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**REGULATION OF CXCR4-MEDIATED NUCLEAR TRANSLOCATION OF ERK1/2**

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**Running Title:** CXCR4-mediated nuclear translocation of ERK1/2

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**Abbreviations:** 7TM-R, seven transmembrane receptor; EGF, epidermal growth factor; ERK1/2, extracellular signal-regulated kinases 1 and 2; DN-Rho, dominant negative Rho; FCS, fetal calf serum; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HMEC, human microvascular endothelial cells; MEK, mitogen-activated protein kinase/ERK1/2 kinase; NLS, nuclear localization signal; PMSF, phenylmethanesulfonyl fluoride; RFP, red fluorescent protein; SDF-1, stromal cell-derived factor 1.

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### Abstract

Activation of the chemokine receptor CXCR4 by its agonist SDF-1 has been associated with cell migration and proliferation in many cell types, but the intracellular signaling cascades are incompletely defined. Here we show that CXCR4-dependent ERK1/2 phosphorylation was mediated through the Ras/Raf pathway as demonstrated with a dominant negative Ras mutant and pharmacological inhibitors. The Src inhibitor, PP1, and the Rho-kinase (ROCK) inhibitor, Y27632, also attenuated SDF-1 induced ERK1/2 phosphorylation. Involvement of Src could furthermore be demonstrated by Src phosphorylation and by the shortened ERK1/2 phosphorylation in SYF cells, which are Src/Yes/Fyn deficient compared to Src reconstituted Src<sup>++</sup> cells. Membrane translocation of RhoA could similarly be detected. A large portion of the SDF-1-mediated ERK phosphorylation was detected in the nucleus, as shown by Western blotting and confocal microscopy, and resulted in the phosphorylation of the transcription factor Elk. Interestingly, the nuclear accumulation of ERK1/2 and Elk phosphorylation was completely blocked by DN-Rho, Y27632, PP1 and latrunculin B, indicating that the Rho/Rock pathway, Src kinase and the actin cytoskeleton were required in this process. In accordance, neither nuclear ERK phosphorylation nor Elk phosphorylation were observed in SYF cells stimulated with SDF-1, but were reconstituted in Src<sup>++</sup> cells. In summary, these results demonstrate that Src, Rho/ROCK and an intact cytoskeleton contribute to overall ERK1/2 activation in SDF-1 stimulated cells, and are indispensable for nuclear translocation of ERK1/2 and activation of transcription factors.

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## Introduction

The chemokine stromal cell-derived factor 1 (SDF-1/CXCL12) and its receptor, CXCR4, play an important role in various cellular responses, including chemotaxis, angiogenesis, and cell proliferation (Rossi and Zlotnik, 2000). CXCR4 also serves as a coreceptor of T-tropic HIV-1 entry into mononuclear cells. Numerous signaling molecules including  $G_i$ , Ras, PI-3 kinase, NF- $\kappa$ B and ERK, have been implicated in CXCR4 signaling in different cell types. Recently, it has been established that CXCR4 signaling pathways are activated in a range of cancer cells, where their activation has been associated with proliferation and metastatic dissemination (Kijima et al., 2002; Muller et al., 2001; Schrader et al., 2002; Taichman et al., 2002; Zhou et al., 2002).

Extracellular signal-regulated kinases 1 and 2 (ERK1/2) activation is mediated by numerous seven transmembrane spanning receptors (7TM-Rs), but the activation mechanism varies for different receptors (Luttrell et al., 2001). In a classical module, ligand stimulation of 7TM-Rs activates Ras on the plasma membrane, which in turn activates c-Raf, MEK1/2, and ultimately ERK1/2. Other signaling regulators have been implicated in control of ERK1/2 activation, including Src,  $\beta$ -arrestin, PKC, PI-3 kinase, and Rho (Luttrell et al., 2001). These different mechanisms contribute to fine-tuning of ERK1/2 activation in response to diverse stimuli. Duration, intensity and subcellular compartmentalization of ERK1/2 play roles in numerous cellular activities, including cell proliferation. ERK1/2 mediated cell proliferation is dependent on the activation of transcription factors following nuclear translocation of ERK1/2.

ERK1/2 does not possess a nuclear localization signal (NLS), and the nuclear entry of ERK1/2 is not an obligatory consequence following activation (Osawa et al., 2004; Whitehurst et al., 2004a). ERK1/2 accumulation in the nucleus depends on the nature of the stimulus, but is independent of the intensity of ERK1/2 activation (Whitehurst et al., 2004b). Although the underlying mechanism of ERK1/2 translocation into the nucleus remains unclear, the sub-cellular distribution of ERK1/2 can be regulated by a number of

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signaling molecules, e.g.  $\beta$ -arrestin, PEA-15, and MEK. MEK is reported to interact with ERK, maintaining ERK in the cytoplasm (Fukuda et al., 1997). Upon growth factor stimulation, ERK undergoes phosphorylation and dissociates from MEK. Similarly,  $\beta$ -arrestin has been shown to mediate ERK activation by acting as a scaffolding protein. It, too retains ERK1/2 in the cytoplasm instead of promoting ERK translocation to nucleus (DeFea et al., 2000). This cytoplasmic ERK activation has different functions from nuclear activation, as it has been implicated in Ras-induced senescence (Gaumont-Leclerc et al., 2004), but it can also be anti-apoptotic (Ajenjo et al., 2004). Recently, the nuclear export factor PEA-15 was reported to regulate the actions of the ERK MAP kinase cascade. PEA-15, which contains a nuclear export sequence, binds ERK1/2 and sequesters it in the cytoplasm, blocking ERK-dependent transcription and proliferation (Formstecher et al., 2001). In contrast, growth factor receptor-bound protein 2-associated binder-1 (Gab1) has a putative NLS, and its interaction with ERK1/2 plays a role in ERK1/2 translocation and Egr-1 accumulation in the nucleus (Osawa et al., 2004). Recent reports also suggest that RhoA/ROCK activation is involved in regulation of ERK1/2 translocation to the nucleus, but the details of this pathway have not been elucidated (Kawamura et al., 2003; Liu et al., 2004).

After entry into the nucleus, phosphorylated ERK1/2 can activate several transcription factors including Elk and early growth response factor-1 (Egr-1). Elk is responsible for transcriptional activation of the immediate early gene *c-fos*. Thus Elk activation reflects the entry of ERK1/2 from the cytoplasm to the nucleus. A second transcription factor, Egr-1, which is up-regulated by nuclear ERK activation, plays a regulatory role in the expression of growth factors, cytokines and adhesion molecules. It is responsible for increased expression of growth factors, such as platelet-derived growth factor (PDGF), cytokines, and adhesion molecules.

CXCR4-mediated ERK1/2 activation has been reported in several cell types, and CXCR4 signaling has been associated with cell proliferation in normal and cancer cells. However, the intracellular signaling pathways, which are elicited in ERK1/2 activation-mediated cell proliferation during these processes, are

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poorly understood. Activation of CXCR4 in human megakaryoblasts led to the phosphorylation of the nuclear transcription factor Elk (Majka et al., 2000). Similarly, Egr-1 expression was increased by SDF-1 in arterial endothelial cells, thus enhancing VEGF-mediated cell proliferation (Neuhaus et al., 2003). Both these observations imply nuclear translocation of ERK, but this has not been experimentally confirmed. Here, we report that CXCR4 activation induced nuclear translocation of ERK1/2 and phosphorylation of the transcription factor Elk. This pathway is dependent on Src kinase, Rho/ROCK activation and an intact actin cytoskeleton, a pathway that has previously been described for integrin-dependent nuclear translocation of ERK1/2 (Aplin et al., 2001), but not for 7TM-Rs.

## Materials and Methods

**Materials.** CXCR4 cDNA was PCR-amplified and inserted into pcDNA3.1 (Invitrogen, Carlsbad, CA). The plasmid encoding CXCR4-GFP and the expression vector encoding red fluorescence protein (RFP)-tagged ERK2 have been described (Zhao et al., 2004). Dominant-negative Rho (DN Rho) was generously provided by Dr. Gary Bokoch (The Scripps Research Institute). FTI-277, Raf-1 kinase inhibitor, PD98059, Y27632, LY294002 and latrunculin B were purchased from Calbiochem (San Diego, CA). PP1 was obtained from Biomol (Plymouth Meeting, PA) and pertussis toxin was from List Biological Laboratories (Campbell, CA). Anti-c-Raf, anti-RhoA, anti-actin, and anti-lamin B antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-Elk (Ser383), anti-Elk, anti-phospho-ERK1/2, anti-ERK1/2 and anti-phospho-Src antibodies were from Cell Signaling (Beverly, MA); anti-Src monoclonal antibody was from Upstate Biotechnology (Lake Placid, NY). Protein A beads were obtained from Amersham Bioscience (Arlington Heights, IL), and [ $\gamma$ -<sup>32</sup>P]ATP was from Perkin-Elmer (Boston, MA). SDF-1 was expressed in *E.coli* and purified as described before (Zhao et al., 2004).

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**Cell culture and transfection.** HEK293 human embryonic kidney epithelial cells, HMECs, a human microvascular endothelial cell line, SYF, Src/Yes/Fyn deficient mouse embryonic fibroblasts and Src<sup>++</sup> cells (Klinghoffer R.A., 1999), their Src-reconstituted counterpart, were obtained from ATCC. HEK293, SYF and Src<sup>++</sup> cells were grown in DMEM containing 10% FCS. HMECs were cultured in EBM (Clonetics). HEK293 cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stable cells lines were selected with 800 µg/ml of G418. All cells were serum-starved overnight prior to SDF-1 stimulation.

**Western blotting.** Monolayer cells were stimulated as described in the figure legends, then lysed with modified RIPA buffer (50 mM Tris-HCl, pH7.4, 10% glycerol, 1% NP-40, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 2 mM EDTA, 2 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 2 mM sodium pyrophosphate, 2 mM sodium vanadate and 10 mM NaF) and clarified by centrifugation. Immunoprecipitation assays were performed as previously described (Zhao et al., 2004). The clarified cell lysates or immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked with 3% dry milk in TBS-Tween, and exposed to specific primary antibodies as described for each experiment. Antibody binding was detected using horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse secondary antibodies and enhanced chemiluminescence (ECL, Amersham Bioscience). Phosphoblots were re-probed with a second antibody, e.g. anti-ERK1/2 antibody to assure equal loading. UN-SCAN-IT gel digitizing software (Silk Scientific, Orem, UT) was used to quantify results.

**Preparation of membrane fraction.** To isolate membrane fractions, cells were stimulated for the indicated times and lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH7.4, 50 mM NaCl, 1mM MgCl<sub>2</sub>, 2 mM EDTA, 1 mM DTT, 2 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin) by sonication. Nuclei and cell debris were removed by centrifugation at 500 x g for 10 min. The supernatants were subjected to ultracentrifugation at 100,000 x g for 40 min and the pellets were washed once. Supernatant fractions (cytoplasm) and pellet fractions (membranes) were separated by SDS-PAGE.

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**Isolation of cell nuclear fraction.** Cells were vortexed in buffer A (10 mM HEPES, pH 8.0, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 2 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin) containing 0.5% NP-40, then microfuged at 12,000 g for 2 min. The pellets were washed with buffer A, then resuspended in buffer B (20 mM HEPES, pH8.0, 250 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 2 mM PMSF, 2 µg/ml leupeptin, 2 µg/ml aprotinin), incubated on ice for 15 min followed by another 2 min centrifugation at 4°C. The supernatants (nuclear fraction) were transferred to clean tubes containing 4 x Laemmli sample buffer, boiled and separated by SDS-PAGE. To exclude cross-contamination between nuclear and cytoplasmic fractions, fractionated samples were probed with a nuclear marker (lamin B) and with actin, which is excluded from the nucleus.

**Raf kinase assay.** Experiments were carried out as described (Zhao et al., 2004). Briefly, washed c-Raf immunoprecipitates were suspended in 40 µl of kinase buffer (30 mM HEPES, pH7.4, 10 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 25 mM β-glycerophosphate, 50 µM ATP) containing 15 µCi [ $\gamma$ -<sup>32</sup>P] ATP and 0.4 µg of purified kinase-inactive MEK1 (Upstate Biotechnology), and then incubated at 30°C for 30 min. Kinase reactions were stopped by adding Laemmli sample buffer and boiling, followed by separation by SDS-PAGE electrophoresis, gel transfer, and autoradiography. Membranes were probed with anti-c-Raf antibody to assure equal loading.

**Actin polymerization.** Serum-starved cells on collagen-coated coverslips were pretreated with various inhibitors prior to stimulation with SDF-1, and fixed with 4% paraformaldehyde in PBS for 20 min followed by permeabilization with 0.2% Triton X-100 for 5 min. Cells were then stained with Alexa 488 phalloidin (Molecular Probes, Eugene, OR) according to the manufacturer's instruction. Images were taken on an Olympus FV1000 confocal microscope (Melville, NY).



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**Fluorescence Microscopy.** HEK293 cells expressing CXCR4-GFP were grown on collagen-coated glass coverslips. Following SDF-1 stimulation, cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with methanol for 10 min at -20°C. After blocking with 3% BSA for 2 h at room temperature, cells were incubated with anti-phospho-ERK1/2 for 1 h, followed by staining with Alexa Fluor 568 anti-mouse IgG<sub>1</sub> monoclonal antibody (Molecular Probes) in 1% BSA for 1 h at room temperature. Cells were then stained with DAPI (Sigma Chemical Co.) for 10 min and washed twice with PBS before mounting with AntiFade (Molecular Probes). Images were taken on an Olympus FV1000 confocal microscope.

To examine translocation of total ERK to the nucleus, HEK293 cells transiently co-transfected with different combinations of GFP and RFP plasmids were treated with SDF-1 for 5 min in the presence or absence of inhibitors, and confocal images were captured as above.

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## Results

**CXCR4-mediated ERK1/2 phosphorylation depends on the Ras/Raf pathway.** To determine ERK1/2 signaling pathways used by CXCR4, HMEC cells, which express the CXCR4 constitutively, or HEK293 cells stably transfected with CXCR4 were stimulated with SDF-1 for the indicated times. In both cell types, SDF-1 elicited a rapid and robust ERK1/2 phosphorylation, reaching a maximum at 5 min (Fig 1A, 1B). Pretreatment with the farnesyl transferase inhibitor FTI277 which blocks Ras, or expression of a dominant-negative mutant of Ras in CXCR4-293 cells attenuated this response (Fig 1C). As expected a Raf-1 inhibitor and the MEK inhibitor PD98059 significantly reduced ERK1/2 phosphorylation (Fig 1D). In addition, c-Raf activity could also be detected directly in a peptide kinase assay in SDF-1 stimulated CXCR4-293 cells (Fig 1E). These results indicate that CXCR4-elicited ERK1/2 activation is mediated by the Ras/Raf pathway.

**Involvement of multiple signal regulators in CXCR4-mediated ERK1/2 activation.** To explore the signaling pathways involved in CXCR4-mediated ERK1/2 activation, inhibitor screening was performed. Pertussis toxin (PTX) abolished ERK1/2 phosphorylation (Fig 2A), indicating  $G_i$  was essential in this process. Since Src has been shown to play a role in MAPK activation of some 7TM-Rs (Klinger et al., 2002; Luttrell et al., 1999), the effect of the Src inhibitor, PP1, was tested next. Inhibition of Src family kinases largely blocked SDF-1-induced ERK1/2 activation in a dose-dependent fashion (Fig 2B). To exclude that this effect was due to non-specific effects of PP1, ERK1/2 phosphorylation was also assessed in SYF cells, a mouse embryonic fibroblast cell line derived from Src/Yes/Fyn triple knockout embryos that expresses CXCR4 constitutively. In these cells, SDF-1-dependent ERK1/2 phosphorylation was more short-lived than in Src<sup>++</sup> cells, which are SYF cells reconstituted with Src (Fig. 2C). Since Src phosphorylation is a complex process, phosphorylation of a single amino acid usually shows only a subtle response. Auto-phosphorylation of Tyr416 in the activating loop of the kinase domain on Src is known to be required for Src kinase activation. In Fig 2D, SDF-1 stimulation caused a visible increase in phosphorylation of Src on Tyr416 in both CXCR4-293 and HMECs. Since focal

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adhesion kinase, FAK is a kinase that is downstream of Src activation, tyrosine phosphorylation of FAK was also determined and was found to increase following the addition of SDF-1 (Fig 2D).

The PI-3-kinase inhibitor LY294002 showed only a minor effect on ERK1/2 activation in HEK293 cells, and the EGF receptor blocker AG1478 failed to inhibit the ERK1/2 phosphorylation (results not shown), even though it has been reported that the EGF receptor can be transactivated by SDF-1 (Porcile et al., 2004).

More surprisingly, the ROCK inhibitor Y27632 was found to attenuate SDF-1-induced ERK1/2 phosphorylation in CXCR4-HEK293 cells (Fig 2E). DN-Rho also showed partial inhibition of SDF-1-induced ERK1/2 activation (Fig. 2E). These results strongly suggested that the Rho/ROCK pathway participated in the regulation of CXCR4-mediated ERK1/2 activation. Since inhibition of the Rho cascade attenuated ERK1/2 activation, RhoA translocation to the membrane, an indication of RhoA activation (Hirakawa et al., 2004), was determined next to confirm RhoA involvement. In isolated plasma membrane fractions from CXCR4-293 cells stimulated with SDF-1, increasing RhoA was detected by Western blotting (Fig 2E), suggesting that RhoA was activated by SDF-1. Since the RhoA/ROCK pathway is usually linked with actin cytoskeletal changes, we next determined if an intact actin cytoskeleton is required for SDF-1 induced ERK1/2 phosphorylation. Cytoskeletal disruption by pretreatment of cells with latrunculin B (latB) largely reduced SDF-1-induced ERK1/2 phosphorylation (Fig 2F), suggesting an essential role of the intact actin cytoskeleton in ERK1/2 activation. To exclude a general toxic effect of latB, the effect of latB on ERK1/2 phosphorylation was also determined in HEK293 cells stimulated with EGF. As shown in Fig 2F, EGF-induced ERK1/2 phosphorylation was barely affected in the presence of latB. This result indicated HEK293 cells retained the ability to respond with ERK activation in the presence of latB, and ruled out a non-specific effect of latB on the MAPK cascade.

**SDF-1 induced nuclear translocation of ERK1/2.** Since CXCR4 can mediate cell proliferation through ERK1/2 activation, we next determined if ERK translocation to the nucleus could be detected. To exclude cross-contamination of the nuclear and cytoplasmic fractions, both fractions were immuno-blotted with actin as

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a cytoplasmic marker and lamin-B as a nuclear marker. As shown in Fig 3A, carry-over of proteins was negligible. In CXCR4-293 cells, SDF-1 induced rapid and strong ERK1/2 phosphorylation in the nucleus as shown by Western blot in Fig. 3B. A sizeable portion of nuclear ERK1/2 was phosphorylated as evident from the additional bands of slightly higher molecular weight representing the phosphorylated forms of the protein which are visible in the nuclear fractions in blots of total ERK1/2, for instance in Fig. 3B. PP1, an inhibitor of Src family kinases, largely inhibited ERK1/2 translocation (Fig. 3C), suggesting that Src family kinases were required in this process. As before, the inhibitor study was complemented by genetic evidence. In SYF cells deplete of Src family kinases, no nuclear accumulation of ERK1/2 could be observed following stimulation with SDF-1 (Fig. 3D). This behavior was reversed in the Src<sup>++</sup> cell line in which Src expression is restored (Fig. 3D), and this activation could be overcome by PP1 (Fig. 3D). These results indicated that Src expression was sufficient to mediate this effect, but does not rule out an overlapping or redundant role of Yes and Fyn in this process. Moreover, Latrunculin B abolished the nuclear import of ERK1/2 (Fig. 3E), suggesting that an intact actin cytoskeleton was required in this process. Similarly, both DN-Rho and Y27632 largely blocked ERK1/2 phosphorylation in the nucleus (Fig. 3E).

To confirm the biochemical analysis, nuclear translocation of ERK1/2 was also detected by confocal microscopy. HEK293 cells were cotransfected with ERK2-RFP and CXCR4-GFP using a minimal amount of DNA to achieve low level expression. In these cells, CXCR4-GFP was internalized following stimulation with SDF-1 and often co-localized with ERK2-RFP in discrete vesicles in the cytoplasm. Over time ERK2-RFP accumulated in the nucleus (Fig. 4A). In the presence of inhibitors that had been shown to block nuclear translocation by Western blot, nuclear entry of RFP-ERK2 was prevented (Fig. 4A). SDF-1-induced phosphorylation of nuclear ERK1/2 was also detected by immune-fluorescence with anti-phospho-ERK1/2 antibody. Following stimulation with SDF-1, increased phospho-ERK1/2 could be detected in the nucleus counterstained with DAPI (Fig 4B). Again, this was prevented in the presence of the inhibitors described above (Fig. 4B). Based on these results, it appears that SDF-1 induced ERK1/2 translocation from the cytoplasm to the nucleus involves RhoA/ROCK, Src and the actin cytoskeleton.

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**Stress fiber formation.** Disruption of the actin cytoskeleton prevented SDF-1-induced nuclear translocation of ERK1/2 as indicated by the effect of latrunculin B (Fig 4). RhoA/ROCK involvement also suggested that the actin skeleton was required for CXCR4-mediated nuclear translocation of ERK1/2. Although SDF-1 induced actin polymerization in HEK293 cells (Fig 5A), these cells do not show prominent stress fiber formation in general. Therefore HMECs, which respond with prominent stress fiber formation, were used for inhibitor studies. In these cells, SDF-1 induced actin polymerization was easily detectable and lasted for at least 20 min (Fig 5B). Inhibition studies showed that PP1, Y27632, DN-Rho, and latrunculin B blocked SDF-1-induced stress fiber formation (Fig 5C). These results were consistent with the above conclusion, and suggested Src and the RhoA/ROCK pathways were intimately linked in CXCR4 signaling, coordinating ERK1/2 activation and nuclear translocation.

**Nuclear translocation of ERK1/2 increased phosphorylation of transcription factors.** Nuclear ERK1/2 activation has been shown to activate a number of transcription factors, including Elk and Egr-1. The transcription factor Elk is a direct target of ERK1/2 and is phosphorylated at Ser382 when activated. To assess the consequence of CXCR4-mediated ERK1/2 entry into the nucleus, phosphorylation of Elk was determined in cell lysates from CXCR4-293 cells, SYF and Src<sup>++</sup> cells stimulated with SDF-1 for various times by using anti-phospho-Elk antibody. In agreement with the above results that SDF-1 induced nuclear import of ERK1/2, Elk phosphorylation was observed as early as 5 min after stimulation, showing a similar time course as ERK1/2 activation (Fig 6A). Pretreatment of cells with the MEK inhibitor, PD98059, abolished Elk phosphorylation, which was direct evidence that Elk phosphorylation was dependent on ERK1/2 activation. All inhibitors that had been shown to prevent nuclear translocation of ERK1/2 (PP1, Y27632, DN-Rho, and latB) completely blocked SDF-1 induced Elk phosphorylation (Fig 6B). As expected, SDF-1-induced Elk phosphorylation could be detected in Src<sup>++</sup> cells, but not in SYF cells (Fig 6C), which was strong evidence that Src is required for CXCR4-mediated nuclear ERK1/2 and Elk activation.

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## Discussion

In this study, we found that SDF-1 can induce ERK1/2 nuclear translocation and Elk activation in a Src, Rho/ROCK and F-actin dependent manner. This explains the ability of CXCR4 to promote cell proliferation. We also observed higher levels of ERK phosphorylation in the nucleus than in the cytoplasm, suggesting that the nuclear ERK activation is a major target in CXCR4-mediated MAPK signaling.

In general, the mechanisms of nuclear translocation of ERK1/2 are incompletely understood. They are particularly intriguing, however, because of the opposite effects of nuclear phospho-ERK1/2, which promotes proliferation (Brunet et al., 1999), and cytoplasmic phospho-ERK1/2, which can induce senescence (Gaumont-Leclerc et al., 2004) and differentiation (Smith et al., 2004). This ERK activation in the nucleus appears of particular significance in the case of CXCR4, since this receptor is expressed by a variety of metastatic cancer cell. Although internalization of CXCR4 depends on the presence of  $\beta$ -arrestin (Orsini et al., 1999), it is difficult to show complex formation between the CXCR4,  $\beta$ -arrestin and the MAPK cascade (Zhao, et al., unpublished results), as is e.g. possible for the  $\beta_2$ -adrenergic receptor (Luttrell et al., 1999) or CXCR2 (Zhao et al., 2004). Since  $\beta$ -arrestin complexes with the MAPK cascade retain ERK1/2 in the cytoplasm (DeFea et al., 2000), it is perhaps the transiency or low affinity of these complexes in the case of CXCR4 that allows nuclear translocation of ERK1/2.

While nuclear ERK phosphorylation is a common response for 7TM-Rs, we are only aware of a single report in which nuclear transport of ERK1/2 was shown to be secondary to Rho/ROCK activation. In serotonin stimulated smooth muscle cells, ROCK inhibition blocked nuclear ERK1/2 activation and Elk phosphorylation, but in contrast to our study showed no effect on overall ERK phosphorylation (Liu et al., 2004). It is possible that in the case of the serotonin stimulated cells a larger fraction of the ERK1/2 activation occurred in the

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cytoplasm, whereas in our study the majority of ERK1/2 phosphorylation took place in the nucleus. It has also been shown by confocal microscopy that RhoA or Rac1 activation is involved in stretch-induced nuclear translocation of ERK1/2 (Kawamura et al., 2003). The Rho/ROCK pathway was also reported to regulate nuclear export signal through the nuclear membrane (Lee et al., 2004). It appears Rho/ROCK can act as a switching molecule that manages the influx or efflux of signal molecules to the nucleus, thus regulating various cell activities.

The RhoA/ROCK pathway plays an important role in regulation of the cytoskeleton, which in turn plays a role in cell migration, cell cycle progression, apoptosis and cancer development. While  $G_{\alpha_{12}}$  and  $G_{\alpha_{13}}$  are best known as the G-proteins that cause Rho/ROCK activation and actin stress fiber formation, activation of these pathways in SDF-1 stimulated cells was downstream of  $G_{\alpha_i}$  as indicated by the inhibitory effect of pertussis toxin. The ROCK inhibitor Y27632 attenuated SDF-1-induced overall ERK activation, but almost completely prevented the nuclear translocation of ERK1/2 and abrogated Elk phosphorylation. While ROCK activation appears to be upstream of ERK phosphorylation, one may speculate that its effect is indirect. One possible explanation would be that actin polymerization was necessary for translocation of signaling molecules to the plasma membrane. It has been described that translocation of Src to the plasma membrane is mediated by the actin cytoskeleton under the control of Rho family proteins (Fincham et al., 1996). Alternatively, ROCK activity may promote nuclear translocation of ERK and/or of transcription factors such as myocardin-related transcription factors (called MRCF or MAL) (Miralles et al., 2003).

The Rho/ROCK pathway has been implicated in CXCR4-mediated lymphocyte organization, chemotaxis, calcium mobilization and cancer cell invasion (Bartolome et al., 2004; Bug et al., 2002). In CXCR4-293 cells, the Rho/ROCK pathway and an intact actin cytoskeleton not only influenced nuclear translocation of ERK1/2, but also regulated the overall ERK1/2 activation. This regulation may rely on Raf-1 phosphorylation, since it

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has been reported that stretch-induced Raf-1 activation requires integrity of the actin cytoskeleton (Krepinsky et al., 2005).

The actin cytoskeleton has been implicated in numerous cellular activities, including chemotaxis, and exocytosis. Disruption of the actin cytoskeleton with latrunculin B abolished CXCR4-mediated ERK1/2 activation, indicating a critical role of the intact cytoskeleton in CXCR4 signaling. Disruption of caveolae in neonatal rat cardiomyocytes abolished stretch-induced RhoA and Rac activation, impaired actin polymerization and ERK1/2 translocation to nucleus, but had no effect on overall ERK activation (Kawamura et al., 2003). Although an intact actin cytoskeleton has been shown to be required for ERK1/2 translocation to the nucleus in this case, disruption of the cell cytoskeleton had no effect on ERK1/2 phosphorylation or nuclear translocation of ERK1/2 induced by activation of the serotonin receptor 1B/1D in smooth muscle cells (Liu et al., 2004). Finally cytoskeletal disruption can even have the opposite effect. In retinoic acid treated cells an intact cytoskeleton is required to restrict activated ERK1/2 to the cytoplasm, and actin disrupting agents lead to increased nuclear ERK1/2 activation (Smith et al., 2004). The role of the cytoskeleton in nuclear translocation of ERK1/2 clearly needs further investigation. Actin polymerization is not a general requisite for nuclear translocation of ERK1/2, and inhibition of the actin response had no effect on EGF-mediated nuclear ERK activation (Fig 2F).

We hypothesize that the intact cytoskeleton played a role in the initial translocation of signaling molecules including Src to the plasma membrane (Fincham et al., 1996). Involvement at this stage is also suggested by the observation that inhibitors that blocked nuclear translocation of ERK1/2 (PP1, Y27632, latrunculin B) also inhibited internalization of SDF-1 stimulated CXCR4-GFP as detected by confocal microscopy (Zhao et al., unpublished results), indicating that the Rho/ROCK pathway and the actin cytoskeleton were involved in the initial assembly of signaling molecules at the plasma membrane. This signaling cascade resembles that reported for integrin-dependent ERK1/2 activation (Fincham et al., 2000), a notion that is supported by the previously described integrin-activating effect of SDF-1 (Burger et al., 2003; Cardones et al., 2003). Integrin engagement



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has furthermore been shown to be involved in the nuclear translocation of ERK1/2 (Aplin et al., 2001). In this scenario F-actin serves as a scaffold for efficient signaling.

Previous reports as well as our results indicate that SDF-1 stimulation leads to Src family kinase activation (Chernock et al., 2001; Ptasznik et al., 2002). This occurred upstream of ERK1/2 phosphorylation. While PP1 is a relatively specific inhibitor, it inhibits all Src family kinases. Use of SYF and Src<sup>++</sup> cells, however, allowed proving that Src itself is sufficient for the ERK nuclear translocation, and showed that this pathway is of physiological relevance in cells that express a low copy number of CXCR4 constitutively, underscoring that the nuclear translocation of ERK and Elk activation are not the consequence of over-expression of the receptor. There remained, however a fraction of ERK phosphorylation that was not amenable to inhibition by Src family inhibitors/knockouts, which was retained in the cytoplasm. Since it was shown previously that dominant negative arrestin can attenuate SDF-1-mediated ERK phosphorylation (Zhao et al., 2004), this fraction of phospho-ERK may have resulted from complex formation with  $\beta$ -arrestin as has been shown for the PAR-2 (DeFea et al., 2000) and angiotensin type 1A receptors (Tohgo et al., 2002). As expected, FAK, another downstream signal of Src, was also phosphorylated following SDF-1 stimulation, as previously described for a human hematopoietic progenitor cell line (Wang et al., 2000). Since FAK can cause paxillin recruitment to the plasma membrane followed by activation of Rho family proteins (Igishi et al., 1999), FRNK, a FAK inhibitor protein, was transfected together with CXCR4-GFP and RFP-ERK1/2. FRNK did, however not seem to block nuclear translocation of RFP-ERK1/2 (results not shown), suggesting that FAK activation and ERK1/2 nuclear translocation were both downstream of Src activation, but independent from each other.

Nuclear ERK activation stimulates transcription factors such as Elk and Egr-1 which are known to regulate cell proliferation. In addition to the Elk phosphorylation described in this study, we also observed moderately increased Egr-1 expression in 293 cells upon stimulation of CXCR4 by using a luciferase activity assay, which was inhibited in the presence of Y27632 (results not shown). In agreement with the dependence of nuclear translocation of ERK1/2 on Src, Rho/ROCK and polymerized actin, Elk phosphorylation was blocked by the

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same inhibitors. In contrast, activation of Elk following stimulation with lysophosphatidic acid depended on Rho, but not on F-actin (Gineitis and Treisman, 2001).

In summary, we showed evidence that CXCR4 activation mediated nuclear translocation of ERK1/2 by using two different methods, Western blotting and confocal microscopy. It appears that the Src and RhoA/ROCK pathways not only regulated overall ERK1/2 activation mediated by CXCR4, but primarily controlled the nuclear translocation of ERK/2 and thereby influenced the activity of transcription factors. Moreover, these results suggest that cytoskeletal reorganization following agonist stimulation may play an important role in regulating the subcellular distribution of ERK1/2.

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## References

- Ajenjo N, Canon E, Sanchez-Perez I, Matallanas D, Leon J, Perona R and Crespo P (2004) Subcellular localization determines the protective effects of activated ERK2 against distinct apoptogenic stimuli in myeloid leukemia cells. *J Biol Chem* **279**(31):32813-32823.
- Aplin AE, Stewart SA, Assoian RK and Juliano RL (2001) Integrin-mediated adhesion regulates ERK nuclear translocation and phosphorylation of Elk-1. *J Cell Biol* **153**(2):273-282.
- Bartolome RA, Galvez BG, Longo N, Baleux F, van Muijen GNP, Sanchez-Mateos P, Arroyo AG and Teixido J (2004) Stromal cell-derived factor-1 $\alpha$  promotes melanoma cell invasion across basement membranes involving stimulation of membrane-type 1 matrix metalloproteinase and Rho GTPase activities. *Cancer Res* **64**(7):2534-2543.
- Brunet A, Roux D, Lenormand P, Dowd S, Keyse S and Pouyssegur J (1999) Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO J* **18**(3):664-674.
- Bug G, Rossmannith T, Henschler R, Kunz-Schughart LA, Schroder B, Kampfmann M, Kreutz M, Hoelzer D and Ottmann OG (2002) Rho family small GTPases control migration of hematopoietic progenitor cells into multicellular spheroids of bone marrow stroma cells. *J Leukoc Biol* **72**(4):837-845.
- Burger M, Glodek A, Hartmann T, Schmitt-Graff A, Silberstein LE, Fujii N, Kipps TJ and Burger JA (2003) Functional expression of CXCR4 (CD184) on small-cell lung cancer cells mediates migration, integrin activation, and adhesion to stromal cells. *Oncogene* **22**(50):8093-8101.
- Cardones AR, Murakami T and Hwang ST (2003) CXCR4 enhances adhesion of B16 tumor cells to endothelial cells in vitro and in vivo via  $\beta$ 1 integrin. *Cancer Res* **63**(20):6751-6757.
- Chernock RD, Cherla RP and Ganju RK (2001) SHP1 and cbl participate in alpha-chemokine receptor CXCR4-mediated signaling pathways. *Blood* **97**:608-615.

MOL # 16923

- DeFea KA, Zalevsky J, Thoma MS, Dery O, Mullins RD and Bunnett NW (2000) beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 Is required for intracellular targeting of activated ERK1/2. *J Cell Biol* **148**:1267-1281.
- Fincham V, Unlu M, Brunton V, Pitts J, Wyke J and Frame M (1996) Translocation of Src kinase to the cell periphery is mediated by the actin cytoskeleton under the control of the Rho family of small G proteins. *J Cell Biol* **135**(6):1551-1564.
- Fincham VJ, James M, Frame MC and Winder SJ (2000) Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. *EMBO J* **19**(12):2911-2923.
- Formstecher E, Ramos JW, Fauquet M, Calderwood DA, Hsieh JC, Canton B, Nguyen XT, Barnier JV, Camonis J, Ginsberg MH and Chneiweiss H (2001) PEA-15 mediates cytoplasmic sequestration of ERK MAP kinase. *Dev Cell* **1**(2):239-250.
- Fukuda M, Gotoh Y and Nishida E (1997) Interaction of MAP kinase with MAP kinase kinase: its possible role in the control of nucleocytoplasmic transport of MAP kinase. *EMBO J* **16**(8):1901-1908.
- Gaumont-Leclerc MF, Mukhopadhyay UK, Goumard S and Ferbeyre G (2004) PEA-15 is inhibited by adenovirus E1A and plays a role in ERK nuclear export and Ras-induced senescence. *J Biol Chem* **279**(45):46802-46809.
- Gineitis D and Treisman R (2001) Differential usage of signal transduction pathways defines two types of serum response factor target gene. *J Biol Chem* **276**(27):24531-24539.
- Hirakawa M, Oike M, Karashima Y and Ito Y (2004) Sequential activation of RhoA and FAK/paxillin leads to ATP release and actin reorganization in human endothelium. *J Physiol (Lond)* **558**(2):479-488.
- Igishi T, Fukuhara S, Patel V, Katz B-Z, Yamada KM and Gutkind JS (1999) Divergent signaling pathways link focal adhesion kinase to mitogen-activated protein kinase cascades. Evidence for A role of paxillin in c-Jun NH2-terminal kinase activation. *J Biol Chem* **274**(43):30738-30746.
- Kawamura S, Miyamoto S and Brown JH (2003) Initiation and transduction of stretch-induced RhoA and Rac1 activation through caveolae: Cytoskeletal regulation of ERK translocation. *J Biol Chem* **278**(33):31111-31117.

MOL # 16923

- Kijima T, Maulik G, Ma PC, Tibaldi EV, Turner RE, Rollins B, Sattler M, Johnson BE and Salgia R (2002) Regulation of cellular proliferation, cytoskeletal function, and signal transduction through CXCR4 and c-Kit in small cell lung cancer cells. *Cancer Res* **62**(21):6304-6311.
- Klinger M, Kudlacek O, Seidel MG, Freissmuth M and Sexl V (2002) MAP kinase stimulation by cAMP does not require RAP1 but Src family kinases. *J Biol Chem* **277**(36):32490-32497.
- Klinghoffer R.A., Sachsenmaier C., Cooper J.A., and Soriano P. (1999) Src family kinases are required for integrin but not PDGFR signal transduction. *EMBO J* **18**:2459-2471.
- Krepinsky JC, Li Y, Tang D, Liu L, Scholey J and Ingram AJ (2005) Stretch-induced Raf-1 activation in mesangial cells requires actin cytoskeletal integrity. *Cell Signal* **17**(3):311-320.
- Lee HH, Chien CL, Liao HK, Chen YJ and Chang ZF (2004) Nuclear efflux of heterogeneous nuclear ribonucleoprotein C1/C2 in apoptotic cells: a novel nuclear export dependent on Rho-associated kinase activation. *J Cell Sci* **117**(23):5579-5589.
- Liu Y, Suzuki YJ, Day RM and Fanburg BL (2004) Rho kinase-induced nuclear translocation of ERK1/ERK2 in smooth muscle cell mitogenesis caused by serotonin. *Circ Res* **95**(6):579-586.
- Luttrell LM, Ferguson SSnG, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F-T, Kawakatsu H, Owada K, Luttrell DK, Caron MG and Lefkowitz RJ (1999)  $\beta$ -arrestin-dependent formation of beta-2 adrenergic receptor-Src protein kinase complexes. *Science* **283**(5402):655-661.
- Luttrell LM, Roudabush FL, Choy EW, Miller WE, Field ME, Pierce KL and Lefkowitz RJ (2001) Activation and targeting of extracellular signal-regulated kinases by  $\beta$ -arrestin scaffolds. *Proc Natl Acad Sci* **98**(5):2449-2454.
- Majka M, Ratajczak J, Kowalska MA and Ratajczak MZ (2000) Binding of stromal derived factor-1alpha (SDF-1 $\alpha$ ) to CXCR4 chemokine receptor in normal human megakaryoblasts but not in platelets induces phosphorylation of mitogen-activated protein kinase p42/44 (MAPK), ELK-1 transcription factor and serine/threonine kinase AKT. *Eur J Haematol* **64**(3):164-172.

MOL # 16923

- Miralles F, Posern G, Zaromytidou AI and Treisman R (2003) Actin dynamics control SRF activity by regulation of its coactivator MAL. *Cell* **113**(3):329-342.
- Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E and Zlotnik A (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**(6824):50-56.
- Neuhaus T, Stier S, Totzke G, Gruenewald E, Fronhoffs S, Sachinidis A, Vetter H and Ko YD (2003) Stromal cell-derived factor 1 (SDF-1) induces gene-expression of early growth response-1 (Egr-1) and VEGF in human arterial endothelial cells and enhances VEGF induced cell proliferation. *Cell Prolif* **36**(2):75-86.
- Orsini MJ, Parent J-L, Mundell SJ and Benovic JL (1999) Trafficking of the HIV coreceptor CXCR4. Role of arrestins and identification of residues in the C-terminal tail that mediate receptor internalization. *J Biol Chem* **274**(43):31076-31086.
- Osawa M, Itoh S, Ohta S, Huang Q, Berk BC, Marmarosh NL, Che W, Ding B, Yan C and Abe J-i (2004) ERK1/2 associates with the c-Met-binding domain of growth factor receptor-bound protein 2 (Grb2)-associated binder-1 (Gab1): Role in ERK1/2 and early growth response factor-1 (Egr-1) nuclear accumulation. *J Biol Chem* **279**(28):29691-29699.
- Porcile C, Bajetto A, Barbero S, Pirani P and Schettini G (2004) CXCR4 activation induces epidermal growth factor receptor transactivation in an ovarian cancer cell Line. *Ann N Y Acad Sci* **1030**(1):162-169.
- Ptasznik A, Urbanowska E, Chinta S, Costa MA, Katz BA, Stanislaus MA, Demir G, Linnekin D, Pan ZK and Gewirtz AM (2002) Crosstalk between BCR/ABL oncoprotein and CXCR4 signaling through a Src family kinase in human leukemia cells. *J Exp Med* **196**(5):667-678.
- Rossi D and Zlotnik A (2000) The biology of chemokines and their receptors. *Annu Rev Immunol* **18**:217-242.
- Schrader A, Lechner O, Templin M, Dittmar K, Machtens S, Mengel M, Probst-Kepper M, Franzke A, Wollensak T, Gatzlaff P, Atzpodien J, Buer J and Lauber J (2002) CXCR4/CXCL12 expression and signalling in kidney cancer. *Br J Cancer* **86**(8):1250-1256.

MOL # 16923

- Smith ER, Smedberg JL, Rula ME and Xu XX (2004) Regulation of Ras-MAPK pathway mitogenic activity by restricting nuclear entry of activated MAPK in endoderm differentiation of embryonic carcinoma and stem cells. *J Cell Biol* **164**(5):689-699.
- Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS and McCauley LK (2002) Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. *Cancer Res* **62**(6):1832-1837.
- Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, and Juttrel LM (2002)  $\beta$ -Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transacritpion following angiotensin AT1a receptor stimulation. *J Biol Chem* **277**:9429-9436
- Wang JF, Park IW and Groopman JE (2000) Stromal cell-derived factor-1 -alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. *Blood* **95**:2505-2513.
- Whitehurst A, Cobb MH and White MA (2004a) Stimulus-coupled spatial restriction of extracellular signal-regulated kinase 1/2 activity contributes to the specificity of signal-response pathways. *Mol Cell Biol* **24**(23):10145-10150.
- Whitehurst AW, Robinson FL, Moore MS and Cobb MH (2004b) The death effector domain protein PEA-15 prevents nuclear entry of ERK2 by inhibiting required interactions. *J Biol Chem* **279**(13):12840-12847.
- Zhao M, Wimmer A, Trieu K, DiScipio RG and Schraufstatter IU (2004) Arrestin regulates MAPK activation and prevents NADPH oxidase-dependent death of cells expressing CXCR2. *J Biol Chem* **279**(47):49259-49267.
- Zhou Y, Larsen PH, Hao C and Yong VW (2002) CXCR4 is a major chemokine receptor on glioma cells and mediates their survival. *J Biol Chem* **277**(51):49481-49487.

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### Footnotes

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### Figure legends

**Fig. 1.** CXCR4-mediated ERK1/2 activation through the Ras/Raf pathway. Cells were serum-starved overnight, then stimulated with 500 ng/ml SDF-1 for the indicated times, whole cell lysates were separated by SDS-PAGE and ERK1/2 activation was determined by immunoblotting with anti-phospho-ERK1/2 antibody (upper panels) followed by total ERK1/2 detection (lower panels) to assure equal loading. **A**, Time-course of SDF-1 induced ERK1/2 phosphorylation in HEK293 cells expressing CXCR4. **B**, Time-course of ERK1/2 phosphorylation in HMECs stimulated with SDF-1. **C**, Effect of Ras inhibition on ERK1/2 phosphorylation. CXCR4-293 cells were cotransfected with DN-Ras or pretreated with FTI277, a farnesyltransferase inhibitor, and stimulated with SDF-1 for 5 min. Left panel: Western blot, right panel: quantification (mean  $\pm$  S.D., n=3). **D**, Effect of inhibition of Raf and MEK. HEK293 cells expressing CXCR4 were pre-incubated with Raf-1 inhibitor or the MEK inhibitor PD98059 and stimulated with SDF-1 for 5 min. Again, the left panel shows a representative Western blot, and the right panel the quantification (mean  $\pm$  S.D., n=3). **E**, MEK1 phosphorylation assay. CXCR4-expressing HEK293 cells were stimulated with SDF-1 for the indicated times. Raf was immunoprecipitated and a kinase assay was performed as described under methods. Left panel, top row: autoradiography of  $^{32}$ P-phosphorylated p-MEK, bottom row: Western blot loading control. Right panel: quantification of the results of three experiments (Mean  $\pm$  S.D.).

**Fig. 2.** Signaling pathways involved in SDF-1 induced ERK1/2 activation. HEK293 expressing CXCR4 were cotransfected with DN-Rho or pretreated with various inhibitors for 30 min prior to the addition of SDF-1. ERK1/2 phosphorylation was detected by Western blotting. **A**, Effect of pertussis toxin (PTX) on ERK1/2 phosphorylation. CXCR4-293 cells were incubated with 200ng/ml PTX overnight prior to SDF-1 stimulation. **B**, Effect of the Src inhibitor PP1 on ERK1/2 phosphorylation in 293 cells. **C**, Time-course of SDF-1-induced ERK1/2 phosphorylation in Src/Yes/Fyn deficient SYF cells, and Src-reconstituted Src<sup>++</sup> cells. A representative Western blot is shown on the right, quantification to the right, closed circles: SYF cells, open circles: Src<sup>++</sup> cells (mean  $\pm$  S.D., n=3). **D**, Demonstration of Src activation: Src phosphorylation is shown in SDF-1 stimulated

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CXCR4-293 cells (left panel) and HMECs (middle panel); Western blots are shown on top, and quantification of each data point below (mean  $\pm$  S.W.D., n=3). The activation of FAK, a downstream target of Src, was determined by immunoprecipitation (IP) (right panel). FAK immunoprecipitates from CXCR4-293 cells were detected with phospho-tyrosine antibody to reflect FAK activation. **E**, Role of Rho on ERK1/2 phosphorylation: Effect of the ROCK inhibitor Y27632 (top left) and of dominant negative Rho (top right) on ERK1/2 phosphorylation in CXCR4-293 cells. Results were quantified in the left lower panel (mean  $\pm$  S.D., n=3). Right lower panel: SDF-1-induced RhoA translocation to the plasma membrane. CXCR4-293 cells were serum-starved overnight prior to stimulation of SDF-1 for the indicated times, then lysed and the plasma membrane fraction was isolated. Translocated RhoA was detected by Western blotting. **F**, CXCR4-293 cells were pretreated with the cytoskeleton disrupting agents latrunculin B (latB, 0.5  $\mu$ M), then stimulated with SDF-1 or 20 ng/ml EGF for 5 min. ERK1/2 phosphorylation was detected by Western blotting. One experiment representative of 3 is shown in all cases.

**Fig. 3.** SDF-1-induced nuclear translocation of ERK1/2. CXCR4-293 cells, SYF and Src<sup>++</sup> cells were serum-starved overnight and stimulated with SDF-1 for various times, cell lysates were separated into nuclear and cytoplasmic fractions, and immunoblotted with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. **A**, Immunoblot of fractionated samples using anti-lamin B (a nuclear marker) and anti-actin (a cytoplasmic marker) antibodies to exclude cross-contamination between nuclear and cytoplasmic fractions. **B**, Time-course of SDF-1-induced nuclear translocation of ERK1/2 in CXCR4-293 cells. **C**, Effect of the Src inhibitor PP1 on SDF-1-induced nuclear translocation of ERK1/2 in CXCR4-293 cells. **D**, SDF-1-induced nuclear translocation of ERK1/2 in SYF and Src<sup>++</sup> cells. **E**, Effect of the actin-disrupting agent, latrunculin B and of inhibition of Rho/ROCK with Y27632 or dominant negative Rho on SDF-1-induced nuclear translocation of ERK1/2 in CXCR4-293 cells.

**Fig 4.** Confocal fluorescence microscopy of ERK1/2 cellular distribution. **A**, HEK293 cells on coverslips were transiently transfected with a minimum amount of ERK2-RFP and CXCR4-GFP. Cells were pretreated with

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various inhibitors for 30 min prior to the addition of SDF-1 for 5 min. Nuclei were stained with DAPI, and ERK2-RFP entry into the nucleus was detected by confocal microscopy. **B**, HEK293 cells expressing CXCR4-GFP cultured on coverslips were pretreated with various inhibitors as indicated, and SDF-1-induced nuclear ERK1/2 phosphorylation was detected by immunofluorescence confocal microscopy as described in the method section. Cellular distribution of CXCR4-GFP, Alexa 568-stained phospho-ERK1/2 and DAPI-stained nuclei are shown in single channel and overlay images.

**Fig. 5.** SDF-1 induced stress fiber formation in CXCR4-293 and HMEC cells. Cells on coverslips were stimulated with 500 ng/ml SDF-1 as indicated, fixed and stained with Alexa 488 phalloidin. Actin polymerization was viewed by confocal microscopy. **A**, Cytoskeletal reorganization in HEK293 expressing CXCR4. **B**, SDF-1 induced actin polymerization in HMEC cells. **C**, HMEC cells were incubated with various inhibitors for 30 min prior to stimulation with SDF-1 for 10 min. Actin polymerization was detected as above.

**Fig. 6.** Phosphorylation of the transcription factor Elk. **A**, Time course of Elk phosphorylation: HEK293 expressing CXCR4 were stimulated with SDF-1 for different times, whole cell lysates were separated by SDS-PAGE, and Elk phosphorylation was detected by Western blotting (left panel). The right panel shows quantification of these results (mean  $\pm$  S.D., n=3). **B**, Effect of inhibitors on Elk phosphorylation: Cells were cotransfected with DN-Rho or pretreated with various inhibitors for 30 min prior to the addition of SDF-1, Elk activation was evaluated by Western blotting. The left panel shows a representative blot and the right panel quantification (mean  $\pm$  S.D., n=3). **C**, Time course of Elk phosphorylation in SYF and Src<sup>++</sup> cells: SYF and Src<sup>++</sup> cells were serum-starved overnight and then treated with SDF-1 for the indicated times, Elk phosphorylation was examined as above. Left panel: Western blot, right panel quantification (mean  $\pm$  S.D., n=3).

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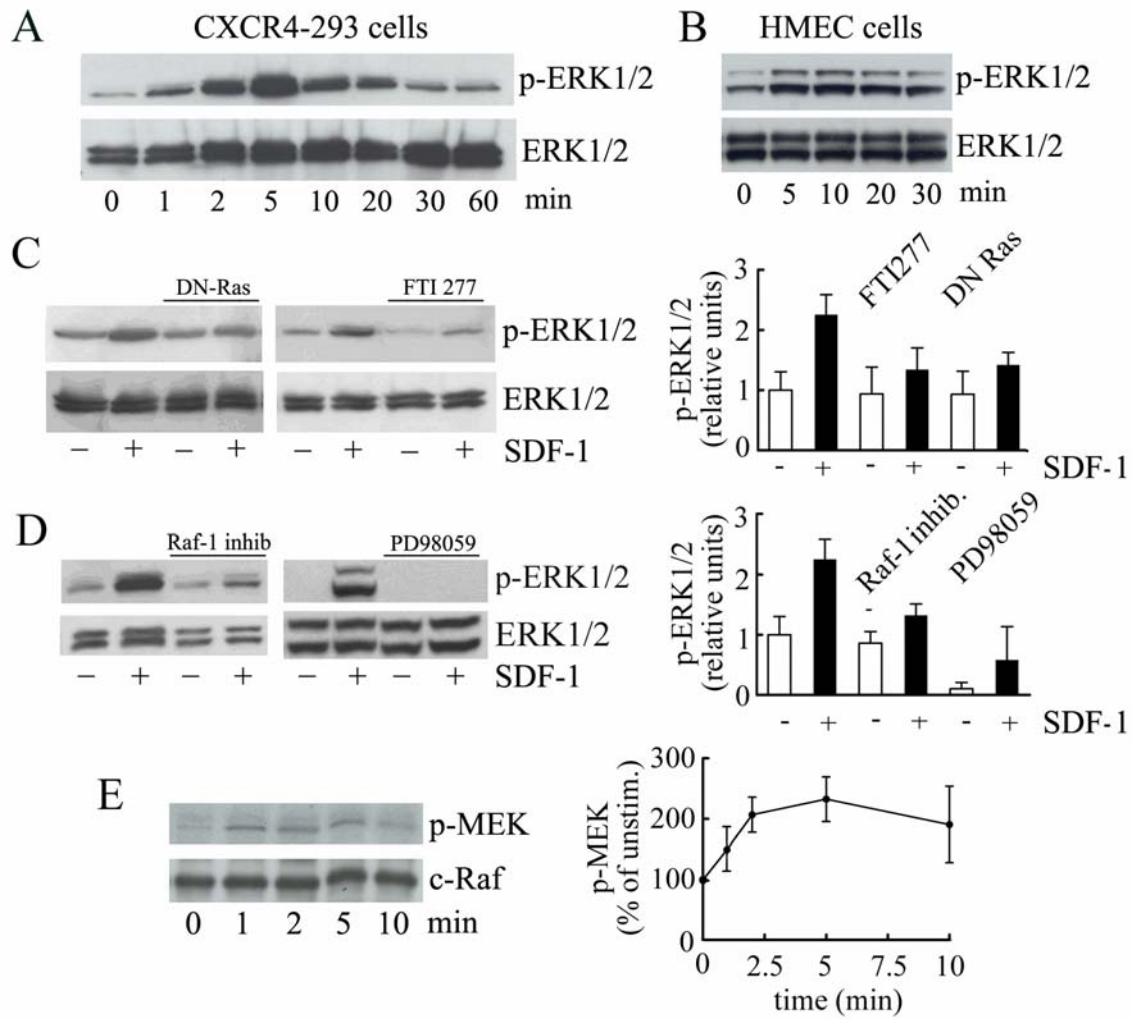


Fig. 1

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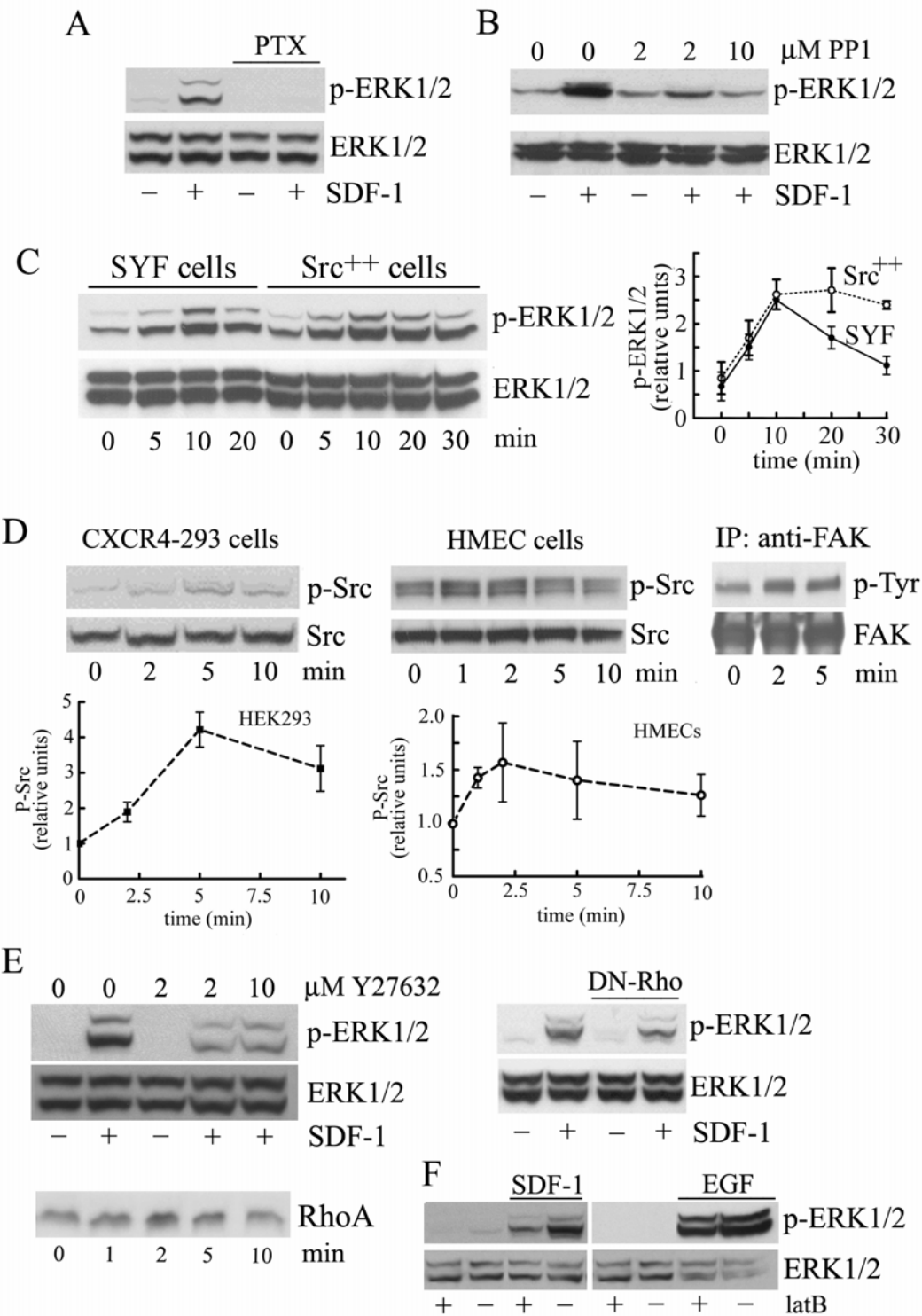


Fig. 2

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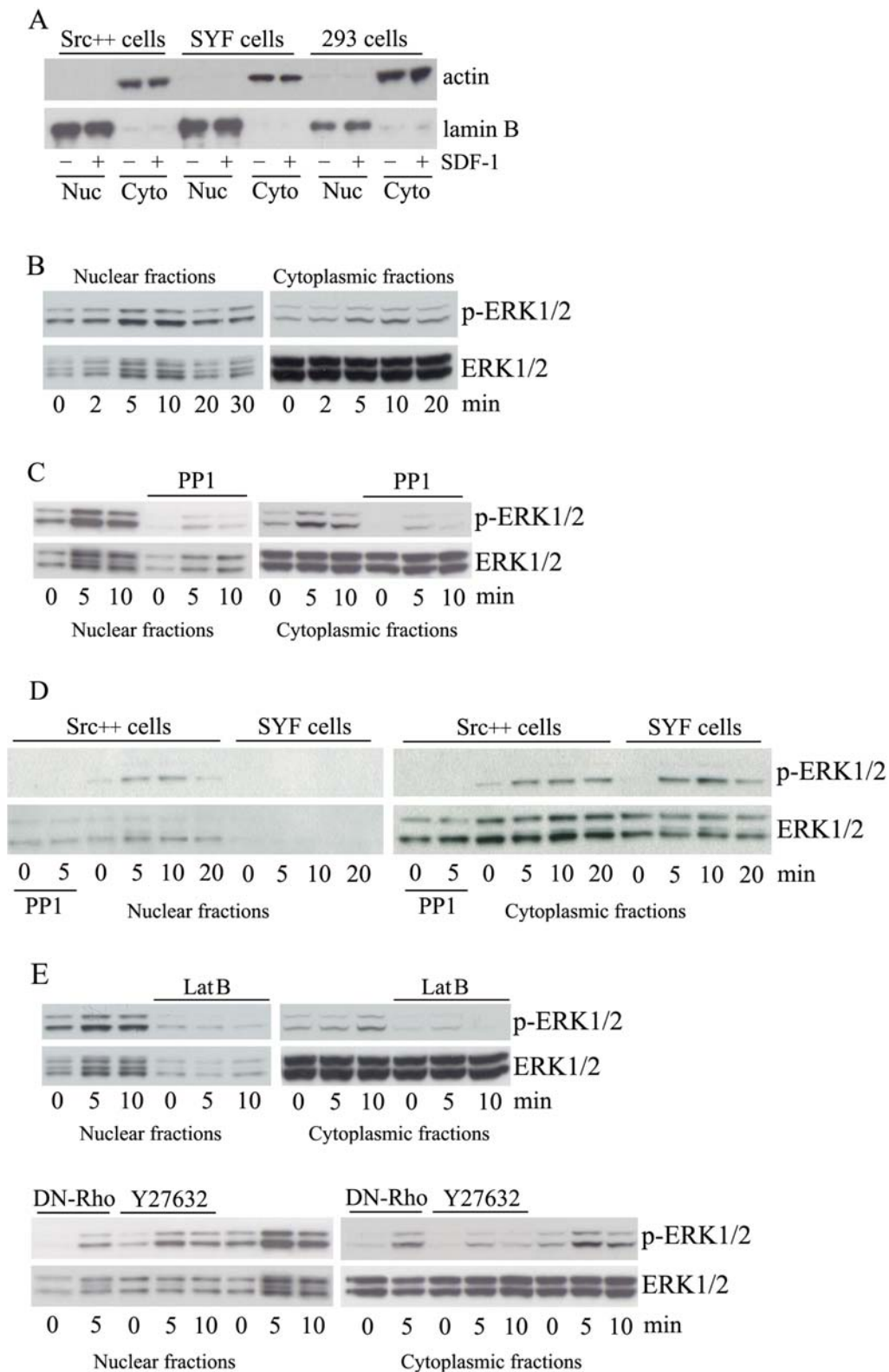


Fig. 3

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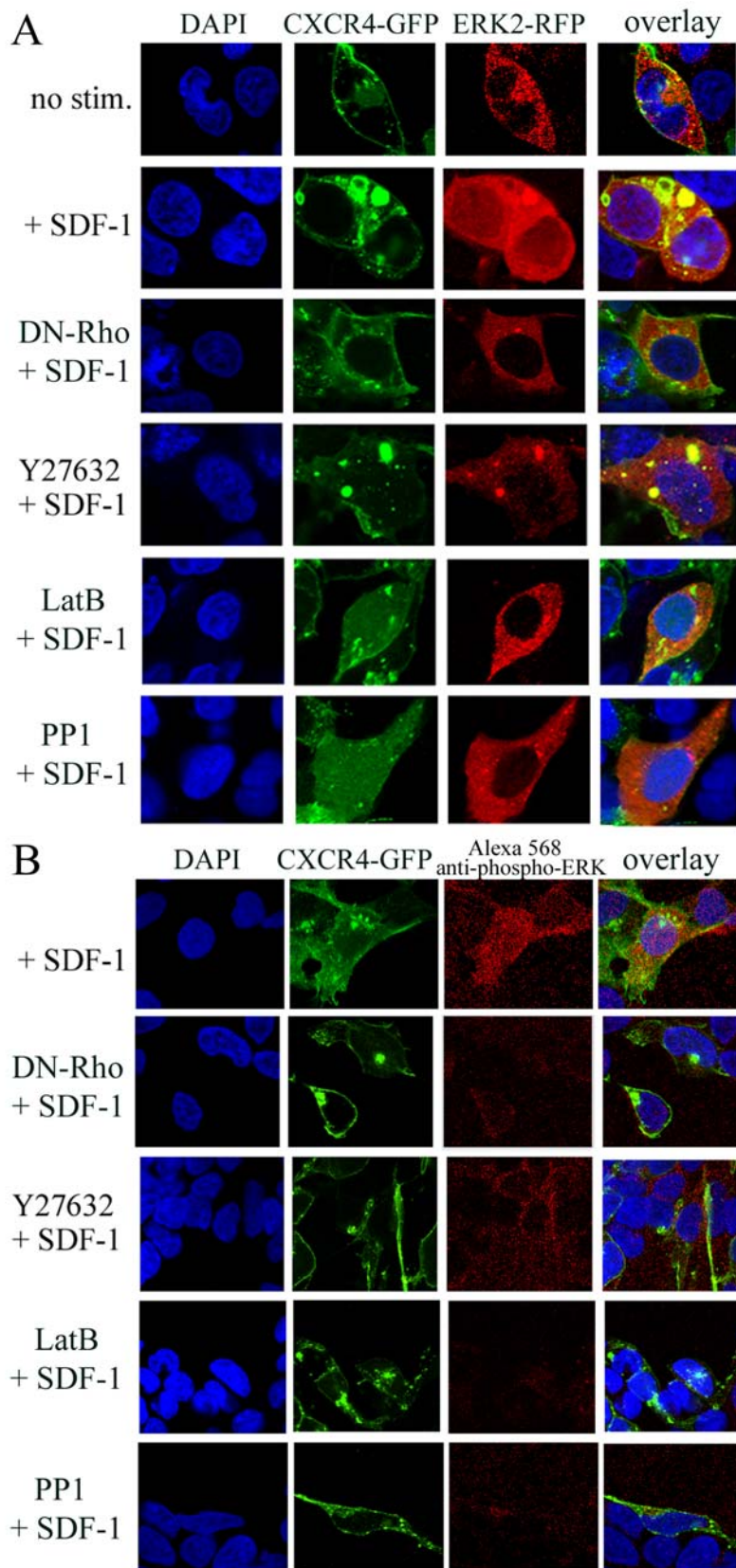


Fig. 4

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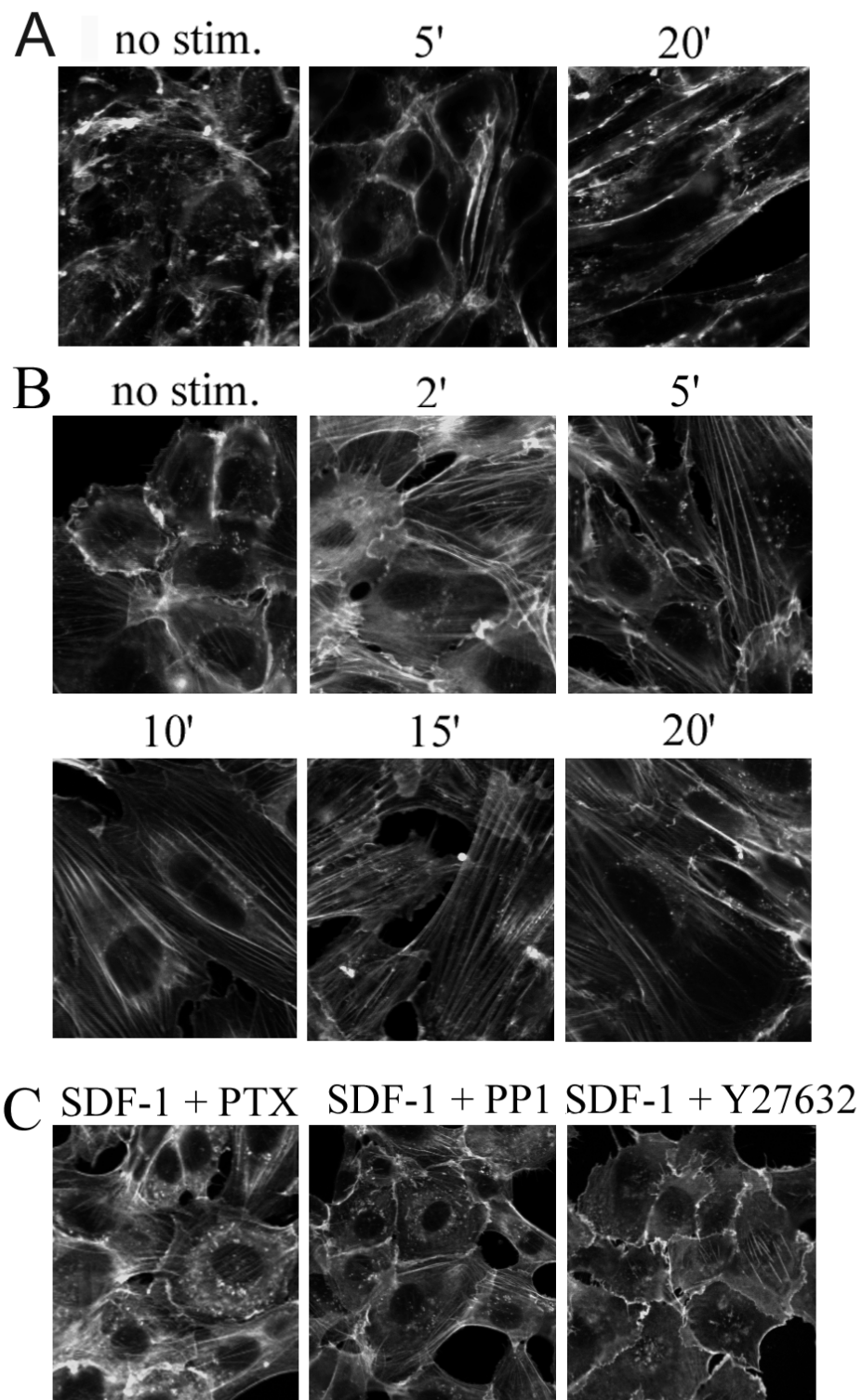


Fig. 5



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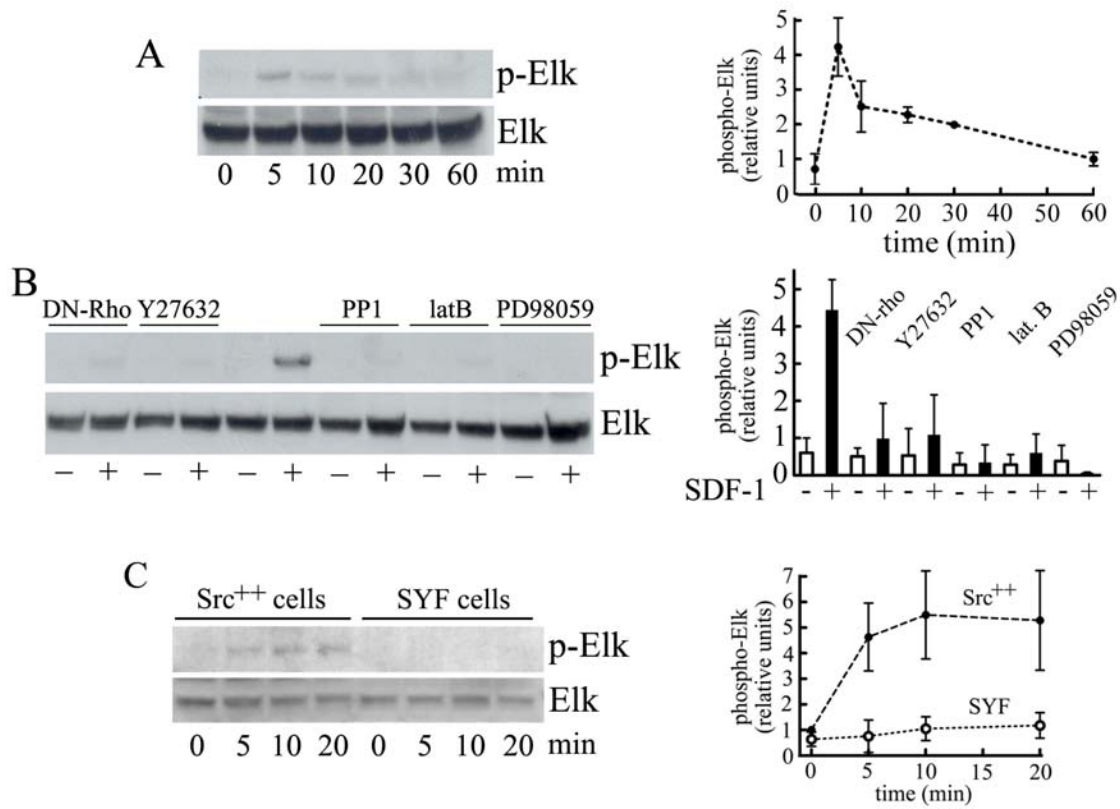


Fig. 6