Neural cell adhesion molecule (NCAM) associates with fibroblast growth factor (FGF) receptor-1 (FGFR1). However, the biological significance of this interaction remains largely elusive. In this study, we show that NCAM induces a specific, FGFR1-mediated cellular response that is remarkably different from that elicited by FGF-2. In contrast to FGF-induced degradation of endocytic FGFR1, NCAM promotes the stabilization of the receptor, which is recycled to the cell surface in a Rab11- and Src-dependent manner. In turn, FGFR1 recycling is required for NCAM-induced sustained activation of various effectors. Furthermore, NCAM, but not FGF-2, promotes cell migration, and this response depends on FGFR1 recycling and sustained Src activation. Our results implicate NCAM as a nonconventional ligand for FGFR1 that exerts a peculiar control on the intracellular trafficking of the receptor, resulting in a specific cellular response. Besides introducing a further level of complexity in the regulation of FGFR1 function, our findings highlight the link of FGFR recycling with sustained signaling and cell migration and the critical role of these events in dictating the cellular response evoked by receptor activation.

The binding of NCAM to FGFR1 induces a specific cellular response mediated by receptor trafficking

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Introduction

FGF receptors (FGFRs) are cell surface receptor tyrosine kinases (RTKs) that, upon binding of FGFs, undergo dimerization and trans-phosphorylation (Beenken and Mohammadi, 2009), which generates multiple docking sites for several adaptor and effector proteins, thus resulting in the activation of various signaling pathways (Eswarakumar et al., 2005; Furdui et al., 2006). Typical effectors of FGFR activity are Shc and FGFR substrate-2α (FRS-2α) that, by recruiting the Grb2–SOS complex, induce the activation of the Ras–Raf–Erk1/2 pathway (Eswarakumar et al., 2005). As for most RTKs, ligand binding induces FGFR internalization and Cbl-mediated ubiquitination followed by lysosomal degradation (Wong et al., 2002).

In addition to heparan sulfate proteoglycans (Yayon et al., 1991), FGF signaling can also be modulated by several membrane proteins (Polanska et al., 2009), including cell adhesion molecules (CAMs) of the cadherin and immunoglobulin (Ig-CAMs) superfamilies (Cavallaro and Christofori, 2004). Among the Ig-CAMs that functionally interact with FGFR, the best characterized is neural CAM (NCAM), a cell surface glycoprotein whose extracellular portion contains five Ig-like domains and two FNIII (fibronectin type III) repeats (Hinsby et al., 2004). In the central nervous system, NCAM enhances intercellular adhesion, axonal growth, and neuronal migration through both homophilic NCAM-mediated cell–cell adhesion and heterophilic interactions with other membrane proteins or extracellular matrix components (Hinsby et al., 2004). After the pioneering work that implicated NCAM-mediated FGFR signaling in neurite outgrowth (Williams et al., 1994), the NCAM–FGFR association has been demonstrated in several cell types, including nonneural cells (Cavallaro et al., 2001; Kos and Chin, 2002; Sanchez-Heras et al., 2006; Francavilla et al., 2007). Recently, NCAM-derived peptides or protein domains have been reported to interact with FGFR1 and FGFR2 (Kiselyov et al., 2003; Christensen et al., 2006) and to modulate various FGFR-mediated neuronal functions (Hansen et al., 2008). Nevertheless, the biological significance...
of FGFR activation by NCAM has remained largely elusive, especially in nonneural cell types.

In this study, we have investigated the outcome of NCAM–FGFR interplay in fibroblasts and epithelial cells. To this goal, we used soluble versions of NCAM, which enabled us to perform a direct comparison with FGF, the classical FGFR ligand that acts as a soluble growth factor. Our data show that (a) NCAM is a novel, noncanonical ligand for FGFR1 and induces a specific set of FGFR-dependent biochemical events, leading to cell migration; (b) soluble NCAM stimulates FGFR1 signaling in the absence of cell surface NCAM; (c) NCAM induces the internalization of FGFR1 and, unlike FGF, promotes its recycling to the cell surface, resulting in sustained signaling; and (d) NCAM stimulates cell migration, and this effect requires FGFR1 recycling. These data provide novel insights into the regulation and function of FGFR.

Results

Soluble, NCAM-derived fragments mimic cell surface NCAM in activating FGFR

To gain insights into the functional outcome of the NCAM–FGFR interplay in nonneuronal cell types, we asked whether NCAM and FGFs, the classical FGFR ligands, elicit the same cellular response downstream of FGFR. We reasoned that, for a direct comparison with FGF, NCAM must be presented to FGFR as a soluble ligand rather than as a membrane protein. However, in most cases, NCAM occurs as a cell surface molecule, and therefore, we initially verified whether soluble NCAM-derived molecules recapitulated the FGFR-mediated function of membrane-associated NCAM.

First, by using the whole ectodomains of NCAM and FGFR1 in surface plasmon resonance and solid phase–binding assays (Fig. S1, A and B), we confirmed and extended previous data on the binding of recombinant or synthetic fragments of NCAM to FGFR1 and FGFR2 (Kiselyov et al., 2003; Christensen et al., 2006). We previously reported that the reconstitution of pancreatic β-tumor cells from NCAM knockout mice with full-length NCAM rescues both cell matrix adhesion and neurite outgrowth but only in the presence of an intact FGFR-mediated signaling, thus implicating an interplay between NCAM and FGFR (Cavallaro et al., 2001). The treatment of NCAM-deficient β-tumor cells with soluble NCAM-Fc, consisting of the extracellular portion of NCAM fused to the Fc fragment of IgG, rescued both cell matrix adhesion (Fig. S1 C) and neurite outgrowth (not depicted). In contrast, no effect was observed with an NCAM-Fc version deleted of the second FNIII repeat (ΔFN2-Fc), which is where the FGFR-binding motif is located (Kiselyov et al., 2003). Very similar results were obtained on mouse fibroblast L cells, which express no endogenous NCAM (Cavallaro et al., 2001; Francavilla et al., 2007). On one hand, the forced expression of trans-membrane NCAM stimulated matrix adhesion of L cells, an effect that was abolished by the FGFR inhibitor PD173074 (Fig. S1 D). On the other hand, soluble NCAM-Fc promoted matrix adhesion of L cells via FGFR signaling, whereas ΔFN2-Fc had no effect (Fig. S1 E). Thus, a soluble version of NCAM’s ectodomain was able to recapitulate the FGFR-dependent function of membrane-associated NCAM.

These observations validated NCAM-Fc as a suitable tool to study the impact of NCAM on FGFR function, and the physiological relevance of this approach is further supported by the notion that NCAM also occurs as a soluble protein naturally released by various cell types in vivo (Secher, 2008). However, the ectodomain of NCAM contains several modules that engage in both homophilic (i.e., NCAM–NCAM) and heterophilic interactions with various cell surface and extracellular matrix molecules (Nielsen et al., 2008). Thus, to focus specifically on the effect of NCAM binding to FGFR, the experiments with NCAM-Fc were complemented with the NCAM-derived FGL peptide, which mimics the binding to and activation of FGFR1 (Kiselyov et al., 2003). Indeed, both in cell matrix adhesion (Fig. S1 E) and in the assays described in the following paragraphs, we obtained convincing evidence that FGL recapitulates the FGFR-dependent function of NCAM-Fc.

NCAM and FGF stimulate different FGFR1-mediated pathways

Membrane-associated NCAM inhibits FGF signaling in different cell types (Francavilla et al., 2007). Therefore, to compare the impact of NCAM on FGFR function with that of FGF, we selected the HeLa epithelial cell line, which does not express NCAM (Fig. S1 F). Furthermore, HeLa cells express all FGFR family members (Fig. 1 A) and are amenable to biochemical and imaging approaches that were undertaken to this purpose. Finally, we verified that the ectopic expression of membrane-associated NCAM in HeLa cells did not affect the ability of soluble NCAM fragments to induce FGFR-mediated signaling (Fig. S1 F), further supporting the choice of this cell line as a model system suitable to investigating the effect of NCAM versus FGF.

When HeLa cells were treated for 10 min with FGF-2, NCAM-Fc, or FGL, each of these ligands stimulated the phosphorylation of FGFR1 to a similar extent (Fig. 1 A). In contrast, none of the ligands stimulated the phosphorylation of FGFR2 or FGFR3, suggesting that NCAM, similar to FGF-2 (Itoh and Ornitz, 2004), does not activate the epithelial isoforms of these FGFR family members. FGFR4 underwent phosphorylation in response to FGF-2, but not to FGL or NCAM-Fc, thus indicating that NCAM-derived ligands selectively induced the activation of FGFR1. The stimulation of HeLa cells with FGF-2, FGL, or NCAM-Fc led to the activation of FRS-2α and PLC-γ, two classical FGFR substrates (Eswarakumar et al., 2005), which is an effect abolished by PD173074 (Fig. 1 B). FGF-2 induced the phosphorylation of Shc adaptor proteins, whereas no effect was observed with either NCAM-Fc or FGL (Fig. 1 B). Non-RTKs of the Src family have been implicated in NCAM signaling (Williams et al., 1994; Kiryushko et al., 2006), and, indeed, the treatment of HeLa cells with NCAM-Fc or FGL but not with FGF-2 induced FGFR-dependent Src activation (Fig. 1 B). However, FGF-2 did induce Src phosphorylation in NIH-3T3 cells (unpublished data), thus pointing to cell type–specific activities of this growth factor (Dailey et al., 2005). Finally, the activation of Erk1/2 was induced by FGF-2, NCAM-Fc, and...
NCAM induces sustained activation of FGFR effectors

We investigated the kinetics of FGFR signaling upon stimulation of HeLa cells with FGF-2, NCAM-Fc, or FGL for different time periods. As shown in Fig. 2 (left), FGF-2 induced a transient activation of FRS-2α that declined after 30 min. Weak phosphorylation of Akt, another classical FGFR effector (Eswarakumar et al., 2005), was observed at 5 min but became undetectable at later time points. Immunoblotting for phospho-Src showed a very weak band after 5 min of FGF-2 stimulation, which was no longer detectable after longer treatments, confirming and extending the data shown in Fig. 1 A. A prolonged phosphorylation was instead observed for Shc and Erk1/2 (Fig. 2, left). In contrast to FGF-2, both FGL (Fig. 2, middle) and NCAM-Fc (Fig. 2, right) induced sustained phosphorylation of FRS-2α, Akt, and Src, whereas no phosphorylation of Shc was detected. Instead, Erk1/2 activation occurred in a transient manner, declining after 1 h of treatment (Fig. 2, middle and right). Therefore, the interaction of NCAM or FGF with FGFR induced the activation of signaling cascades with remarkably different kinetics, with NCAM stimulating the sustained activation of the FGFR effectors FRS-2α, Src, and Akt and transient activation of Erk1/2.

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readily detected the Cbl–FRS-2α complex in FGF-2–stimulated HeLa cells. Instead, cell treatment with FGL did not induce any association between Cbl and FRS-2α (Fig. 4 B). Thus, unlike FGF-2, NCAM stimulation of FGFR1 does not result in Cbl-mediated receptor ubiquitination, likely accounting for the stabilization of the receptor itself.

A clear indication of the cellular fate of stabilized FGFR1 came from the immunofluorescence analysis of HA-FGFR1–transfected HeLa cells (Fig. 3 A). At 2 and 4 h, neither surface nor cytosolic HA-FGFR1 was detected in FGF-2–treated cells, which is consistent with FGF-induced degradation of FGFR. In contrast, massive recycling of HA-FGFR1 to the cell surface occurred in cells stimulated with FGL or NCAM-Fc (Fig. 3 A, left). The quantification of the immunofluorescence results confirmed that both NCAM and FGF-2 promote the internalization of FGFR1 (Fig. S3 B, middle), but receptor recycling is only observed upon NCAM stimulation (Fig. S3 B, bottom). The dramatic decrease in overall HA-FGFR1 signal in FGF-2–treated cells (Fig. S3 B, top) is consistent with receptor degradation. We also used a modified version of the biochemical method used for the internalization assay (see Materials and methods) to investigate the fate of endogenous FGFR1 after cell stimulation. This technique confirmed that NCAM stimulation results in FGFR1 stabilization and recycling to the cell surface as opposed to FGF-induced degradation of the receptor (Fig. 3 C). The apparent delay in FGFR1 degradation observed in immunofluorescence-based as compared with biochemical assays is likely caused by the antibody prebound to FGFR1 (see Materials and methods). Indeed, when we performed the biochemical assay in the presence of the antibody, both FGF-induced degradation and FGL-dependent recycling of FGFR1 were delayed (Fig. S3 C). The differential fate of internalized FGFR1 upon stimulation with FGF versus NCAM was further confirmed by...
recycling (Jones et al., 2006). In cells stimulated with FGL or NCAM-Fc, HA-FGFR1 showed extensive colocalization with Rab11, whereas no costaining was observed in FGF-2–treated cells (Fig. 5 D).

Collectively, these results indicate that FGFR1 enters early endosomes upon both FGF-2 and NCAM stimulation but is then sorted to divergent routes. In FGF-2–stimulated cells, FGFR1 is targeted for lysosomal degradation, whereas NCAM promotes the stabilization of FGFR1 followed by its recycling to the cell surface via Rab11-positive vesicles.

As a possible mechanism accounting for FGFR1 recycling in NCAM-stimulated cells, we focused on Src activity based on the considerations that (a) sustained Src activation was an NCAM-specific effect (Figs. 1 and 2) and that (b) Src has been implicated in the trafficking of FGFR1 (Sandilands et al., 2007). Thus, HA-FGFR1–transfected HeLa cells were pretreated with either PP1 or SU6656 before monitor receptor trafficking in FGF-2– or NCAM-stimulated cells. Receptor recycling was
no longer observed in NCAM-stimulated cells that were preincubated with Src inhibitors (Fig. 6 A), and the lack of either cell surface or cytosolic staining for HA-FGFR1 pointed to receptor degradation. Because these findings implicate Src in NCAM-dependent stabilization of FGFR1, we determined the effect of inhibiting Src on the recruitment of Cbl to FRS-2α upon FGFR stimulation with FGF-2 versus FGL. Intriguingly, in PP1- or SU6656-treated cells, Cbl formed a complex with FRS-2α even upon FGL stimulation (Fig. 6 B). This resulted in FGL promoting FGFR1 ubiquitination to a level comparable with FGF-2 (Fig. S4 A). Thus, the stabilization and recycling of FGFR observed upon NCAM stimulation implicates an active role of Src in preventing the association of Cbl with FRS-2α.

**NCAM stimulates cell migration, which requires FGFR1 recycling**

The dichotomy in FGFR signaling and trafficking induced by NCAM versus FGF raised the possibility that the two ligands elicit different FGFR-mediated cellular responses. To verify this hypothesis, we focused on cell migration and proliferation, two processes linked with FGFR function (Boilly et al., 2000). Monolayer-wounding assays combined with time-lapse video microscopy revealed that both FGL and NCAM-Fc promoted the migration of HeLa cells, whereas FGF-2 did not (Fig. 7, A and B; and Videos 1–4). Both the covered distance and the speed of migrating cells were enhanced by NCAM-derived ligands (Fig. 7, A and B). Similar results were obtained with a 3D assay for cell migration based on modified Boyden chambers, with FGL and NCAM-Fc inducing the migration of HeLa (Fig. 7 C) and L cells (Fig. S4 B and not depicted), whereas FGF-2 failed to do so. In contrast, ΔFN2-Fc did not promote cell migration (Fig. 7 C), supporting the key role of NCAM interaction with FGFR. Along the same line, NCAM-induced cell migration was abolished by either a pretreatment of cells with PD173074 (Fig. 7 C) or by the transfection with dn-FGFR1 (Fig. 7 D). The compound AG1478, a chemical inhibitor of EGF receptor (EGFR), showed no effect on FGL-induced cell migration, whereas it blocked the migration of EGF-stimulated cells (Fig. S4 D), thus supporting the specificity of the NCAM interaction with FGFR. Moreover, PP1, SU6656, dn-Src, and PD98059 repressed NCAM-induced cell migration (Fig. 7 C and Fig. S4 E), which indicated the requirement for both Src and Erk1/2 activity.

In agreement with our previous results (Francavilla et al., 2007), FGF-2 exerted a strong proliferative effect on both HeLa and L cells. In contrast, FGL had no impact on cell proliferation (Fig. 7 F and Fig. S4 C), confirming previous observations with NCAM-Fc (Francavilla et al., 2007). Thus, NCAM and FGF elicit distinct, FGFR-mediated cellular responses in both epithelial cells and fibroblasts, with NCAM promoting cell migration and FGF inducing cell proliferation.

Based on the promigratory activity of NCAM and on the stabilization (and thus recycling) of FGFR1, we verified whether NCAM-induced migration requires its ability to promote FGFR1 recycling. To this goal, HeLa cells were transfected with dn-Rab11, which blocks the recycling pathway (Ren et al., 1998). Indeed, HA-FGFR1 recycling was no longer observed in NCAM-stimulated HeLa cells transfected with dn-Rab11, whereas it was not affected in cells expressing wild-type Rab11 (Fig. 8 A). In agreement with this, dn-Rab11 caused the retention of internalized HA-FGFR1 in early endosomes (Fig. S5 A). To confirm the role of Rab11 in FGFR1 recycling, the expression of the three members of the Rab11 subfamily, namely Rab11a, Rab11b, and Rab25 (Prekeris, 2003), was ablated by using the RNAi technology. Similar to dn-Rab11, the knockdown of Rab11 genes resulted in the block of HA-FGFR1 recycling in FGL-stimulated cells (Fig. 8 B). Thus, we used both the ectopic expression of dn-Rab11 and the knockdown of endogenous Rab11 genes to investigate the contribution of Rab11 GTPases to NCAM–FGFR-dependent cell migration. Although dn-Rab11 abrogated the migratory response of HeLa cells to FGL (Fig. 8 C, left), it failed to inhibit cell migration in response to EGF, which is consistent with previous results (Palmieri et al., 2006). Analogous results were obtained when Rab11 expression was reduced by siRNA (Fig. 8 C, right). Thus, the recycling of FGFR1 via Rab11-dependent pathway is a specific prerequisite for NCAM-stimulated cell migration. In addition, the inactivation of endogenous Rab11 with either dn-Rab11 or siRNA-mediated knockdown caused the loss of sustained Src activation in response to NCAM stimulation, whereas no effect was observed on FGF-2–induced activation of Erk1/2.
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transfected with wild-type Cbl (Fig. 9 B). Notably, the expres-
sion of dn-Cbl enabled FGF-2 to induce HeLa cell migration,
although not to the same extent as FGL (Fig. 9 C). Furthermore,
under these conditions, FGF-2 stimulated sustained Src activa-
tion to a similar level as FGL (Fig. S5 E). Thus, the inhibition of
Cbl-dependent ubiquitination and degradation of FGFR1 was
sufficient to switch the functional properties of FGF-2, enabling
it to mimic NCAM-induced cell migration and sustained signal-
ing. This implies that the biological activity of FGFR1 is strictly
dependent on its stability and trafficking. However, although
interfering with FGFR1 degradation resulted in a migratory
response to FGF-2, the blockade of FGFR1 recycling with
dn-Rab11 (Fig. S5 F) did not confer proliferative function to
NCAM (Fig. 9 D). This suggests that additional events are re-
quired to trigger the FGFR1-mediated signaling cascade that
underlies the proliferative response.

Discussion

The functional cross talk between NCAM and FGFR signaling
in neurons has long been described (Williams et al., 1994).
However, the molecular aspects of the interaction between the
two molecules and, more importantly, the cellular response elic-
ted by this interaction in nonneural cells, have remained elu-
sive. This study shows that NCAM acts as a noncanonical ligand
for FGFR and stimulates an FGFR-mediated cellular response
that is remarkably distinct from that elicited by FGF. In particu-
lar, NCAM promotes sustained FGFR signaling and cell migra-
tion, which are two processes that require NCAM-dependent
recycling of endocytic FGFR1 to the cell surface (whereas the
receptor is degraded upon FGF stimulation). The dichotomy

(Fig. 8, D and E). To further validate and extend these findings,
FGFR1 recycling was inhibited by two additional approaches,
namely cell pretreatment with monensin (Mitchell et al., 2004)
and a temperature shift to 16°C (Ren et al., 1998). Both strate-
gies showed efficient repression of NCAM-dependent recycling
of HA-FGFR1 (Fig. S5, B and C), and in both cases, FGL was
no longer able to stimulate cell migration, whereas recycling in-
hibition had no effect on EGF-induced migration (Fig. 8, F and G).
Furthermore, blocking FGFR1 recycling at 16°C resulted in the
loss of NCAM-stimulated activation of Src (Fig. S5 D). This
was not caused by temperature-dependent inactivation of
FGFR1, as under the same conditions, FGF-2 retained the abil-
ity to induce sustained Erk1/2 phosphorylation (Fig. S5 D).
Thus, NCAM-induced, FGFR1-mediated signal transduction
underlying cell migration relies on the recycling of the receptor.
Collectively, these results support the notion that NCAM pro-
motes cell migration by favoring a sustained and efficient recy-
cling of internalized FGFR1 to the cell surface.

If the different cellular response to NCAM and FGF de-
pends on the different stability and intracellular fate of FGFR1
imposed by the two ligands, one should be able to switch FGF
into an NCAM-like stimulus by modulating FGFR1 stability.
To verify this hypothesis, HeLa cells expressing a dn version
of Cbl (Penengo et al., 2006) or wild-type Cbl as a control were
stimulated with either FGF-2 or FGL. The inhibitory effect of
dn-Cbl was confirmed by the fact that FGFR1 level was no
longer reduced after a 120-min treatment with FGF-2 (Fig. 9 A).
The dn-Cbl–dependent stabilization of FGFR1 resulted in the
recycling of the receptor to the cell surface even after FGF-2
stimulation (Fig. 9 B), which is in contrast to the receptor deg-
radation observed in parental HeLa cells (Fig. 3) or in cells

![Figure 5.](image-url)
with FGF-induced response did not depend on the ligand concentration used in the experiments (unpublished data).

To perform a direct comparison with the extracellular, soluble factor FGF-2, most of the experiments described in this study were conducted using either the FGL peptide or NCAM-Fc, namely soluble versions of NCAM-derived ligands. Despite NCAM acting mainly as a cell surface molecule, there is evidence indicating that FGL and NCAM-Fc recapitulate physiological activities of membrane-associated NCAM, thus supporting the biological relevance of our observations. First, soluble NCAM fragments mimicked cell surface NCAM in inducing FGFR-dependent matrix adhesion in different cell types (Cavallaro et al., 2001; this study). Second, in line with our findings on NCAM-Fc and FGL as promigratory factors, membrane NCAM induces cell migration in neurons (Maness and Schachner, 2007) and during epithelial–mesenchymal transition (Lehembre et al., 2008). Finally, as discussed in Results, NCAM’s ectodomain or fragments thereof are also released by certain cell types in vivo as soluble molecules, thus generating potential FGFR ligands analogous to NCAM-Fc and FGL. The oligomeric (FGL) or dimeric (NCAM-Fc) state of NCAM fragments are also likely to mimic physiological conditions, as NCAM is known to oligomerize by means of cis-homophilic interactions (Kiselyov et al., 2005).

The stimulation of FGFR with soluble NCAM underscores the importance of trans-interactions between the two molecules, as it would occur upon contact between NCAM- and FGFR-expressing cells or upon binding of shed extracellular domains of NCAM to cell surface FGFR. Nevertheless, the detection of NCAM–FGFR complexes on the surface of single cells (Cavallaro et al., 2001; Sanchez-Heras et al., 2006; Francavilla et al., 2007) indicates that the two proteins can also...
FGFR1, impacting on ligand-dependent signaling (Suyama et al., 2002; Bryant et al., 2005). Unlike cadherins, the clustering of integrins does stimulate ligand-independent activation of RTKs (including EGFR, VEGFR, and PDGF receptor) as a result of the cis-interactions between integrins and RTKs themselves (Walker et al., 2005). Because NCAM-Fc is expected to act as a dimer (as a result of spontaneous Fc dimerization) and the FGL peptide was used in its dendrimeric form (Kiselyov et al., 2003), both molecules could induce FGFR1 activation by clustering, which is similar to cis-interacting integrins.

The molecular basis of the divergence between NCAM- and FGF-induced FGFR signaling remains elusive and could entail different mechanisms. For example, N- and E-cadherin regulate FGF-induced endocytosis of FGFR1, impacting on ligand-dependent signaling (Suyama et al., 2002; Bryant et al., 2005). Unlike cadherins, the clustering of integrins does stimulate ligand-independent activation of RTKs (including EGFR, VEGFR, and PDGF receptor) as a result of the cis-interactions between integrins and RTKs themselves (Walker et al., 2005). Because NCAM-Fc is expected to act as a dimer (as a result of spontaneous Fc dimerization) and the FGL peptide was used in its dendrimeric form (Kiselyov et al., 2003), both molecules could induce FGFR1 activation by clustering, which is similar to cis-interacting integrins.

The molecular basis of the divergence between NCAM- and FGF-induced FGFR signaling remains elusive and could entail different mechanisms. For example, the two molecules could promote the autophosphorylation of different FGFR’s tyrosine residues and, therefore, the activation of different docking sites for the specific effectors. Also, it remains to be clarified whether NCAM induces the recruitment of the receptor to

**Figure 7.** NCAM induces cell migration via FGFR1, Src, and Erk1/2, whereas FGF promotes cell proliferation. (A) Monolayers of HeLa cells were scratch wounded as described in Materials and methods and left untreated (control) or stimulated with FGF-2, FGL, or NCAM-Fc. Time-lapse microscopy was performed as described in Materials and methods. Images were taken from Videos 1–4. Colored lines show five representative tracks of single cells. (B) Mean distance covered (top) and velocity (bottom) of cells stimulated with the different ligands. Data represent the mean ± SEM from 45 individually tracked cells over the 24-h period (see Materials and methods). Values are expressed in micrometers. Bar, 30 µm.

Bar, 30 µm. (B) Mean distance covered (top) and velocity (bottom) of cells stimulated with the different ligands. Data represent the mean ± SEM from 45 individually tracked cells from three independent experiments. (C) HeLa cells stimulated with FGF-2, FGL, NCAM-Fc, ΔFN2-Fc, or Fc were subjected to migration assays in modified Boyden chambers (see Materials and methods). FGL stimulation was also performed in the presence of PD173074, PP1, or PD98059. (D) HeLa cells transfected with an empty vector (mock) or with Myc-tagged dn-FGFR1 (transfection efficiency was nearly 100%; not depicted) were stimulated with either FGF-2 or FGL and subjected to migration assay as for C. (E) HeLa cells were subjected to cell proliferation assay in the presence of FGF-2 or FGL as described in Materials and methods. (C–E) Data represent the mean ± SEM from at least three independent experiments. *, P < 0.005 relative to untreated cells.
Figure 8. Inhibition of FGFR1 recycling represses NCAM-induced cell migration. (A) HeLa cells cotransfected with HA-FGFR1 (red) and either Rab11-GFP or dn-Rab11–GFP (green) were processed as for Fig. 3 A. Arrowheads indicate the colocalization of Rab11-GFP with HA-FGFR1 (yellow staining), whereas arrows indicate the lack of colocalization of HA-FGFR1 with dn-Rab11–GFP. Asterisk indicates a Rab11-GFP–expressing cell with recycled HA-FGFR1, whereas # shows a dn-Rab11–GFP-expressing cell with no recycling of HA-FGFR1. (B) HeLa cells were transfected with control siRNA (top) or with a mixture of siRNA targeting the Rab11 family (bottom) before transfection with HA-FGFR1 (green) and processing as for Fig. 3 A. Arrows indicate cells transfected with anti-Rab11 siRNA with no recycling of HA-FGFR1 upon FGL stimulation, whereas the asterisk shows a control cell where HA-FGFR1 has recycled to the cell surface. Bars, 10 µm. (C, left) HeLa cells transfected with either Rab11-GFP or dn-Rab11–GFP were stimulated with FGF-2, FGL, or EGF, and the migration of GFP-positive cells in modified Boyden chambers was measured. (right) HeLa cells were transfected with either control or anti-Rab11 siRNA and stimulated with FGF-2 and FGL, and subjected to migration assay as described for the left panel. *, P < 0.005 relative to cells transfected with either Rab11-GFP (left) or control siRNA (right) and stimulated with FGL. (D) HeLa cells transfected with either Rab11-GFP or dn-Rab11–GFP were stimulated with FGL (top) or FGF-2 (bottom) for the indicated time lengths. Lysates from FGL-stimulated cells were immunoblotted for phospho-Src and total Src, whereas lysates from
specific membrane compartments where the repertoire of adaptors/effectors would be different from that normally affected by FGF stimulation.

The dichotomy in FGFR signaling between NCAM and FGF is best exemplified by the pathway of Erk1/2 activation (Ras-dependent for FGF and Ras-independent and Src-dependent for NCAM) and by the differential involvement of Shc and Src downstream of FGFR (specifically activated by FGF and NCAM, respectively). Src-mediated activation of Erk1/2 can result from integrin signaling through focal adhesion kinase (Mitra and Schlaepfer, 2006). Because NCAM–FGFR signaling stimulates β1-integrin function in pancreatic β-tumor cells (Cavallaro et al., 2001), NCAM-induced activation of FGFR could trigger an integrin–FAK–Src–Erk1/2 pathway. This model would be supported by the observation that, similar to NCAM, integrin-mediated activation of Erk1/2 is independent of Ras (Chen et al., 1996), although this issue is still controversial (Clark and Hynes, 1996).

The divergence between NCAM and FGF signaling is accompanied by a dramatic difference in the intracellular trafficking of FGFR1, with FGF inducing the classical route of rapid internalization and lysosomal degradation, whereas NCAM promotes FGFR1 stabilization and recycling to the cell surface. We have provided evidence that NCAM stimulation uncouples receptor internalization from ubiquitination, most likely because NCAM does not induce the recruitment of Cbl to FRS-2α, which is required for FGFR1 ubiquitination and degradation upon FGF stimulation (Wong et al., 2002). Recent studies showed that preventing ubiquitination targets FGFR1 to recycling endosomes instead of lysosomes (Haugsten et al., 2008), further supporting the view that this is the mechanism underlying NCAM-dependent recycling of FGFR1. The sharp dichotomy in the intracellular fate of FGFR1 after FGF versus NCAM stimulation is likely determined by Src activity. Indeed, Src prevents the association of Cbl with FRS-2α and, thus, the ubiquitination of FGFR1, thus accounting for the lack of receptor degradation and for its recycling to the cell surface. However, our unpublished results ruled out the possibility that Src induces the phosphorylation of Cbl in NCAM-stimulated cells, thus hindering its recruitment to FRS-2α (unpublished data). It is conceivable that NCAM stimulation promotes Src-dependent phosphorylation of one or more substrates that in turn interfere with the formation of the Cbl–FRS-2α complex, which is a hypothesis that deserves further investigation. In parallel with Src-dependent FGFR recycling, we also observed that the sustained activation of Src induced by NCAM requires FGFR recycling itself. This points to a mutual regulation between the two events as a key step in the cellular response elicited by the NCAM–FGFR interplay.

Our study revealed the tight connection between recycling of FGFR1 to the plasma membrane and NCAM-induced cell migration. Interestingly, transmembrane NCAM itself undergoes endocytosis and recycling in neurons (Diestel et al., 2007). Although the biological significance of these processes remains elusive, it is tempting to speculate that NCAM could act as a carrier for other proteins and in particular for FGFR, thus favoring their recycling to the cell surface. Despite the copious evidence that endocytosis is used by cells to propagate RTK-mediated signaling from endosomal compartments (Hoeller et al., 2005), very few studies have focused on RTK recycling (Marmor and Yarden, 2004), and little information is available on its biological significance. Recycling of EGFR and PDGF/VEGFR is critical for the spatial redistribution of RTK signaling during the directional migration of border cells in Drosophila melanogaster (Jékely et al., 2005). The role of recycling in ensuring localized signaling during cell migration has also been reported for the small GTPase Rac (Palamidessi et al., 2008) and for integrins (for review see Caswell and Norman, 2008). Future research should clarify whether FGFR1 recycling in NCAM-stimulated migratory cells is necessary to restrict receptor localization to specific membrane compartments, or if it rather represents a mechanism to avoid degradation and ensure the sustained signaling required to maintain a motile phenotype. In this context, it appears that the duration of NCAM-dependent FGFR signaling is critical to confer a migratory phenotype to cells. This was further confirmed by our observation that preventing FGFR1 ubiquitination and degradation, thus promoting its recycling, was sufficient to confer promigratory activity to FGF-2. Thus, the cellular response to FGF-2 is dictated by the stability of FGFR1, which in turn affects the duration of downstream signaling. In agreement with this view, forcing the sustained activation of FGFR2 induces epithelial to mesenchymal transition and cellular invasion (Xian et al., 2007).

Our results on the NCAM–FGFR1 interplay that induces recycling-dependent cell migration have broad physiopathological implications. For example, NCAM-dependent sustained activation and recycling of FGFR are very likely to underlie axonal growth, a process that requires an intact FGFR signaling downstream of NCAM both in vitro and in vivo (Saffell et al., 1997). NCAM knockout mice exhibit various developmental and behavioral defects, including the impaired migration of neuronal precursors to the olfactory bulb (Cremer et al., 1994). Our findings imply that these defects might depend on the disruption of promigratory FGFR signaling upon loss of NCAM. In this context, NCAM-deficient mice exhibit a depression-like phenotype that is reverted by the treatment with FGL (Aonurm-Helm et al., 2008). These findings support the physiological relevance of the NCAM–FGFR interaction for brain development.
acting as an unconventional ligand, stimulates a signaling cascade remarkably distinct from that induced by FGF. Furthermore, our experiments revealed that NCAM induces sustained FGFR activation by uncoupling receptor internalization from ubiquitination and promoting Rab11-dependent recycling of the receptor, and this results in cell migration. Besides uncovering a further level of complexity in the regulation of RTK activity, our data could contribute to elucidate the pathogenesis of those disorders characterized by dysregulated function of NCAM and/or FGFR.

Materials and methods

Reagents

The following commercial reagents were used: FGF-2 (PeproTech), EGF (Inalco), the MEK inhibitor PD98059, the inhibitor of transport to plasma membrane monensin, the protein synthesis inhibitor cycloheximide...
The following antibodies were used: rabbit anti-phospho-Akt and anti-Akt; mouse anti-phospho-Erk1/2, rabbit anti-phospho-FRS2-α, rabbit anti-phospho-Src (recognizing the activated form of most Src kinases); rabbit anti-phospho–PLCγ, anti-phospho-Shc, and anti-Shc (Cell Signaling Technology, Boston, MA); mouse anti-Rab25, anti-Rab11, and anti-Rab25; goat anti-EEA1 (Santa Cruz Biotechnology, Inc.); mouse anti-phospho-ERK1/2 and mouse anti-aubiquitin and antivinculin (Sigma-Aldrich); mouse anti-HA (clone F7; used in immunoprecipitation and Western blot analysis); rabbit anti-FGR1, anti-FGR2, anti-FGR3, anti-FGR4, rabbit anti-FRS2-α, mouse anti-NCAM (clone 123C3), anti-Src kinases, anti-GFP, anti-aubiquitin, and anti-Rab25; goat anti-EAA1 (Santa Cruz Biotechnology, Inc.); mouse anti-phospho-ERK1/2, anti-Cbl, and anti-phosphotyrosine (BD); rabbit anti–PLCγ, anti-Cbl, and anti-phosphotyrosine. The mouse anti-HA tag (used in immunofluorescence; HA.11; Covance); and rabbit anti-Rab11, cross-reacting with both Rab11a and Rab11b (Invtrogen). Rabbit anti–LAMP-2 was provided by G. Griffiths (Cambridge Institute for Medical Research, Cambridge, England, UK).

The Myc-tagged scFv against FGFR1 was isolated and used in immunoblotting as described previously (Francavilla et al., 2007). Peroxidase-conjugated streptavidin was obtained from Jackson ImmunoResearch Laboratories, and TRITC-conjugated Tf (TRITC-Tf) was obtained from Invitrogen. The FGL peptide from the second FNII module of NCAM and its mutated version, FGLmut, which carries two alanine substitutions that abolish its binding to FGFR (Kiselyov et al., 2003), were provided by ENKAM Pharmaceuticals. Peptides were synthesized as dendrimers, with four peptides attached to a three-lysine backbone (Kiselyov et al., 2003).

Expression vectors
The pl3 vectors containing the cDNA for the ectodomain of human NCAM, either full-length or deleted of the second FNIII repeat (ΔFN2), fused to the Cc fragment of human IgG (NCAM-Fc and ΔFN2-Fc, respectively) were provided by L. Needham (Duke University, Durham, NC). The cDNA for full-length, transmembrane NCAM-140 was subcloned into a pRSet vector encoding N-terminally HA-tagged FGFR1 (Zhang et al., 2001) was provided by G. David (White et al., 2007; Palamidessi et al., 2008) with slight modifications. In brief, cells were plated at 80% confluence on 100-mm-diameter dishes and incubated on ice for 60 min in the presence of 0.5 mg/ml thiol-cleavable sulfo-NHS-S-Sbiotin (Thermo Fisher Scientific). After washing, labeled cells were incubated at 37°C for the indicated time periods in the presence of FGF-2 or FGL to allow internalization. Cells were incubated on ice twice for 20 min with 45 mM GSH (glutathione; EMD), a membrane-nonpermeable reducing agent, to remove the biotin label from surface proteins. Free sulfo-reactive groups were quenched with iodoacetamide (Sigma-Aldrich).

Total labeling was determined in samples not treated with GSH, whereas background values were obtained from samples not subjected to incubation at 37°C.

Immunoprecipitation of FGFR1 from cell extracts was performed as described previously (Cavallaro et al., 2001) using anti–FGFR1 (C15). After SDS-PAGE, immunoprecipitates were probed with HRP-conjugated streptavidin (to visualize biotinylated FGFR1) followed by stripping and immunoblotting for total FGFR1. Densitometric analysis was performed with the Image software (National Institutes of Health). Internalization was calculated as a percentage of the total amount of labeled receptor.

FGFR1 recycling and degradation (Fig. 3 C) was determined as described previously (Fabbri et al., 1999). In brief, cells were labeled with sulfo-NHS-S-Sbiotin (as described in the previous paragraph), and internalization was allowed for 30 min at 37°C in the presence of the stimuli. Cells were treated with GSH (as described in the previous paragraph) to remove the label from the residual cell surface receptor. Recycled fraction was chased by reincubation at 37°C for the indicated time points in duplicate samples. One sample (Fig. 3 C, +GSH) was treated with GSH to determine the amount of FGFR1 that recycled back to the plasma membrane, whereas the other sample (−GSH) was left untreated to determine the total level of labeled receptor at each time point. The samples were subjected to FGFR1 immunoprecipitation and immunoblotting as described in the previous paragraph. In −GSH samples, HRP-conjugated streptavidin recognized residual biotinylated FGFR1 after incubation at 37°C without GSH treatment (i.e., internalized + recycled − degraded). In +GSH samples, HRP-conjugated streptavidin recognized residual biotinylated FGFR1 after incubation at 37°C and GSH treatment (i.e., internalized − recycled − degraded). FGFR1 degradation was calculated by subtracting the densitometric value of residual biotinylated receptor in −GSH samples from the total pool of internalized receptor. FGFR1 recycling was calculated by subtracting both the degradation value and the value of residual biotinylated receptor in +GSH samples from the total pool of internalized receptor (Iampugnani et al., 2006). Values represent the means ± SD from at least three independent experiments.

Cell proliferation assays
Cells were seeded in triplicate on 24-well plates at 8 × 10^3 cells/well, serum starved overnight, and treated for 1–4 d with FGF-2 or FGL replenished every 24 h. At each time point, viable cells were counted using the Trypan blue exclusion method, and the ratio with nonstimulated cells at time 0 was determined for each time point. Values represent the means ± SEM from at least three independent experiments performed in triplicate.

Cell migration
Time-lapse video microscopy was performed as described previously (White et al., 2007; Palamidessi et al., 2008) with slight modifications. In brief, confluent monolayer cultures of HeLa cells were wounded with a plastic pipette tip to induce migration into the wound. Cells were incubated in serum-free, Hepes-buffered L15 medium containing the different stimuli and placed on the stage of an inverted motorized microscope (IX81: Olympus) in a cage incubator (Okolab) at 37°C. Phase-contrast images were collected with a 10x NA 0.3 Plan lens (FLN; Olympus) every 15 min over a 24-h period using a camera (Orcsa-AG; Hamamatsu Photonics) and the cell*R software (Olympus). Videos were generated using the

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