

Src kinase modulates the activation, transport and signalling dynamics of fibroblast growth factor receptors

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The non-receptor tyrosine kinase Src is recruited to activated fibroblast growth factor receptor (FGFR) complexes through the adaptor protein factor receptor substrate 2 (FRS2). Here, we show that Src kinase activity has a crucial role in the regulation of FGFR1 signalling dynamics. Following receptor activation by ligand binding, activated Src is colocalized with activated FGFR1 at the plasma membrane. This localization requires both active Src and FGFR1 kinases, which are inter-dependent. Internalization of activated FGFR1 is associated with release from complexes containing activated Src. Src-mediated transport and subsequent activation of FGFR1 require both RhoB endosomes and an intact actin cytoskeleton. Chemical and genetic inhibition studies showed strikingly different requirements for Src family kinases in FGFR1-mediated signalling; activation of the phosphoinositide-3 kinase–Akt pathway is severely attenuated, whereas activation of the extracellular signal-regulated kinase pathway is delayed in its initial phase and fails to attenuate.

Keywords: Src; FGFR; phosphorylation; RhoB; actin

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INTRODUCTION

The dynamics of signal propagation by receptor tyrosine kinases (RTKs) has a crucial role in cellular responses to ligands (Marshall, 1995). Mutations in fibroblast growth factor receptors (FGFRs), which slow ligand dissociation rates and extend signal duration, lead to phenotypic effects in skeletal development (Wilkie *et al*, 1995; Hajihosseini *et al*, 2004) and act as ‘driver’ mutations in various types of common tumour (Greenman *et al*, 2007). RTKs

are activated by ligand-mediated receptor homo- or heterodimerization (Schlessinger 2000; Pellegrini *et al*, 2000; Furdui *et al*, 2006); however, there is evidence that receptor activation might occur indirectly or be modified by recruitment and activation of other tyrosine kinases (Halford & Stacker, 2001). In the case of FGFR, Src kinase is recruited, by receptor-mediated phosphorylation, to the adaptor protein factor receptor substrate 2 (FRS2), influencing signalling dynamics by phosphorylation of the attenuator Sprouty (Li *et al*, 2004).

This finding prompted further analysis of the relationship between Src activity, FGFR1 activation and downstream signalling. As Src regulates endosomal transport of tyrosine kinases, including Src itself, through RhoB- and Rab11-containing endosomes (Sandilands *et al*, 2004), we reasoned that Src activation might have a role in FGFR activation through a directed transport mechanism. By using phospho-specific antibodies and Src inhibition, we show that Src activity, through a RhoB and actin-dependant pathway, controls FGFR activation and transport to and from the plasma membrane, and has both positive and negative roles in the activation and termination of FGFR signalling.

RESULTS AND DISCUSSION

Localization of activated FGFR and Src

Previous biochemical studies have shown that, following FGF2 stimulation, Src is recruited to FRS2 in a kinase-dependant manner, but the cellular location of this co-recruitment is unknown (Li *et al*, 2004). In the first set of experiments, phospho-specific antibodies directed against FGFR1/2-Tyr463 (characterized in supplementary Fig 1 online) and the activated form of Src were used to define the spatial relationship between Src localization, and Src and FGFR activation.

Mouse embryo fibroblasts (MEFs) were stimulated with FGF2, and endogenous FGFR1 and activated FGFR were detected at the plasma membrane and in endosomal vesicles throughout the cytoplasm (Fig 1A, upper left panels). In Src/Fyn/Yes^{-/-} (SYF) MEFs, endogenous FGFR1 was also detected at the plasma membrane and in endosomes; however, a lower basal level of

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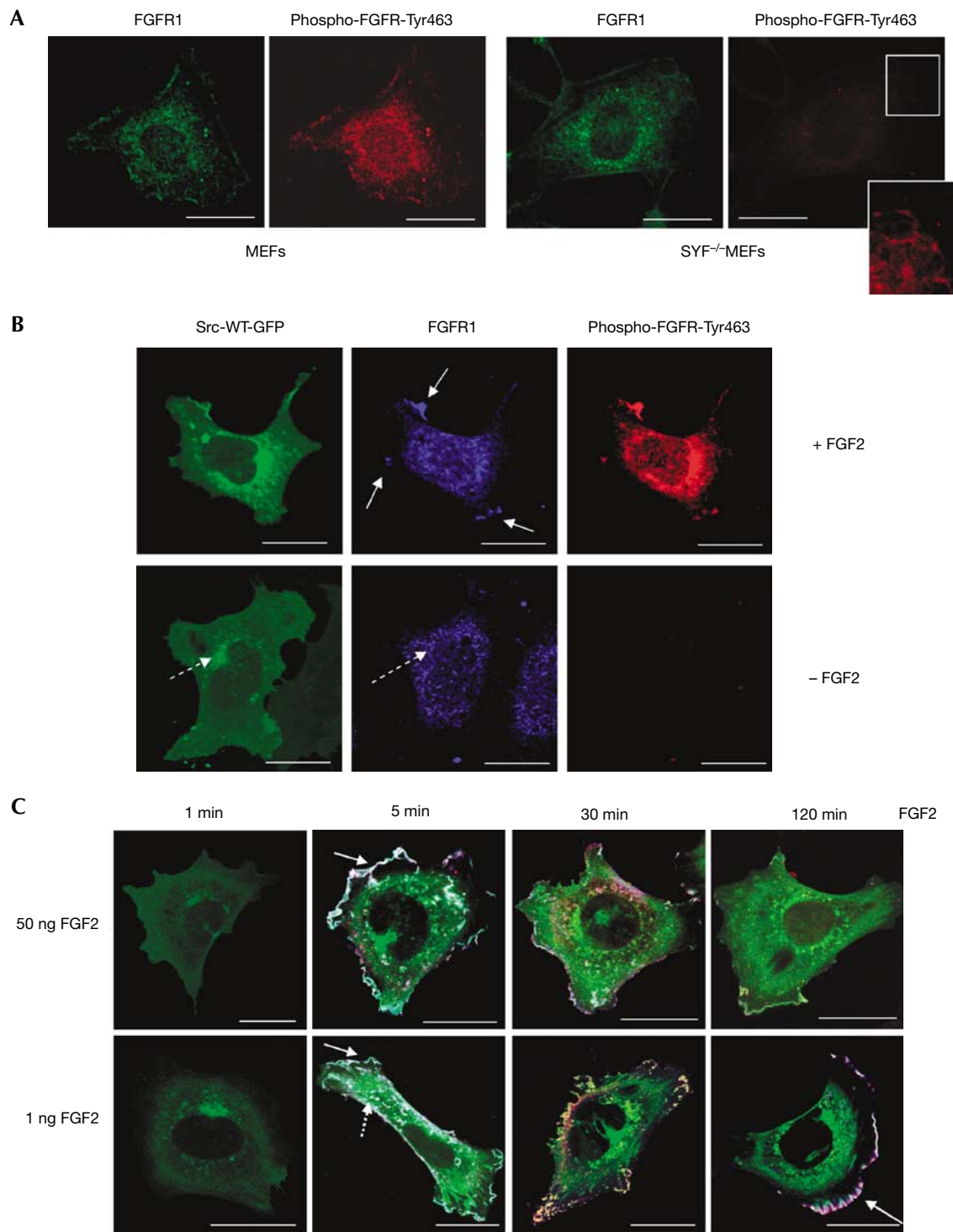


Fig 1 | FGF2 induces co-recruitment of active Src and active FGFR to the plasma membrane. (A) MEFs or SYF^{-/-}MEFs were maintained in serum-free media and then stimulated with FGF2 (50 ng/ml⁻¹) for 30 min. SYF^{-/-}MEFs expressing Src-WT-GFP were maintained in serum-free media overnight and then stimulated with FGF2 at (B) 50 ng/ml⁻¹ for 30 min or (C) 1 ng/ml⁻¹ and 50 ng/ml⁻¹ for 1, 5, 30 or 120 min. Total FGFR1 was detected with an FGFR1 antibody and active proteins were detected using anti-phospho-FGFR-Tyr463 or anti-phospho-Src-Tyr416. Solid arrows indicate protein localized to the plasma membrane, dashed arrows indicate protein maintained in the perinuclear region and dotted arrows show endosomes. Green, blue and red colours represent Src, phospho-Src-Tyr416 and phospho-FGFR-Tyr463, respectively. Scale bars, 25 µM. FGFR, fibroblast growth factor receptor; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; SYF^{-/-}MEF, Src/Yes/Fyn-deficient MEF; WT, wild type.

FGFR activation was detected in endosome-like structures in the perinuclear region of cells. On closer inspection, we also found that there was a trace of active FGFR at the plasma membrane (Fig 1A, upper right panels). When SYF^{-/-}MEFs were reconstituted with Src-WT-GFP (Sandilands *et al*, 2004), there was a substantial increase in the intensity of staining of activated FGFR, both at the plasma membrane and in endosomal structures (Fig 1B, upper panels). These findings show that Src protein is required for enhanced activation of FGFR at the plasma membrane in FGF2-stimulated cells, and that in the absence of Src, there is only a minor pool of Src-independent activated FGFR constitutively localized at the plasma membrane. This implies that FGF2 ligand stimulates an 'amplification' of active FGFR at the plasma membrane.

In the absence of FGF2 stimulation, Src-WT-GFP was localized in a cytoplasmic and tight perinuclear position, and no remarkable reactivity with anti-phospho-Src-Tyr416 or anti-phospho-FGFR-Tyr463 was observed, indicating that neither protein is activated under unstimulated conditions (Fig 1B; supplementary Fig 2 online). This experiment indicates that activity, and not simply overexpression of Src, is linked to the enhanced activation of FGFR at the plasma membrane. Furthermore, in unstimulated cells, total FGFR1 was retained in the cytoplasm in most of the cells (Fig 1B, lower panels), until stimulation with FGF2 when total FGFR1 was readily detected at the plasma membrane (Fig 1B, upper panels).

When SYF^{-/-}MEFs expressing Src-WT-GFP were stimulated for varying time durations with two different concentrations of FGF2, the location of Src-WT-GFP, activated Src and activated FGFR was visualized (Fig 1C). The results showed that, following FGF2 stimulation, there was a rapid (5 min) and prominent accumulation of both active Src and active FGFR at the plasma membrane. At higher concentrations of FGF2 (50 ng/ml), this localization and phospho-specific staining decayed over the following 120 min—implying receptor degradation. At low concentration (1 ng/ml), the colocalization of active Src and FGFR in the peripheral membrane location persisted for longer—for the duration of the time course (Fig 1C, solid arrows). At early time points, active Src and active FGFR were also observed in cytoplasmic vesicular structures (Fig 1C, dotted arrows). This colocalization occurred only in a sub-fraction of total cellular Src, as a significant proportion of Src-WT-GFP (Fig 1C, left panel) remained in a cytoplasmic and perinuclear location following stimulation, which is similar to the findings of Sandilands *et al* (2004). Thus, only a small proportion of Src is activated in a very tightly spatially regulated manner in response to FGF2 stimulation and what is activated is co-recruited with activated FGFR to the plasma membrane.

Together, these results show that FGF2 stimulation results in the co-activation of Src and FGFR and in the co-recruitment of the activated kinases to the peripheral plasma membrane, most likely through cytoplasmic endosomes. The peripheral signals derived from FGF2 decay in a manner that is inversely related to the concentration of FGF2 used. These findings raise the question of the mechanism of Src dependency of FGFR kinase activation at the plasma membrane.

The role of Src kinase activity in FGFR activation

SYF^{-/-}MEFs were reconstituted with either a dominant-negative kinase-deficient Src-251-GFP or a constitutively active Src-Tyr527Phe-GFP (Timpson *et al*, 2001; Sandilands *et al*, 2004). In the absence of functional Src kinase, FGF2 stimulation

failed to activate peripheral FGFR kinase activity, as determined by anti-phospho-FGFR staining (Fig 2A, upper panels). In the presence of constitutively active Src, FGFR was obviously phosphorylated (Fig 2A, lower panels) at peripheral membrane structures in a ligand-independent manner. One characteristic of these observations, as reported previously, is the localization of constitutively active Src in sub-membrane aggregates containing focal adhesion proteins (Avizienyte *et al*, 2002), whereas activated FGFR is correctly located in the peripheral membrane. These data imply that constitutively activated Src drives activated FGFR to its peripheral membrane sites of activity, even in the absence of ligand.

We also confirmed the requirement for Src kinase activation in FGFR activation by treating SYF^{-/-}MEFs expressing Src-WT-GFP with the selective Src inhibitor dasatinib (Lombardo *et al*, 2004; Serrels *et al*, 2006) before stimulation with FGF2. These results show that inhibition of Src kinase in the presence of FGF2 suppresses phosphorylation of both Src and FGFR, but does not prevent the translocation of Src-WT-GFP to the cell membrane (Fig 2B). Interestingly, the peripheral targeting of the low level of basal FGFR detected in cells at high laser intensities is also uninhibited (Fig 2B, right panel). By using a high-intensity CCD camera under fixed laser settings, images were captured that allowed quantification of the intensity of phospho-FGFR staining at the plasma membrane in the presence of Src-WT-GFP (Fig 2C). The results of this analysis showed that the active FGFR signal was reduced by approximately 80% in the presence of dasatinib. These findings show that Src is not necessary for the transport of the low basal level of active FGFR present in these cells, whereas it is both necessary and sufficient for 'amplification' of active FGFR at the plasma membrane.

The mechanism of Src and FGFR co-recruitment

We were interested to learn more about the mechanism by which Src is responsible for FGFR activation at the peripheral plasma membrane. When we overexpressed the late endosomal marker RhoB, we found that there was some colocalization with FGFR1 (Fig 3A). There was also a striking colocalization of Src, phospho-FGFR and RhoB in cytoplasmic endosomal structures (Fig 3A, right panel). This implies that activated FGFR might be a passenger in the Src/RhoB-dependent endosome delivery pathway previously described (Sandilands *et al*, 2004). We therefore interrogated the requirement for RhoB in FGFR activation and peripheral membrane localization. RhoB^{-/-}MEFs were stimulated with FGF2 and colocalization of Src, FGFR1 and phospho-FGFR examined (Fig 3B). We observed total FGFR1 at the plasma membrane and in intracellular vesicles in these cells. However, the translocation of Src-WT-GFP was inhibited by RhoB deficiency, as was the increase in phospho-FGFR normally detected in FGF2-stimulated MEFs expressing Src-WT-GFP (Fig 1A). Interestingly, a lack of RhoB also inhibited the transport of the low level of basal phospho-FGFR detected previously (Figs 1A,2C), as no signal was detected at the plasma membrane of RhoB^{-/-} cells (Fig 3B). Thus, peripheral membrane localization of activated FGFR is dependent on the presence of endosomal RhoB protein, and FGFR activation is suppressed in the absence of RhoB.

We have recently shown that RhoB is involved in the transport and activation of Src, using a farnesyl transferase inhibitor (FTI; Sandilands *et al*, 2007). FTIs are a class of biologically active anticancer drugs that inhibit farnesylation of many proteins such

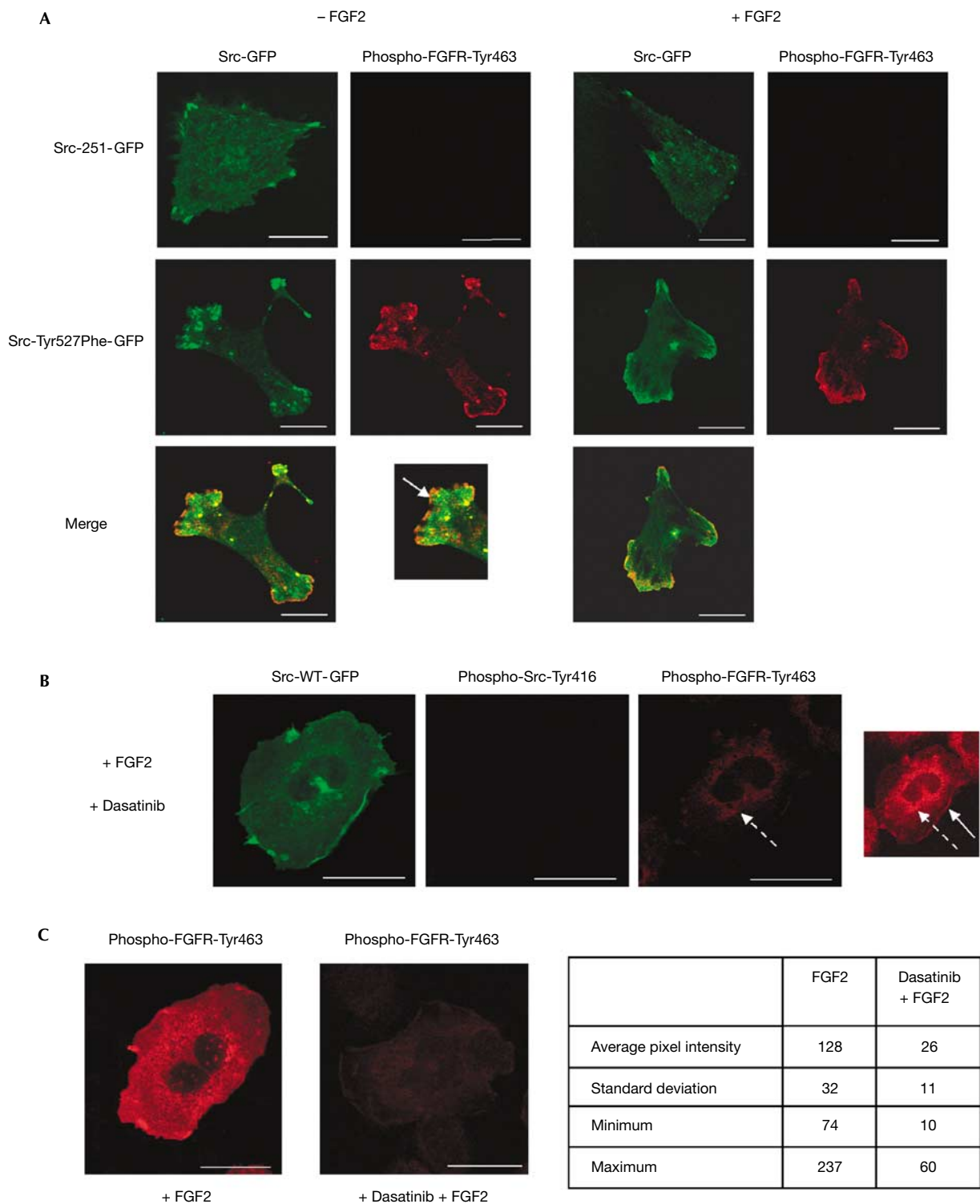


Fig 2 | Src activity is sufficient for increased peripheral membrane targeting of active FGFR. (A) *SYF*^{-/-} MEFs expressing Src-251-GFP or Src-Tyr527Phe-GFP were maintained in serum-free media or stimulated with FGF2. (B) *SYF*^{-/-} MEFs expressing Src-WT-GFP were treated with dasatinib (200 nM) for 2 h before stimulation with FGF2. (C) Images of 50 cells from (B) were taken under exactly the same conditions and the average pixel intensity at the membrane was calculated and compared. Active proteins were detected using anti-phospho-FGFR-Tyr463 or anti-phospho-Src-Tyr416. Solid arrows indicate protein localized to the plasma membrane and dashed arrows indicate protein maintained in the perinuclear region. Scale bars, 25 μ M. FGFR, fibroblast growth factor receptor; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; *SYF*^{-/-} MEF, Src/Yes/Fyn-deficient MEF.

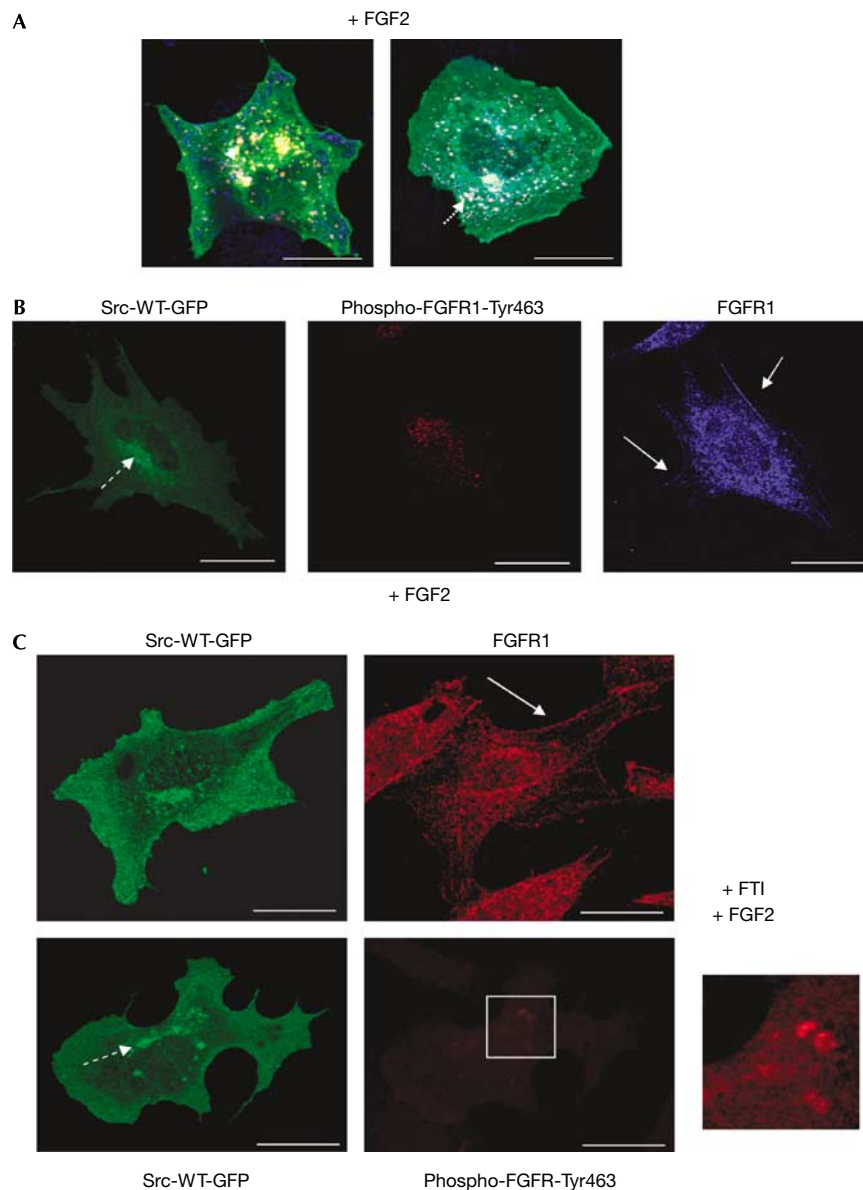


Fig 3 | Targeting of activated FGFR after FGF2 stimulation is RhoB dependent. (A) SYF^{-/-} MEFs expressing Src-WT-GFP and Myc-RhoB were stimulated with FGF2. Left panel: green, Src-WT-GFP; blue, FGFR; red, RhoB. Right panel: green, Src-WT-GFP; blue, phospho-FGFR-Tyr463; red, RhoB. (B) RhoB^{-/-} MEFs expressing Src-WT-GFP were stimulated with FGF2. (C) SYF^{-/-} MEFs expressing Src-WT-GFP were treated with L744832 (10 μM) for 2 h before stimulation with FGF2. RhoB, FGFR and active FGFR were detected with Myc, FGFR1 and phospho-FGFR1-Tyr463 antibodies, respectively. Solid arrows indicate protein localized to the plasma membrane, dashed arrows (B, left panel; C, bottom left panel) indicate protein maintained in the perinuclear region, whereas the dotted arrow (A, right panel) indicates endosomal proteins. Scale bars, 25 μm. FGFR, fibroblast growth factor receptor; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; SYF^{-/-} MEF, Src/Yes/Fyn-deficient MEF.

as Ras and RhoB through inhibition of the enzyme farnesyl transferase (Gibbs *et al*, 1994). Administration of an FTI, such as L744832, has been shown to affect the function of RhoB (Du & Prendergast, 1999), and therefore provides a test of the RhoB dependence of membrane targeting. To ascertain whether FTIs would influence the intracellular transport and activation of FGFR, we treated SYF^{-/-} MEFs expressing Src-WT-GFP with L744832 before stimulation with FGF2. The results indicate that although total FGFR1 could be detected at the plasma membrane, increased

activation of this protein was not detected (Fig 3C). We could, however, detect a low level of activation in endosomes throughout the cytoplasm (Fig 3C, right panel). Together, these data strongly indicate that FGFR activation and peripheral membrane targeting are tightly linked.

RhoB endosomes harbour actin-regulatory proteins, and Src and RhoB coordinate to control the actin assembly required for the transport of RhoB endosomes to the peripheral membrane; in essence, the RhoB/Src endosome shuttle is dependent on an intact

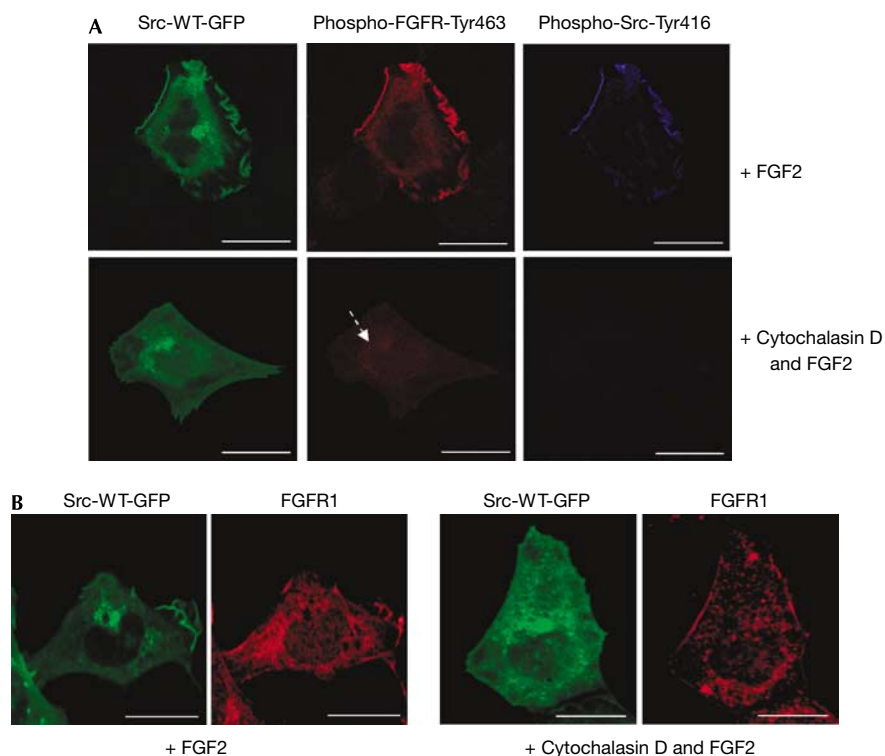


Fig 4 | Targeting of activated FGFR after FGF2 stimulation is actin dependent. SYF^{-/-}MEFs were treated with cytochalasin D (0.3 μg/ml) for 1 h before stimulation with FGF2. Cells were stained with (A) phospho-FGFR-Tyr463 antibody and anti-phospho-Src-Tyr416 or with (B) anti-FGFR1. Dashed arrows indicate protein maintained in the perinuclear region. Scale bars, 25 μm. FGFR, fibroblast growth factor receptor; MEF, mouse embryonic fibroblast; SYF^{-/-}MEF, Src/Yes/Fyn-deficient MEF.

actin cytoskeleton to drive vesicle movement (Sandilands *et al*, 2004). This predicts that FGFR activation might be dependent on actin and so abrogated by dissolution of the actin cytoskeleton. Indeed, we found that pretreatment of FGF2-stimulated MEFs by cytochalasin D inhibited activation of both FGFR and Src (Fig 4A), establishing that enhanced ligand-induced FGFR activation was dependent on both Src and an intact actin cytoskeleton. Low basal levels of activated FGFR were detected in the perinuclear regions of cytochalasin D-treated cells but were not visible at the plasma membrane (Fig 4A, lower middle panel). The localization of total FGFR1 did not seem to be affected by disruption of the actin cytoskeleton (Fig 4B). We have previously shown that the use of an interfering mutant of Scar1, a member of the WASP/Scar family of adaptor proteins, disrupts filamentous actin, and inhibits transport and activation of Src-WT-GFP (Sandilands *et al*, 2004). Here, we show that expression of this protein inhibits the increase in activation of FGFR1 and prevents translocation of basal activated FGFR to the plasma membrane (supplementary Fig 3 online).

Collectively, these data show that there is an essential requirement for the Src kinase/RhoB-dependent shuttle previously identified, which is driven by actin cytoskeleton assembly, in delivery of activated FGFR to the peripheral membrane. In the absence of this, the peripheral targeting steps of FGFR activation are blocked.

Role of Src in signalling pathways downstream of FGFR1

We were interested to learn how the engagement of Src in the process of FGFR activation and peripheral recruitment influenced

control of the dynamics of intracellular signalling pathways downstream of activated FGFR. MEFs were stimulated with FGF2 in the presence of dasatinib (Fig 5A) to block Src activation (data not shown), and we examined the kinetics of activation of the mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase/Akt pathways. This showed a striking dependency of FGFR signalling on Src activity, and this was quite distinct for the two signalling pathways. In the case of Akt activation, FGF2 stimulation induced a rapid rise in Akt activation, which reached a maximum amplitude at about 4 min followed by a period of decay after 8 min (Fig 5A, upper panels). Pharmacological inhibition of Src activity resulted in attenuation of Akt activity. Although much weaker, the initial phase of residual activation reached peak amplitude at similar time points to control cells (Fig 5A, upper panels). An identical pattern was seen following inhibition of FGFR kinase activity (data not shown). This experiment was confirmed using SYF^{-/-}MEFs, which have significantly less active Akt than FGF2-stimulated MEFs (Fig 5B).

These data show that Akt activation is wholly dependent on the activation of Src, and is linked to Src-dependent peripheral membrane targeting of activated FGFR, presumably requiring FGFR to be at the plasma membrane.

A different pattern of kinetic behaviour was seen for the MAPK kinase pathway following inhibition of Src activity. In this case, the rising phase of extracellular signal-regulated kinase-1 (ERK) activation was delayed compared with untreated cells (shown in Fig 5A for treatment with dasatinib and confirmed in Fig 4C with the Src inhibitor SU6656, right panels), consistent with a

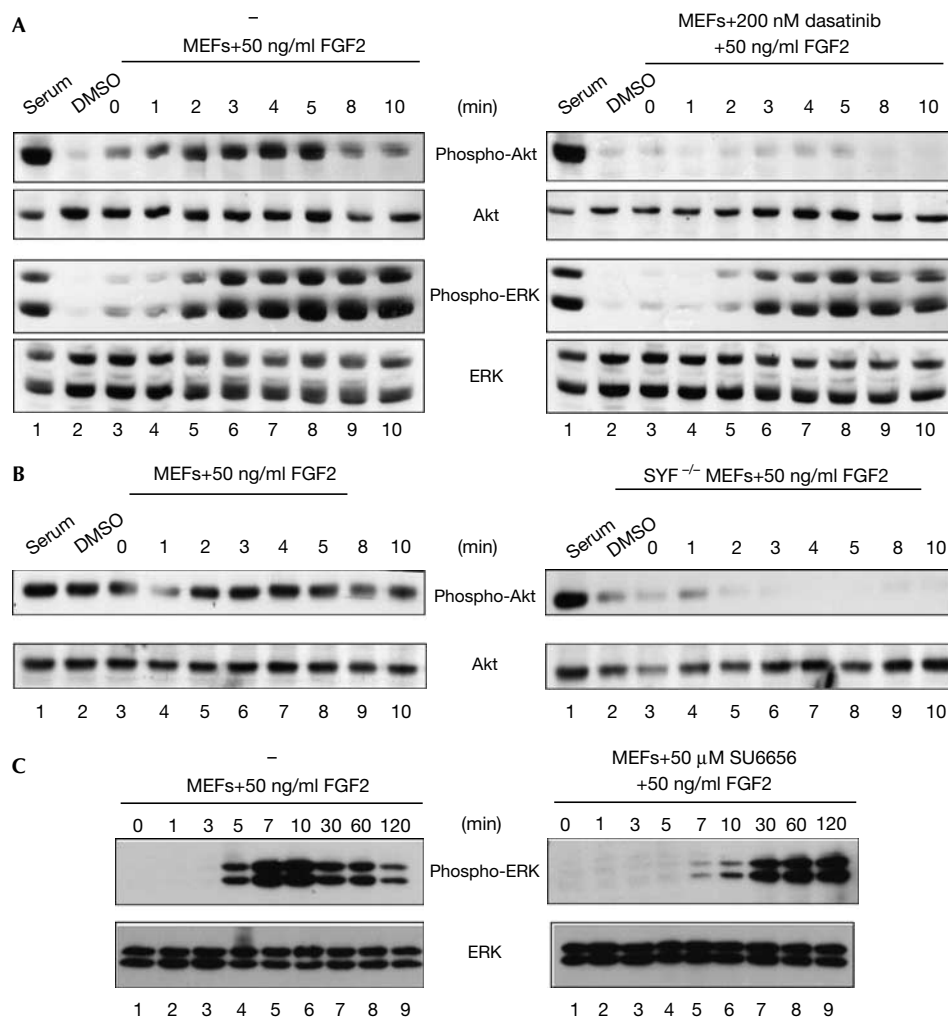


Fig 5 | Signalling from Src-dependent and Src-independent active FGFR pools. (A) MEFs were treated with dasatinib (200 nM) and stimulated with FGF2 for different durations. (B) SYF^{-/-}MEFs and MEFs alone were treated with FGF2. (C) MEFs were treated with 50 μM SU6656 before stimulation with FGF2 for different durations. Western blot analysis was carried out on cell lysates using Akt, phospho-Akt, ERK and phospho-ERK antibodies. ERK, extracellular signal-regulated kinase; FGFR, fibroblast growth factor receptor; MEF, mouse embryonic fibroblast; SYF^{-/-}MEF, Src/Yes/Fyn-deficient MEF.

requirement for Src activity for the correct FGFR-induced signalling response. However, in stark contrast to activation of Akt, robust activation of ERK persisted and it was the decay component of ERK activation that was markedly slowed down by inhibition of Src (Fig 5C, right panels). This inhibition of decay was also observed in SYF^{-/-}MEFs (data not shown). Thus, activation of ERK, which occurs through Grb2/SOS engagement of FRS2 (Ong *et al*, 2001), is delayed in its initial phase, which is consistent with the requirement for Src in FGFR activation, but prolonged in the decay phase, indicating an additional role for Src kinase in ERK attenuation after signal peak.

Several earlier studies have indicated that the Ras/Raf/ERK/MAPK cascade is activated at both the plasma membrane and cytosolic locations following growth factor stimulation (reviewed by Miaczynska *et al*, 2004), and that the two pools of signalling modules show markedly different dynamic properties (Harding *et al*, 2005). On a similar basis, we can explain the requirement for Src activation for attenuating ERK activity as a consequence

of Src releasing the activated ERK module to cytosolic locations where it requires higher signalling inputs to sustain activity; consequently, the amplitude of the signal degrades more quickly when Src is present. When Src is inhibited, the module is not released, continues to respond to inputs and fails to decay.

Given that Src activation is a common, if not indeed ubiquitous, characteristic of signalling from RTK receptors (Parsons & Parsons, 2004), it seems possible that a crucial role of Src in cellular signalling is to tune the dynamics of downstream processes by regulating the spatial localization and confinement of activated signalling complexes. This could also contribute to the functions of oncogenic forms of Src, as one consequence of constitutively activated Src is hypersensitization of RTK-mediated signalling pathways, thereby emulating the consequences of activating mutations in the RTKs themselves or downstream effectors. These considerations indicate that therapeutic intervention by the inhibition of Src might be accentuated in combination with agents that inhibit RTK kinase activity (Mohammadi *et al*, 1997). Indeed,

dasatinib is now licensed for clinical use in the treatment of chronic myeloid leukaemia, and this agent and other Src inhibitors are being clinically tested for activity against common solid tumours. Our work raises the exciting possibility that such agents might be useful in the treatment of cancers in which FGFR mutations are driving proliferation.

METHODS

Cell culture. Src/Yes/Fyn-deficient MEFs (SYF^{-/-}MEFs), control MEFs and RhoB^{-/-}MEFs were routinely grown in DMEM supplemented with 10% FCS and 1% glutamine. Cells were transiently transfected under serum-free conditions using FuGene6 (Roche Diagnostics Ltd, Sussex, UK) or Polyfect Transfection Reagent (Qiagen, Crawley, UK). Cells were treated with L744832 (10 μM) for 2 h (Biomol Int, Exeter, UK), cytochalasin D (0.3 μg/ml) for 1 h (Sigma, Poole, UK), dasatinib (200 nM) for 2 h (BMS, New York, NY, USA) or 50 μM SU6656 (Calbiochem, Nottingham, UK) before stimulation with FGF2 (50 ng/ml⁻¹ for 30 min) and 10 μg/ml heparin (Sigma, Poole, UK).

Immunofluorescence. Cells were fixed in 3% paraformaldehyde, washed in TBS/100 mM glycine and permeabilized with TBS/0.1% saponin/20 mM glycine. After blocking with TBS/0.1% saponin/10% FCS, cells were incubated with primary antibodies. Anti-phospho-Tyr463 FGFR1 was generated in sheep (Diagnostics Scotland, Edinburgh, UK) by immunization with a dendromeric form of the phosphopeptide LAGVSEY(P)ELPED (Alta Biosciences, Birmingham, UK) and purified by three cycles of affinity chromatography on an immobilized form of the phosphopeptide. Antibody specificity was confirmed by ELISA and in western blots, and immunohistochemistry was confirmed by inhibition with the phosphorylated form of the peptide. Anti-FGFR1 was used to detect total FGFR1 (Santa Cruz, Santa Cruz, CA, USA), 9E10 monoclonal antibody was used to detect Myc-tagged RhoB and anti-phospho-Tyr416-Src was used to detect active Src (Upstate Biotechnology, New York, NY, USA). Non-conjugated antibody detection was by reaction with species-specific fluorescein isothiocyanate-, Cy5- or Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch, Luton, UK). Cells were visualized by using a Leica confocal microscope (Leica UK Ltd, Milton Keynes, UK). Each experiment was repeated a minimum of three times, 100 cells were counted for each condition for quantification purposes and an image that represented the phenotype of most of the cells was selected (supplementary Table 1 online).

Protein immunoblotting. Samples were lysed in radioimmuno-precipitation buffer (20 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol, pH 7.4) containing protease and phosphatase inhibitors (2 mM phenylmethylsulphonyl fluoride, 10 μg/ml aprotinin, 1.5 mM sodium fluoride and 300 μM sodium vanadate) and then centrifuged at 40 °C for 15 min. Immunoblotting was carried out using 50–100 μg of lysate per sample. Proteins were separated by SDS–10% polyacrylamide gel electrophoresis, transferred to nitrocellulose, blocked with 5% BSA in TBS–0.2% Tween 20 (Sigma, Poole, UK) and probed with anti-ERK (Sigma, Poole, UK), anti-phospho-p44/42 MAP kinase (Thr202/Tyr204), anti-phospho-Akt (Ser473) and anti-Akt (Cell Signalling, Hertfordshire, UK). Detection was by incubation with horseradish peroxidase-conjugated secondary antibodies and visualization was by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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