies will establish if Rac/Cdc42 activation by NaF proceeds through a mechanism similar to the one described for RhoA. Indeed, the fact that the p190RhoGAP catalytic domain has a primary structure that is not obviously distinct from other reported RhoGAPs (Settleman et al., 1992) suggests that the mechanism by which it interacts with its GTPase target is not atypical. In this scenario, endothelial cells express high levels of a Cdc42GAP, which can be sequestered by fluoride in a manner analogous to p190RhoGAP. Based on our studies, we propose that the expression pattern of RhoGAPs and RhoGTPases determine the actin phenotype induced upon NaF treatment.

Cdc42 is a key player in the process of podosome formation in all cell types examined so far. Exploring the requirement of activated GTPases in the process of podosome formation, we revealed that in addition to Cdc42, Rac1 is also needed for podosome formation when the process is triggered by NaF, whereas Rac1 was found dispensable for podosome induction in HUVECs exposed to PMA. Also in contrast with other models (Berdeaux et al., 2004; Tatin et al., 2006), RhoA is not required for podosome formation upon NaF treatment and Rho protein inhibition favours podosome formation in endothelial cells. These results are consistent with our previous study describing the role of RhoA in V12Cdc42-driven endothelial podosome assembly (Moreau et al., 2003). These results confirm that Cdc42 plays a master role in the general process of podosome formation and that podosome assembly and possibly podosome arrangement are controlled by the integration of several GTPase activities. They also demonstrate that Rac1 and RhoA may or may not play a role, depending on cellular contexts and podosome inducers.

Src, which is activated downstream of growth factor and integrin receptors, is also a master regulator of podosome formation (Linder, 2009). Many podosomal components, such as cortactin, focal adhesion kinase or vinculin are Src substrates. Consequently, pharmacological agents targeting Src activity affect podosome formation (Linder et al., 2000; Tatin et al., 2006; Varon et al., 2006). Indeed, consistent with previous reports, NaF-induced podosomes were found to be sensitive to PP1. At the GTPase level, Src inhibition alters NaF-mediated events in further increasing RhoA activation mediated by NaF, suggesting that Src may operate upstream RhoGTPases. As Src is a positive regulator of p190RhoGAP (Roof et al., 1998), the PP1 inhibitory effect on Src is expected to suppress a negative regulation on RhoA and therefore promote the GTP-bound form of the GTPase. Since Rho protein inhibition increased the number of cells showing podosomes, the PP1 inhibitory effect on NaF-induced podosome assembly could be a direct consequence of alteration in RhoA activity. One explanation is that hyperactivation of RhoA reduces Cdc42 activity as a result of the crosstalk between RhoGTPases (Moorman et al., 1999). Indeed, PP1 inhibitory action lowered basal Cdc42-GTP content and slightly prevented Cdc42-GTP accumulation in response to NaF. This result is also consistent with our previously reported data showing that v-Src induced Cdc42 activation in PAE cells (Tatin et al., 2006). Alternatively, PP1 may simply act by reducing the incoming signals (from integrins or growth factors from the culture medium). These various regulations may explain why NaF-mediated cytoskeletal reorganization remains dependent on Src kinase activity. Thus, besides its obvious inhibitory action on phosphotyrosine-regulated podosomal components, PP1 may affect the balance of RhoGTPase activities in endothelial cells exposed to NaF and thereby contribute to suppress podosome formation.

Given that vanadate, an inhibitor of protein tyrosine phosphatase (Marchisio et al., 1988), drives podosome formation, and given that fluoride is also a general inhibitor of protein phosphoseryl and phosphothreonyl phosphatases, it cannot be ruled out that fluoride targets phosphatases with specificities for major signalling components in podosome formation and induces podosome formation through this route. For instance, constitutively active forms of either PAK1 (p21-activated kinase 1) (Webb et al., 2005) or PKCα (protein kinase Cα) (Gatesman et al., 2004; Tatin et al., 2006) can by themselves trigger podosome assembly. However, phosphatase inhibitors targeting PKC- or PAK-phosphorylated substrates (okadaic acid, cyclosporin) were inefficient in this
Sodium fluoride induces podosomes

regard (Violaine Moreau, unpublished data). Thus the phosphatase inhibitor function of NaF is unlikely to play a major role, if any, in the process of podosome formation.

In the present study we demonstrate that fluoride is capable of triggering the assembly of functional podosomes. Thus fluoride may be a useful tool to study podosome formation and function in cultured cells.

Materials and Methods

Culture conditions
PAE cells, clone p23, and PAE cell lines expressing V12Cdc42 under the control of an IPTG (isopropyl β-D-thiogalactoside)-inducible promoter were described previously (Moreau et al., 2003). HAECS and HUAECs were purchased from Promocell and cultured in the endothelial cell basal medium supplemented with the ‘supplement pack’ recommended by the manufacturer (EGM-MV). PHAECS were purchased from Cell Systems Corporation and cultured in the same medium. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Reagents and antibodies
IPTG and PP1 were purchased from Calbiochem. Rhodamine-phalloidin and Alexa Fluor® 488-labelled secondary antibodies were from Invitrogen. Monoclonal anti-vinculin antibodies (hVIN-1) were from Sigma. Antibodies against MT1-MMP (Lemm-2/15.8) were from Millipore, those against p190A and Cdc42 from BD Biosciences. Anti-RhoA antibodies were from Santa Cruz and anti-p85α antibody was from Serotec (U5 clone). Constructs encoding GST–RBD-rhotekin, GST–CRIB (Cdc42/Rac-interacting binding)-PAK, active mutants of RhoA or Cdc42, GFP (green fluorescent protein)–N-WASP and GFP–WIP were described previously (Moreau et al., 2003). Rho inhibitor C3 transferase was purchased from Cytoskeleton (Tebu). Tat-C3 and Tat–GFP were a gift from Dr. Jacques Bertoglio (INSERM Unit 749, Chatenay-Malabry, France).

Immunofluorescence microscopy
Cells plated onto glass coverslips were prepared for immunofluorescence microscopy as previously described (Moreau et al., 2003). Fluorescent images were recorded on an Eclipse Nikon fluorescence microscope as previously described (Moreau et al., 2003). Immunofluorescence confocal microscopy was performed using a Zeiss LSM 510 inverted laser scanning fluorescence confocal microscope equipped with an acquisition software (LSM 510 acquisition software; Zeiss) and a 63× oil immersion objective. The images were processed using Adobe Photoshop 5.5 (Adobe Systems). Quantification of cells showing podosomes was carried out in three independent experiments in which at least 200 cells were counted.

Rho and Cdc42 activity assays
Rho protein activity assays were performed as described previously (Tatin et al., 2006).

siRNA transfection
siRNAs were chemically synthesized (Qiagen) and introduced into PAE cells (200 pmol) using Lipofectamine™ RNAiMAX (Invitrogen) according to the protocol of the manufacturer. The antisense strand siRNA was targeted against GTPase using a 21-nt sequence (′-AAGAAGTCAAGCATTTGTGTC-3′) for RhoA, (′-AAGTTCTTAAATGGCTTTCC-3′) for Rac1, and (′-AAGATAACTCACCCTGCTCA-3′) for Cdc42 according to published sequences (Deranne et al., 2003). The AllStars negative-control siRNA from Qiagen were used in all siRNA experiments.

Extracellular matrix degradation assay
Cells were seeded on FITC-gelatin coated coverslips prepared as described previously (Tatin et al., 2006). Co-localization between dark areas and podosomes was visualised after merging FITC and rhodamine-phalloidin images.

Statistics
The Student’s t test was employed to calculate P-values. Differences were considered to be statistically significant at P < 0.05.

Author contribution
Violaine Moreau and Elisabeth Genot conceived the project and designed the experiments. Florence Tatin and Violaine Moreau performed the PAE cell experiments. Florence Grise collected data on siRNA-treated cells. Edith Reuzeau collected data on primary cultures. Violaine Moreau and Elisabeth Genot wrote the manuscript. Florence Tatin and Florence Grise reviewed the manuscript.

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**Figure S1 | NaF induces various actin reorganizations in cells**
HAECs, PHAECs and HUAECs were treated with 20 mM NaF for 1 h, and stained for F-actin. Note the presence of podosome-like structures (arrows) or stress fibres (arrowheads). Scale bar, 10 μm for HAECs; 25 μm for PHAECs and HUAECs.

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Figure S2 | N-WASP and WIP localize at NaF-induced podosomes

PAE cells were transfected with either GFP–N-WASP or GFP–WIP. At 24 h later, cells were treated with 20 mM NaF for 1 h. Cells were stained with rhodamine-phalloidin to visualize F-actin (red). Scale bar, 10 μm.

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