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Cooperative Cdc42 and Rho signalling mediates ephrin-B triggered endothelial cell retraction

Gillian Groeger* and Catherine D. Nobes*†‡

Departments of Physiology* and Biochemistry†, School of Medical Sciences, University of Bristol, University Walk, Bristol, BS8 1TD, UK

‡Author for correspondence:

Kate Nobes

Departments of Physiology and Biochemistry

School of Medical Sciences

University of Bristol

University Walk

Bristol BS8 1TD, UK

catherine.nobes@bristol.ac.uk; Telephone: + 44 (0)117 331 7306; Fax: + 44 (0)117 928 8923

Short title: Cdc42 and Rho mediate ephrin-induced retraction

Abbreviations:

HUVECs: Human Umbilical Venous Endothelial Cells

MRCK: myotonic dystrophy kinase-related Cdc42-binding kinase

MLC: myosin light chain

ROCK: Rho Kinase

K44A Dyn: dominant-negative dynamin construct

Abstract

Cell repulsion responses to Eph receptor activation are linked to rapid actin cytoskeletal reorganisations, which in turn are partially mediated by Rho-Rho kinase (ROCK) signalling, driving actomyosin contractility. Here we show that Rho alone is not sufficient for this repulsion response. Rather, Cdc42 and its effector MRCK are also critical for ephrinB-induced cell retraction. Stimulation of endothelial cells with ephrin-B2 triggers rapid, but transient, cell retraction. We show that whilst membrane retraction is fully blocked by blebbistatin (a myosin-II ATPase inhibitor), it is only partially blocked by inhibiting Rho-ROCK signalling, suggesting there is ROCK-independent signalling to actomyosin contractility downstream of EphBs. We find that a combination of either Cdc42 or MRCK inhibition with ROCK inhibition completely abolishes the repulsion response. Additionally, endocytosis of ephrin-Eph complexes is not required for initial cell retraction but is essential for subsequent Rac-mediated re-spreading of cells. Our data reveal a complex interplay of Rho, Rac and Cdc42 in the process of EphB-mediated cell retraction/recovery responses.

Key words: Cdc42, EphB receptor, actomyosin contraction, MRCK, ROCK

Introduction

Eph receptors and their cell-surface anchored ligands, ephrins, regulate a wide variety of cell migration-related processes in developing and adult tissues [1,2]. Signalling pathways triggered by Eph receptors and ephrins promote cell contact-dependent repulsion that prevents mixing of receptor- and ligand-expressing cells. The mechanisms underlying these cell repulsion responses are complex and not fully characterised but are known to involve cell:cell detachment by loss of high-affinity Eph-ephrin interactions, either due to proteolytic cleavage [3] or by their internalisation [4,5], and concomitant increased contractility of Eph-expressing cells resulting in the withdrawal of cell extensions [6-8]. Both of these component steps have been shown to involve activation of the Rho family GTPases, Rho (contractility) and Rac (Eph endocytosis), by Eph receptor recruitment and activation of guanine nucleotide exchange factors (GEFs), ephexin1 and Vav2 [9-11].

Although Rho, acting through ROCK and MYPT1 phosphorylation, was initially established as the Rho GTPase family member associated with actomyosin contractility and cell retraction episodes [12], it has been demonstrated recently that another Rho family GTPase, Cdc42, acting through myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), can also drive cell contractility [13,14] and, indeed, cooperates with Rho-ROCK signalling in mediating cell invasion [15]. Cdc42 has been shown to be activated by Eph receptors but, rather than being linked to contractility, is proposed to regulate actin polymerisation during dendritic spine morphogenesis [16]. Rac also appears not to regulate cell contractility downstream of Eph receptors, but it, and its exchange factor Vav2 mediate repulsion responses to ephrins by regulating endocytosis of ephrin-Eph complexes [4,11].

In this study, we have investigated how Rho family GTPases and ephrin-Eph endocytosis regulate endothelial cell retraction and subsequent membrane re-spreading triggered by ephrin-B2. We show that the retraction response is entirely dependent on myosin-II ATPase activity and, in line with previous studies, is only partially dependent on Rho-ROCK signalling. Microinjection of dominant-negative constructs reveals that Cdc42-MRCK signalling plays a critical role in endothelial cell contractility triggered by ephrin-B2 and, in combination with ROCK inhibition, can entirely prevent Eph-mediated endothelial cell retraction. Interestingly, we find that blocking endocytosis of ephrin-Eph complexes, through expression of dominant-negative dynamin, had no effect on actomyosin driven cell retraction; rather, it inhibits cell re-spreading suggesting that removal of surface active Eph receptors is required to terminate actomyosin contractility signals prior to cell recovery from repulsion responses. Rac does not appear to play a role in the retraction event, but is necessary for the

recovery of cells to a fully spread morphology. These findings suggest an important role for Cdc42 in repulsive signalling responses to activated Ephs.

Materials and methods

Reagents and expression plasmids

Antibodies for cell staining and Western blotting were anti-c-myc (9E10, Serotec, dilution 1:200), anti-dynamin (HUDY1, Upstate, dilution 1:50), anti-FLAG (M2, Sigma, dilution 1:50), anti-tubulin (YL1/2, Serotec, dilution 1:10,000), anti-phospho-MLC2 (ser19; New England Biolabs; dilution 1:1000) and anti-MLC2 (FL-172, Santa Cruz, dilution 1:500). Anti-phospho-Eph receptor was described previously [4]. All secondary antibodies were from Jackson ImmunoResearch. All reagents were purchased from Sigma unless otherwise stated.

The following constructs were received as gifts: pcDNA3-dynamin1 (K44A Dyn; J.M. Edwardson, Cambridge, UK) and pEFLAG-CDC42BPA-isobK106M-FL (MRCK \square kinase-dead [KD]; C.J. Marshall, CRUK, London, UK).

Cell culture and microinjection

Human umbilical cord venous endothelial cells (TCS Cell Works) were cultured as published previously [4] and were used at 50-80% confluency. Cell nuclei were microinjected with DNA expression constructs (100-300 \square g/ml) and an injection marker (FITC-, TRITC- or biotin-conjugated lysinated dextrans [Molecular Probes]) and cells allowed 1.5-4 h to express recombinant protein as indicated.

Cells were stimulated with ephrin-B2-Fc (1 μ g/ml; R+D Systems) or Fc (1 μ g/ml; R+D Systems) pre-clustered (20 min) with goat anti-human IgG (5 μ g/ml; Stratech). Cells were incubated with blebbistatin (100 \square M; Tocris) for 30 min, or with Y-27632 (10 \square M; Calbiochem) for 2 h, prior to, and also during, stimulation.

Time-lapse movies were made using an inverted microscope (Zeiss Axiovert 200M) with a Zeiss, 40x, 0.6 NA lens, a digital camera (C4742-95, Hamamatsu) and Openlab (Improvision) software. Cells were maintained at 37°C with 5% CO₂ during recording.

Immunocytochemistry

Cultured cells were fixed, permeabilised and quenched as published previously [4] and incubated with primary antibodies for 45 min, followed by appropriate secondary antibodies for 25 min. TRITC- or FITC-conjugated phalloidin was used to stain F-actin when required. All antibody dilutions were in PBSA and incubations were at room temperature. Confocal images were collected using a Leica TCS-NT confocal attached to a Leica DM IRBE inverted microscope with a HCX, 63x, 1.32 NA, oil immersion lens using Leica Confocal Software and were subsequently handled with Adobe Photoshop.

To examine internalisation of ephrin/Eph complexes, two protocols were used. A) After fixation, cells were incubated with anti-goat-Cy3 antibodies to label external IgG-ephrin-B2-Eph complexes. Cells were then washed, post-fixed, permeabilised and incubated with anti-goat-FITC antibodies to label internalised IgG-ephrinB2-Fc. B) Alternatively, cells were acid washed (2 x 5 min) at 4°C (0.2% BSA, 10 mM MES, 1 mM sodium pyruvate in DMEM (Gibco), pH=2) prior to fixation to remove any externally bound ephrin-B2-Fc before immunostaining.

Biotinylation and immunoblotting

The biotinylation method used was as published previously [17]. Briefly, following biotinylation of cell surfaces at 4°C for 10 min using EZ-Link NHS-SS-Biotin (Pierce), cells were maintained at 4°C, and incubated in a Tris-Amine Buffer (25 mM Tris, 137 mM NaCl, 5 mM KCl, 2.3 mM CaCl₂, 0.5 mM MgCl₂, 0.143 g/L Na₂HPO₄) for 10 min. Cells were stimulated as normal at 37°C and, after the required internalisation time, any non-internalized biotin was removed from cell surfaces using a glutathione solution (75 mM NaCl, 75 mM NaOH, 1 mM EDTA, 0.1% BSA, 50 mM glutathione, pH=9) (2 x 20 min), which was neutralised by washing with a Tris-Glycine buffer (0.192 M glycine, 25 mM Tris). Cells were washed several times in TBS before lysates were prepared in a modified RIPA buffer (125 mM NaCl, 20 mM Tris-HCl, 10% glycerol, 1% NP40, 50 mM NaF, 200 mM Vanadate, 100 μg/mL PMSF, protease inhibitor cocktail [Roche]). Biotinylated proteins were precipitated using streptavidin-conjugated, agarose beads (Upstate) and were resuspended in Laemmli loading buffer and analysed by SDS PAGE and Western blot.

Analysis

ImageJ (version 1.33U; public domain software available from <http://rsb.info.nih.gov/ij>) was used to quantify results from Western blots. All graphs were drawn using Microsoft Excel.

Results and discussion

Previous studies have shown that ephrin-induced cell retraction and growth cone collapse is only partially blocked by inhibitors of Rho GTPase and its effector ROCK [6,7,18], suggesting that additional parallel pathways regulate this component of the repulsion response. To investigate signalling pathways underlying ephrin-regulated cell retraction, we have stimulated human umbilical vein endothelial cells (HUVECs) with soluble pre-clustered ephrin-B2-Fc. We show that non-confluent HUVECs display a dynamic, reproducible retraction-recovery response within minutes to ephrin-B2-Fc exposure (Fig. 1A and Supplemental Movie 1). Cells rapidly undergo plasma membrane retraction (within 10 min) but then subsequently, despite the continued presence of ephrin, recover to a spread state (within 35 min) by lamellipodial growth along the retraction fibres left behind (Fig. 1A and B). These early changes in cell morphology correlate with the initial phase of activation of Eph receptors as revealed by phosphorylation of Eph juxtamembrane tyrosine residues (Fig. 1C and D). Interestingly, although increased phosphorylation was observed after 5 min, maximal phosphorylation of Eph receptors was not until 40 min after stimulation (Fig. 1C and D), by which time most cells had recovered from their retracted state. This suggests that dephosphorylation of Eph receptors at the juxtamembrane sites is not a driver of, or even required for cell re-spreading.

Since the switch from retraction to re-spreading appears not to involve de-activation of Eph receptors, we investigated whether it requires Eph receptor internalisation. Previously, it has been shown that removal of ephrinB-EphB complexes at cell-cell contacts, through their internalisation, is required for cell separation during the repulsion response [4,5]. More recent evidence suggests that endocytosis of EphA receptors may direct, rather than simply facilitate, ephrin-triggered repulsion responses, since Vav2 both regulates endocytosis of ephrin-Eph complexes and is necessary for growth cone collapse [11]. Using both immunocytochemistry and biochemical techniques we have determined the temporal relationship between ephrin-Eph complex internalisation and membrane retraction/recovery events. HUVECs were stimulated with IgG pre-clustered ephrin-B2-Fc for various times as indicated, fixed in the absence of detergents and immunostained for ephrin-B2-Fc/IgG on the cell surface (red staining). Cells were then post-fixed, permeabilised and stained for internalised ephrin-B2-Fc/IgG (green). Ephrin-Eph complex endocytosis is relatively slow compared with the cell retraction response and most internalised ephrin/IgG appears around 20-45 min after initial exposure to ephrin-B2-Fc (Fig. 2A). These data are corroborated by surface biotinylation studies that also show the time-course of phosphorylated-Eph (p-Eph) internalisation (Fig. 2B and 2C). In these experiments all surface proteins were biotinylated for 10 min at 4°C, washed and returned to

37°C for various times in the presence of ephrin-B2-Fc to allow endocytosis. Biotinylated proteins remaining on the surface after incubation were debiotinylated by incubating with glutathione buffer at 4°C leaving only the endocytosed proteins biotinylated. The amount of biotinylated p-Eph was determined by binding cell lysate to streptavidin-agarose beads and Western blotting with anti-p-Eph. These experiments show that very little p-Eph is internalised within 10 min of ephrin-B2 stimulation (Fig. 2B and 2C), the time at which we observe maximal cell retraction (Fig. 1B). Moreover, most internalisation of activated Eph receptors is between 25-40 min after ephrin-B2 exposure when we observe withdrawn lamellipodia subsequently extending along retraction fibres. These experiments address the temporal relationship between ephrin-B2 triggered repulsive signalling and endocytosis of activated Eph receptors. They show that internalisation of activated Eph receptors coincides with the recovery of retracted cells rather than the retraction phase itself.

Next we tested whether the cell re-spreading response is dependent on internalisation of ephrin-Eph complexes by expressing a dominant-negative mutant form of dynamin, dynamin-1 (K44A). We show that expression of dominant-negative dynamin inhibits internalisation of soluble ephrin-B2 (Fig. 3A). Importantly, blocking dynamin activity had no effect on cell retraction triggered by ephrin-B2, but did abrogate subsequent cell re-spreading (Fig. 3B and 3C). Time-lapse microscopy shows that dominant-negative dynamin expressing cells remain contracted for up to 60 min after stimulation with ephrin-B2-Fc (Fig. 3C and Supplemental Movie 2). These movies demonstrate, however, that contracted cells are able to produce small, localised ruffles but were unable to re-spread lamellipodia. In order to control whether dominant-negative dynamin may be acting by inhibiting actin filament polymerisation required for cell spreading, we tested whether dynamin-1 (K44A) interfered with lamellipodial extension after cell trypsinisation, and found that dynamin-1 (K44A) expressing cells adhere and spread by extending lamellipodia similarly to uninjected controls after re-plating (data not shown). These experiments show that endocytosis of ephrin-Eph complexes is not necessary for ephrin-B2 induced cell retraction in HUVECs, but rather is required for the subsequent recovery of cells to a fully spread morphology. In line with our results, Mann et al., [19] showed that internalisation of EphB receptors is not necessary for collapse of *Xenopus* retinal growth cones. By contrast, Vav2-dependent internalisation of EphA receptors is necessary for collapse of retinal growth cones [11] indicating that there may be differences between EphB and EphA signalling pathways that control internalisation and retraction, in ways that are not cell type specific.

Many cellular contraction events are actomyosin-dependent and can be blocked by the pharmacological agent blebbistatin, which inhibits myosin-II ATPase activity [20,21].

HUVECs treated with blebbistatin (100 μm) lose their stress fibres but remain well spread with membrane ruffles (Fig. 4B). At 10 min post ephrin-B2-Fc stimulation, when we generally see most cell retraction, blebbistatin completely blocked the retraction response (Fig. 4D). These data demonstrate that rapid retraction triggered by ephrin-B2 is strictly dependent on myosin activity and increased actomyosin contractility. We next tested the effect of inhibiting Rho-ROCK signalling on ephrin-induced endothelial cell retraction. Inhibition of ROCK with Y-27632 (10 μm) had the predicted effect of removing actin stress fibres (Fig. 4C), as did expression of C3 transferase (data not shown). In line with previous studies of ephrin-induced collapse of retinal ganglion cell growth cones and rounding of PC-3M cells [6,7,18], endothelial cell retraction was only partially inhibited by Y-27632 or C3 transferase. Pre-treatment of cells for 2h with Y-27632 (10 μm) or microinjection of C3 transferase (20 $\mu\text{g/ml}$; 2h expression time) attenuated the retraction response by about 50% (Fig. 4D) compared with a greater than 90% suppression by blebbistatin. The blebbistatin-sensitive, but Y-27632/C3-insensitive, component of ephrin-triggered cell contractility was most clear at 10 min (Fig. 4D). Actomyosin-driven cell retraction involves phosphorylation of myosin-II light chain kinase (MLC) [22]. We find that MLC was phosphorylated 2 min after stimulation of cells with ephrin-B2, reached a peak at 5-10 min, and then returned to control levels at 20-60 min (Fig. 4E and F). Taken together, these results show that endothelial cell retraction rapidly triggered by ephrin-B2-Fc is strictly actomyosin-dependent, but that there is Rho/ROCK-independent signalling to this actomyosin contractility.

Next we investigated the involvement of other Rho family GTPases in endothelial cell retraction triggered by ephrin-B2. We injected dominant negative expression constructs (pRK5mycN17Rac and pRK5mycN17Cdc42) to inhibit the activity of Rac and Cdc42 and again examined the response to pre-clustered ephrin-B2-Fc stimulation. Expression of dominant negative Rac barely altered the cell retraction phase, but cells remained retracted and did not subsequently re-spread (Fig. 5A). A sustained retraction phase is not unexpected because Rac regulated lamellipodia extension is known to be required for cell spreading from a rounded morphology [23]. By contrast, expression of N17Cdc42 significantly reduced the retraction response. At 10 min post ephrin-B2 exposure, when control injected cells display maximum retraction, expression of N17Cdc42 attenuated the retraction response by about 50%. However, by 20 min the retraction response of N17Cdc42 injected cells was similar to control-injected cells suggesting that Cdc42 signalling to actomyosin contraction is a component of the initial phase of Eph signalling to cell contractility. Recent studies have demonstrated that Cdc42, like Rho, can regulate actomyosin contractility, through its effector MRCK [24], to regulate cell invasion and nuclear movements during cell polarisation [15,25].

MRCK, like ROCK, phosphorylates MLC. We show that expression of dominant negative MRCK (MRCK^{KD} kinase-dead [KD]) inhibits cell retraction (Fig. 5A). To determine whether the Cdc42-MRCK signalling pathway may interact in a parallel, non-redundant role with Rho-ROCK signalling to regulate the cell retraction response, we combined N17Cdc42 and MRCK^{KD} microinjection experiments with the addition of Y-27632 and, in both cases, this resulted in the inhibition of retraction levels to those obtained with blebbistatin (Fig. 5B and C). Taken together, these data imply that Rho-ROCK and Cdc42-MRCK signalling pathways play complementary/additive roles in the retraction of HUVECs in response to EphB activation.

In summary, the data presented demonstrate that Rho and Cdc42 function in concert to mediate ephrin-B2 induced cell retraction. Whilst blocking Rho-ROCK signalling with C3 transferase and Y-27632 and Cdc42-MRCK signalling with dominant negative constructs, only partially inhibited ephrin-triggered cell contractility, blocking both signalling pathways mirrored the absolute inhibition achieved with blebbistatin, a myosin II ATPase inhibitor. The recovery to a fully spread morphology, on the other hand, requires functioning Rac and the internalisation of ephrin/Eph complexes. The removal of activated Eph receptors from the membrane may be vital to enable cells to reset their signalling machinery and to continue migrating after a temporary Eph-mediated contact inhibition response.

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Supplemental Material:

Supplemental Movie S1: Stimulation of HUVECs with pre-clustered ephrin-B2-Fc triggers transient membrane retraction (see also Fig. 1A). Movie commences 5 min before addition of ephrin-B2-Fc (marked by a single white frame) and covers 46 min (140 frames).

Supplemental Movie S2: Dominant-negative dynamin expression in HUVECs blocks re-spreading of cells after retraction triggered by ephrin-B2-Fc (see also Fig. 3C). Movie commences 3h after microinjection of K44A dynamin. Single white frame indicates time of stimulation with ephrin-B2-Fc. Movie corresponds to 66 min (199 frames).

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Figure legends

Figure 1. Retraction-recovery response of HUVECs to ephrin-B2-Fc. A) Time-lapse movie stills (pre-clustered ephrin-B2-Fc added at 0 min; arrows indicate regions of cell retraction; see also Supplemental Movie S1). B) Cell retraction in response to pre-clustered ephrin-B2-Fc or control Fc (n=5, with >100 cells counted /condition). C, D) Time-course of phosphorylation of Eph receptors (pEph) in response to ephrin-B2-Fc (n=3; means \pm S.E.M). Scale bar = 30 μ m.

Figure 2. EphB-ephrinB2 internalisation coincides with cell recovery, and not with cell retraction. A) Schematic shows extracellular ephrin-B2-Fc (red) and intracellular ephrin-B2-Fc (green). Confocal images show that most ephrin-B2-Fc internalisation occurs between 20 and 45 min. B, C) Surface biotinylation experiments to assay time-course of internalisation of active Eph receptors (n=3; means \pm S.E.M). Scale bar = 20 μ m.

Figure 3. Internalisation of ephrin-B2-Fc is necessary for cell re-spreading. A) Cells expressing K44A Dyn (green) do not internalise EphB-ephrinB2 complexes (red); acid washing the cells immediately prior to fixation removed external ephrin-B2-Fc, therefore red staining represents internalised ephrin-B2-Fc. B) Ephrin-B2 triggered retraction in cells injected with K44A Dyn, or with control injection marker (dextran) (n=3-5, >30 cells/condition; means \pm S.E.M). C) Time-lapse stills showing that K44A Dyn injected cells do not re-spread lamellipodia after ephrinB2-Fc stimulation (see Supplemental Movie S2). Scale bar=30 μ m.

Figure 4. Ephrin-B2-Fc induced retraction is myosin II-dependent but only partially blocked by Rho/ROCK inhibition. A-C) Treatment of HUVECs with blebbistatin (100 μ m) or Y-27632 (10 μ m) results in the loss of actin stress fibres. D) Quantification of cellular retraction in response to ephrin-B2-Fc in the presence of blebbistatin and Y-27632 (n=3, >100 cells/time point) and in cells injected with C3 transferase (n=8, with >40 cells/experiment). E and F) Phosphorylation of myosin light chain II (MLC) during the retraction response (n=6; means \pm S.E.M). Scale bar = 40 μ m.

Figure 5. Cdc42 and Rho pathways cooperate in ephrin-B2-Fc induced retraction. A) Time-course of ephrin-B2-induced retraction in cells injected with injection marker (dextran), N17Rac, N17Cdc42 or MRCK \square KD (n=3-7). B) Quantification of the combined effects of

N17Cdc42 with Y-27632 or MRCK \square KD with Y-27632 (n=7) on retraction induced by ephrin-B2-Fc compared to injection marker and cells pre-treated with blebbistatin (means \pm S.E.M.). C) Representative images of cells (actin in red) injected with either N17Cdc42 (middle panel) or MRCK \square KD (bottom panel) and incubated with Y-27632 (10 μ m) as compared to FITC-dextran injected cell (top panel). Green is the expression marker (top panel) or anti-myc or anti-FLAG (in the middle and bottom panels). Scale bar=20 \square m.

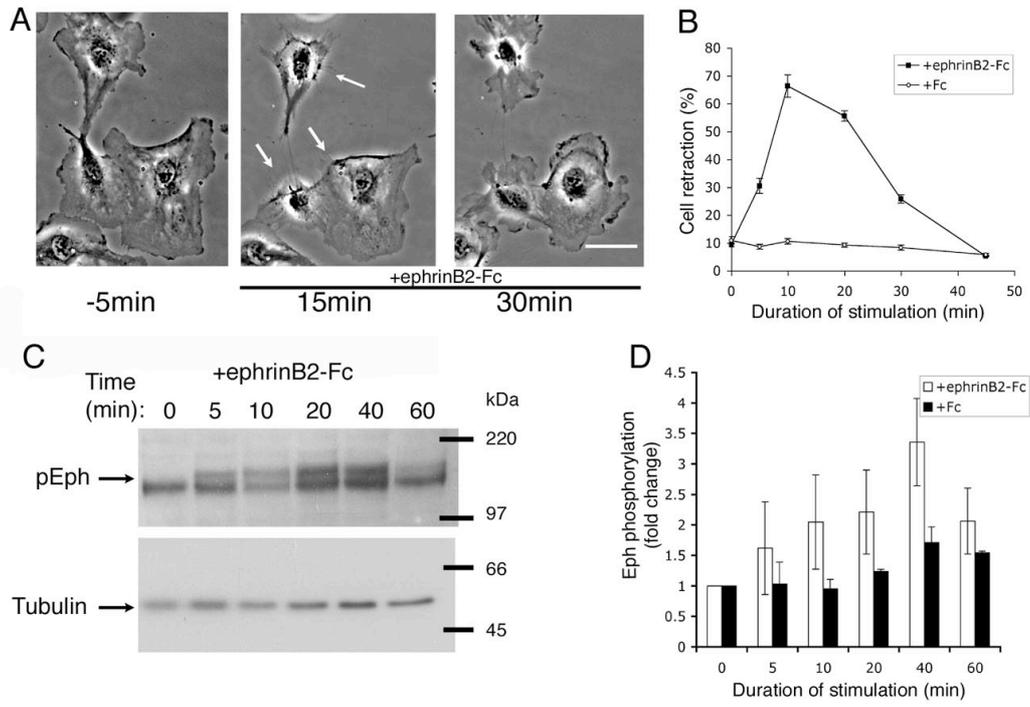


Figure 1

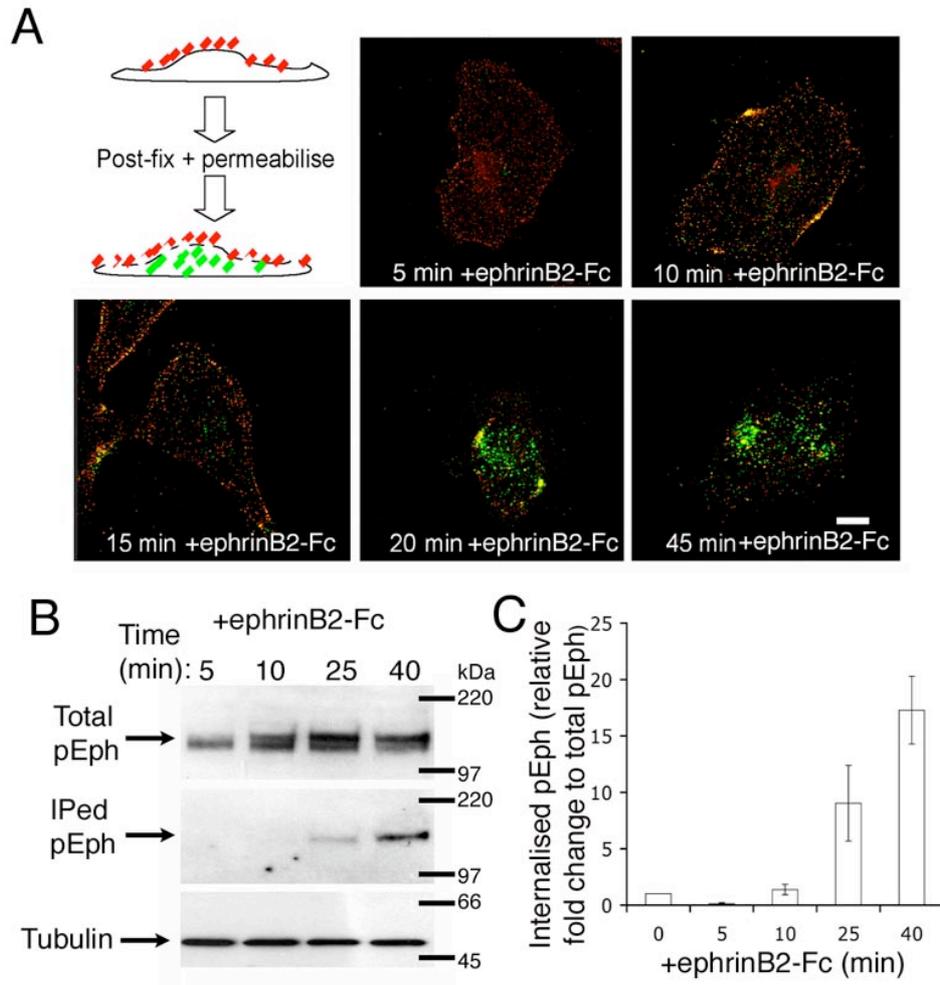


Figure 2

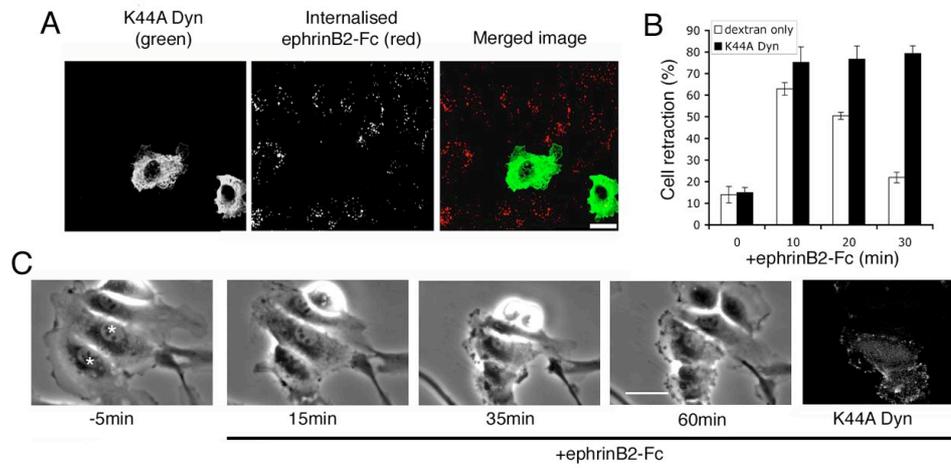


Figure 3

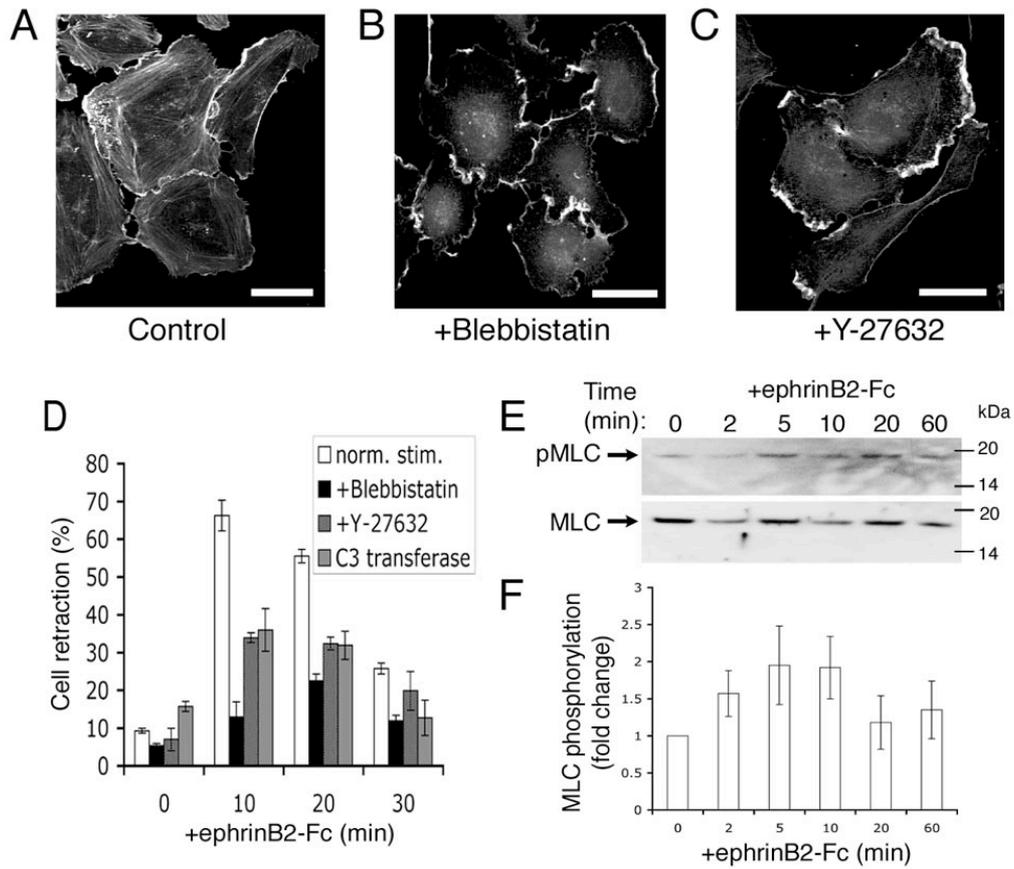


Figure 4

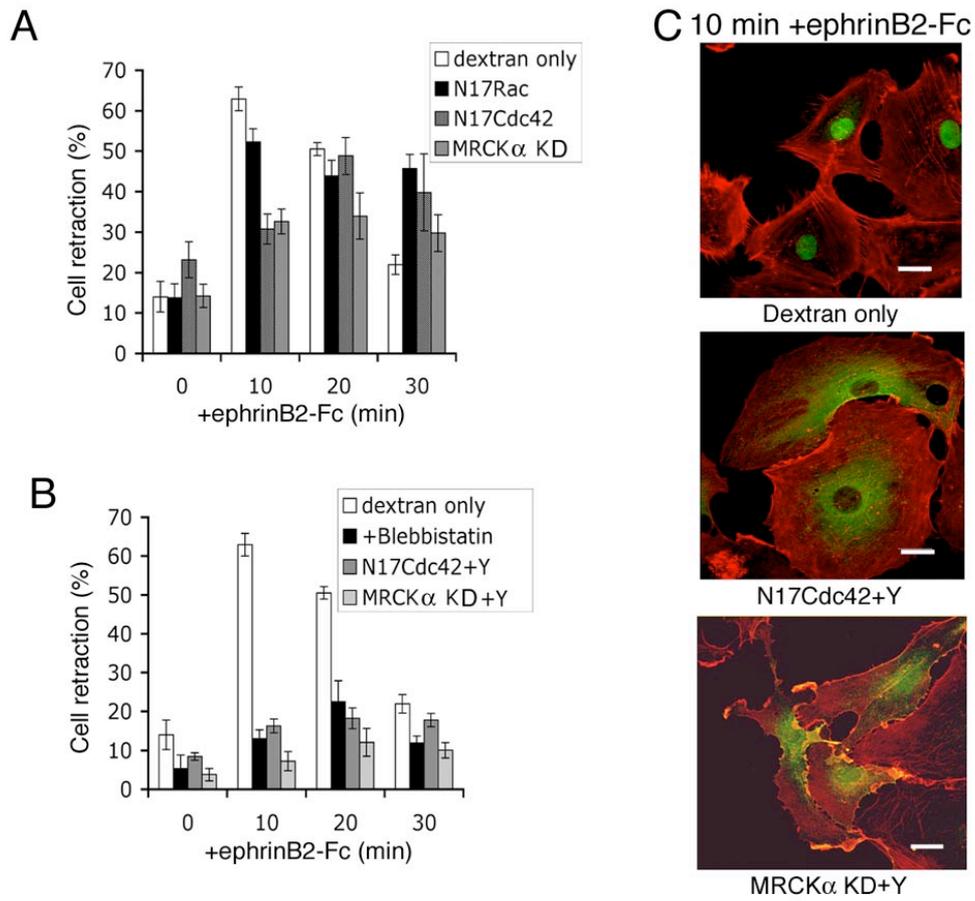


Figure 5