Journal of Cellular Biochemistry 104:879–894 (2008)

Cell Migration by a FRS2-Adaptor Dependent Membrane Relocation of Ret Receptors

T. Kalle Lundgren,[1] Anna Stenqvist,[1] Rizaldy P. Scott,[2] Tony Pawson,[2] and Patrik Ernfors[1]*

[1]Unit of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, 171 77 Stockholm, Sweden
[2]Samuel Lunenfeld Research Institute, Mount Sinai Hospital 600 University Avenue, Toronto, Canada ON M5G 1X5

Abstract During development neural progenitor cells migrate with extraordinary precision to inhabit tissues and organs far from their initial position. Little is known about the cellular basis for directional guidance by tyrosine kinase receptors (RTKs). RET is a RTK with important functions in guiding the migration of neuronal cells, and RET dysregulation leads to clinical disease such as agangliosis of the colon. We show here that RET migration in neuroepitheliomal and non-neuronal cells is elicited by the activation of specific signaling pathways initiated by the competitive recruitment of the FRS2 adaptor molecule to tyrosine 1062 (Y1062) in RET. FRS2 selectively recruited RET to focal complexes and led to activation of SRC family kinases and focal adhesion kinase (FAK). Activation of SRC depended on its direct interaction with RET at a different intracellular tyrosine (Y981) and activation of molecular signaling from these two separate sites in concert regulated migration. Our data suggest that an important function for FRS2 is to concentrate RET in membrane foci, leading to an engagement of specific signaling complexes localized in these membrane domains. J. Cell. Biochem. 104: 879–894, 2008. © 2008 Wiley-Liss, Inc.

Key words: migration; ret; PTB-adaptors; FRS2; SRC; chemotaxis; GDNF

In multicellular organisms morphogenesis and completion of the body structure require precise positioning of specific types of cells, often at distances far from where the cells first emerged. To this end, cellular migration is a fundamental process both during development but also in regeneration and repair of organs, and in disease as exemplified by metastasis and invasion from tumors. To guide movement, extracellular gradients of molecules promote asymmetrically confined signaling inside the cell in order for it to migrate directionally towards a target [Ridley et al., 2003]. How the external environment is processed by the cell and translated into focal signals and precise directionality of movement is incompletely understood, in particular for tyrosine kinase receptors [RTKs; Ridley et al., 2003; Ayala et al., 2007].

The receptor tyrosine kinase rearranged during transformation (RET) has numerous functions during development and in disease [Arighi et al., 2005]. It has been postulated that the diversity in RET mediated functional responses at the cellular level is created at least in part by its competitive interaction with PTB adaptor molecules that activate selective signaling pathways [Lundgren et al., 2006]. Cells expressing RET, similar to many other RTKs that induce chemoattraction, use graded concentrations of ligand molecules as guidance cues for directional migration [Paratcha et al., 2006]. RET is instrumental in migration and organogenesis of the developing kidney where signaling between RET expressing cells in the tip of the ureteric bud and RET ligands Glial cell line-derived neurotrophic factor (GDNF)

This article contains supplementary material, which may be viewed at the Journal of Cellular Biochemistry website at http://www.interscience.wiley.com/jpages/0730-2312/suppmat/index.html.

Grant sponsor: Swedish Cancer Society; Grant sponsor: Swedish Medical Research Council; Grant sponsor: Swedish foundation for Strategic Research (CEDB/DBRM Grant); Grant sponsor: Karolinska Institute MD-PhD program.

*Correspondence to: Patrik Ernfors, Unit of Molecular Neurobiology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, 171 77 Stockholm, Sweden. E-mail: patrik.ernfors@ki.se

Received 3 September 2007; Accepted 26 November 2007
DOI 10.1002/jcb.21671

and the co-ligand GDNF family receptor alpha 1 (GFRα1) released from the surrounding metanephric mesenchyme functions to correctly organize the tubular structures of the developing kidney [reviewed in Shakya et al., 2005]. RET is likewise necessary for establishment of the enteric nervous system as neurons originating from the neural crest migrate great distances to populate the intestinal nervous plexa [Newgreen and Young, 2002]. Down-regulation of RET signaling during development may clinically manifest as CAKUT syndromes of the kidney [Jain et al., 2006] or as agangliosis of the colon (Hirschsprung disease) [Newgreen and Young, 2002] whereas over-activation of the receptor leads to oncogenic syndromes, including Multiple Endocrine Neoplasia type 2a and 2b (MEN2a/2b) [Murakumo et al., 2006]. Recently, regional loss of RET was shown to cause progressive loss of substantia nigra pars compacta neurons in adult mice [Kramer et al., 2007] offering hope to Parkinson disease patients for which GDNF is currently under clinical trials as a drug to mitigate symptoms [Barker, 2006].

In order for any cell to utilize external cues for directional migration a polarization of the cell must occur by rearrangement of the cytoskeleton. During migration, actin containing protrusions at the leading edge are formed [Ponti et al., 2004] that adhere to the substratum via focal adhesions (FAs) which link the substratum to intracellular motility regulatory molecules and the cytoskeleton [Wozniak et al., 2004]. The turnover of FAs and cytoskeletal rearrangement is correlated with the attachment and detachment to the substratum that is necessary for movement of the cell body [Gupton and Waterman-Storer, 2006; Ayala et al., 2007].

The SRC family of cytoplasmic tyrosine kinases (hereafter referred to as SRC) and focal adhesion kinase (FAK) are both implicated in growth factor driven migration [Chang et al., 1995; Sieg et al., 2000]. SRC interacts with membrane bound receptors, integrins and with the actin cytoskeleton as well as with FAK, providing a framework for the signaling events leading to reorganization of actin filaments and FA turnover [reviewed in Thomas and Brugge, 1997]. The activity of SRC and FAK in FA turnover requires that SRC and FAK are localized to focal areas where these two proteins assemble together into signaling complexes along with clusters of integrins, to create actin containing cell protrusions [Cox et al., 2006 and references therein]. SRC and FAK are cytosolic proteins but when active in cytoskeletal remodeling they are distributed close to the cytofacial (subplasma membrane) side of the cell membrane. Notably, FAK associates via its N-terminal end with the EGF receptor and the platelet derived growth factor (PDGF) receptor and bridges the receptors to integrins [Sieg et al., 2000]. SRC family proteins, by means of amino terminal fatty acid acylations, are located in lipid rafts [Sefton et al., 1982; Koegl et al., 1994; Liang et al., 2001] which are integral membrane microdomains (<200 nm) highly enriched in sterols and sphingolipids and constitute platforms for concerted signaling complexes [Jacobson et al., 2007]. The notion of such microdomains is recent and little is known about how inhabitant molecules are brought into or out from rafts. RET can be recruited to, and signal from within rafts, but the mechanism of this RET translocation is unclear [Tansey et al., 2000; Paratcha et al., 2001; Pierchala et al., 2006, 2007].

We have recently shown that RET receptor signaling can be rewired with regards to the binding of PTB domains of adaptor proteins (PTB adaptors) to the intracellular tyrosine 1062 (Y1062) in RET [Lundgren et al., 2006]. This tyrosine is the major executor of RET mediated effects and mice carrying a mutation that replaces Y1062 with a phenylalanine (Y1062F) closely resemble the phenotype of mice with null *ret* alleles [Jijiwa et al., 2004; Wong et al., 2005]. By specifically mutating the PTB adaptor binding domain it is possible to create functional RET receptors in which either the Src homology 2 domain containing (SHC) or the Fibroblast growth factor receptor substrate 2 (FRS2) PTB adaptors exclusively interact with tyrosine 1062 (hereafter referred to as RET^{SHC+} and RET^{FRS+}, respectively) [Lundgren et al., 2006]. These adaptor selective mutants of RET may be used to study adaptor specific functions as compared to wild type RET (RET^{WT}) to which adaptors compete for binding to Y1062 or to RET^{Y1062F} mutants that are incapable of binding the adaptors. PTB adaptors are a family of scaffolding molecules that typically bind to short consensus sequences (NXXY) on receptors [Pawson and Scott, 1997; Smith et al., 2006] and contain motifs that bind and activate further downstream elements that propagate intracellular signaling. Several PTB

adaptor molecules can potentially interact with a particular RTK, but if the receptor only has a single motif, as in the case of RET, only one PTB adaptor can bind at a time. The adaptors may therefore provide a regulatory step in determining which pathways are stimulated by a receptor, which depend on the context in which the receptor is activated [Madhani, 2001; Jain et al., 2006; Lundgren et al., 2006].

In this study we present results that FRS2 binding to RET is necessary for directional migration and suggest that this is due to a relocation of the RET receptor to focal clusters at the cell membrane and activation of specific signaling pathways. Thus, our results suggest a novel function for PTB adaptor proteins to spatially distribute the receptor in the cytoplasmic membrane. Thereby a distinct cooperative signaling complex downstream two different tyrosines of the receptor are generated which elicit RET induced directional migration.

MATERIALS AND METHODS

DNA Constructs and Mutagenesis

Generation of RET vectors has been described [Lundgren et al., 2006]. New mutant constructs were generated by site directed mutagenesis according to Quikchange (Stratagene) protocols. RET^{FRS+} constructs were made through PCR cloning of the FGFR1 juxtamembrane segment (amino acids 410–426) into the RET PJ7Ω construct. RET-eGFP fusion constructs were made by PCR cloning of Ret in-frame with eGFP using Nhe1 and Age1 restriction sites in the eGFP-N1 vectors from the Enhanced FP range of Clontech. SRC constructs were from the Src cDNA allelic kit from Upstate.

Cell Culture and Transfection

SK-N-MC and MDCK cells were maintained in DMEM supplemented with 10% FBS, 2% HBS and 1 mM glutamine. Cos7 and MEF cells [Klinghoffer et al., 1999] were maintained in identical medium but without HBS. Starvations were done in DMEM containing 0.5% total serum. All ligand stimulations were performed using 50 ng/ml recombinant human GDNF and 100 ng/ml recombinant human GFRα1/FC chimera (RnD Systems). Transfections in Cos7 and SK-N-MC for biochemical studies were performed using polyethylenimine at 0.8 μg/ml in PBS (PEI, 25-kDa, Sigma–Aldrich) as described previously (Lundgren et al., 2006). MEF cells and cells used for morphological experiments cells were transfected using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. Growth medium was replaced approx. 7 h after transfection. Transfection efficiency was continuously monitored by eGFP fluorescence.

Antibodies and Reagents

Antibodies against RET9, FAK (H-1), Grb2, Frs2 and phosphotyrosine (PY99) were obtained from Santa Cruz Biotechnology. SRC, phopspho-SRC Tyr 416, phospho-FAK Tyr-576/577, p44/42 MAPK, phospho-p44/42 MAP Kinase, and Actin antibodies were from Cell Signaling. SHC was from Amersham/GE. Paxillin was from Upstate. All phallotoxins were from the Alexa range of Molecular Probes. Cy2-, Cy3- and Cy5-conjugated secondary antibodies were obtained from Jackson laboratories. Cholesterol oxidase (CO; SIGMA) was used as described in [Liu et al., 2000]. Specifically, cultured cells were incubated with CO at 1.8 U/ml for 1 h prior to and during 30 min of ligand stimulation.

Immunoprecipitation, Pulldown Assays, and Protein Immunoblots

Cells were lysed in RIPA buffer and captured with antibodies on protein A/G sepharose beads (GE Healthcare) for immunoprecipitation or directly lysed in Laemmli buffer for phosphoprotein blots. Precipitated proteins were eluted by boiling in Laemmli buffer. Proteins were fractionated on polyacrylamide gels and immobilized on PVDF membranes. Equal RET expression levels were determined by RET Western blot on cell lysates. Western blot detection was carried out by the ECL method (GE Healthcare) according to standard darkroom procedures. Quantifications were done using ImageJ software.

In Vitro Kinase Assay

Assays were performed using the Universal Tyrosine Kinase Assay Kit (TAKARA, Japan.) according to the manufacturer's instructions. Briefly, Cos7 cells were transfected with RET mutants. Twenty-four hour later the cells were ligand stimulated as indicated and harvested in extraction buffer. Endogenous SRC was precipitated with antibodies bound to agarose beads. After washings precipitated material in a volume of 50 μl was added onto immobilized substrate in wells of a 96 well plate.

Ten microlitres of 40 mM ATP solution was added and the kinase reaction was allowed to proceed for 30 min. The wells were thoroughly washed and blocked before incubation with HRP conjugated anti-pY antibodies (PY20, TAKARA, Japan.) for 30 min. Substrate were then added for 12 min before stopping the reaction with 1N H_2SO_4. The absorbance was measured in duplicates in a microplate reader at 450 nm. Controls for linearity of absorbance were the manufacturers supplied PTK control diluted in 10 serials.

Vertical Cell Migration Assays

Cell migration response to GDNF and/or GFRalpha1 was assessed using transwell cell culture inserts (Falcon) or ChemoTX microplates (Neuro Probes) with 12 μm pores. Transfected cells were seeded ($1-5 \times 10^5$ cells) in the chambers and allowed to migrate towards ligands as indicated in figures. For transwell cell culture inserts, quantification was performed under the microscope by counting cells in three visual fields after removal of stationary cells on the upper side of membranes using a cotton-tip or for ChemoTX microplate experiments after de-attaching the cells on the lower side of the membrane into multiwell plates by application of 2 M EDTA to the membrane and subsequent centrifugation. Crystal violet (0.04% (v/w) in 8% ethanol) was applied to cells. Cells were solubilized in 1.5% sodium dodecyl sulfate and absorbance measured at 595 nm in a multiplate reader, as previously described [Cerezo-Guisado et al., 2007]. Experiments with inhibitors were performed as described above using PP2 at 8 or 20 μM, SU6656 at 3 μM and U0126 at 12 μM. Inhibitors were added to starvation media 1 h prior to application of RET ligands.

Wound Healing Assay

Cos7 or MEF cells transfected with RET mutants were seeded on glass slides and in plastic culture dishes respectively and grown to confluence. A tear in the cell layer was made using a 20 μl glass capillary slided along a fastened caliper to ascertain equal width of the tear. Cells were starved for 12 h and then stimulated as indicated with RET ligands. Cells were washed and fixed in paraformaldehyde (PFA) or quantified directly without fixation. Quantification of cells migrating into the wound area was done by counting the number of cells within equal areas for each construct using light microscopy.

siRNA Experiments

Pre-designed anti-FAK siRNA (sc-29310) was purchased from Santa Cruz, resuspended to a stock solution of 10 μM and transfected using Lipofectamine 2000 at a concentration of 60 nM. siRNA against SHC was performed as described previously [Lundgren et al., 2006]. siRNAs against FRS2 were from the pre-designed range of Ambion. Two Sequences 5′-GCAGCACACCAAAAGAAGAtt-3 and 5′-CCAAGUAGUAUUGAGGACAtt-3′ and their corresponding antisense strands were combined and used at a total concentration of 40 nM. Control siRNA [Kim et al., 2005]; 5′-AAGCUGACCCUGAAGUUCAUCUGCACC-3′. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers instruction. The efficacy of the siRNA was examined by Western blotting. Assays were undertaken 48–54 h after siRNA transfection.

Immunocytochemistry

Cells grown on glass coverslips were transfected and the following day after 8 h of starvation stimulated with GDNF and GFRα1. Cells were briefly rinsed with PBS, fixed in 3.8% PFA, permeabilized in 0.2% Triton X-100 in PBS and blocked with 5% goat serum. Fixed and permeabilized cells were then treated with antibodies at 4°C overnight. When applicable, cells were stained with phallotoxins for 30 min at 4°C prior to application of antibodies. After washings in PBS, cells were incubated with fluorescent-conjugated secondary antibodies for 1 h at room temperature washed and mounted in Glycerol–Gelatin (SIGMA).

Imaging/Live Imaging

Cells expressing RET mutants were grown on culture plates with a Poly-D-Lysine coated glass center (MATEK Corp.), starved in media containing 0.2% serum for 10–12 h. During imaging the cells were kept in KREBS buffer at room temperature (RT) or 37°C (intensity quantifications were made at 37°C whereas high resolution imaging was done at 25°C due to focus shifting when heating was applied, no difference in cell behavior between conditions was noted during these short periods of imaging). For live imaging pictures were automatically

taken every 20–30 s for 20 min. Quantification of local fluorescent intensity was done using imageJ analysis software. Cluster index quantification was done by measuring eGFP intensity in 12×12 pixels quadrants. When the intensity in one 12×12 pixel quadrant was measured as 2.5 greater than each equal sized areas immediately nearby in four directions, the cluster index was assigned one point. Clusters larger than 12×12 pixels were still assigned only one point per cluster. Thus, focal staining intensity compared to the immediate surroundings were measured. The number of clusters were counted for 25 cells per condition ($n = 4$) and averaged for each condition. All pictures and movies were taken on Zeiss Axiovert 200 M microscopes with LSM 510 LIVE fastscan assembly and LSM510 software (ZEISS). Movies were exported as Apple QuickTime files.

RESULTS

RET Mediated Cell Motility and Directional Migration Are Dependent Upon FRS2 Adaptor Interaction With Tyrosine 1062

RET is known to mediate directional migration in epithelial cells [Tang et al., 1998] and neural crest cells [Young et al., 2004], as well as autonomic neuroblasts [Enomoto et al., 2001]. RET also produces directional axon outgrowth of neurons towards RET ligands [Tang et al., 1998; Ledda et al., 2002]. To investigate if such cell motility is dependent on the recruitment of specific PTB adaptors to the phosphorylated Y1062 motif in Ret we created mutant RET receptors and expressed these in Cos7 cells. RET^{SHC+} has two amino acid substitutions in the PTB domain binding site (W1056A, E1058D) just N-terminal to Y1062 which leads to a preferential SHC-PTB interaction, whereas RET^{FRS+} is equipped with the juxtadomain segment of the FGF receptor in place of the PTB domain of RET, thereby allowing interaction only with the FRS2-PTB adaptor. The SHC and FRS2 adaptor proteins are able to outcompete each other for binding to RET^{SHC+} and RET^{FRS+} respectively [Lundgren et al., 2006] (Fig. 1A). All mutants display intact levels of tyrosine phosphorylation compared to RET^{WT} (Fig. 1B). We compared cells expressing these mutants to RET^{WT} and to a RET^{Y1062F} receptor, the latter which cannot recruit any PTB adaptors to Y1062 but is otherwise functionally intact. In a wound healing experiment Cos7 cells were grown to confluence on glass slides and a mechanical tear was made by scratching a caliper across the glass, creating an artificial wound. The RET ligands GDNF (50 ng/ml) and GFRα1 (100 ng/ml) were added to the cultures and the number of cells migrating from the edges into the wound area were quantified (Fig. 1C,D). The number of cells migrating into a 1.2 mm long wound field, were quantified after 6 h of RET ligand application. Reduced migration was seen in RET^{SHC+} and RET^{Y1062F} mutants, as only 64% and 60% of the cells, respectively, had moved into the wound area compared to the number of migrating RET^{WT} expressing cells (i.e., 100%). The FRS2 signaling RET mutant, RET^{FRS+} on the other hand showed increased number of cells migrating into the wound area compared to RET^{WT} (148% of WT). The enhancement compared to RET^{WT}, we reason, is a result of RET^{FRS+} mutants not allowing SHC to compete for binding at all whereas some SHC is likely to interact with tyrosine 1062 in RET^{WT} in these cells. Cells transfected with an empty vector as a negative control were similar to RET^{SHC+} and RET^{Y1062F} cells (Fig. 1C,D).

The role of RET in directional cellular migration was next studied in a chemotaxis assay. We expressed either RET^{WT}, RET^{SHC+}, RET^{FRS+}, or RET^{Y1062F} in human peripheral neuroblastoma SK-N-MC cells, which do not express endogenous RET and because of their neuroectodermal origin are relevant for studies of many Ret functions [Fukuda et al., 2002]. To investigate if the motility produced by FRS2 interactions with RET was directional. Chemotaxis was quantified by seeding cells on top of a synthetic porous membrane (upper compartment), soluble ligand was applied in the lower compartment. Both RET^{WT} and RET^{FRS+} cells migrated towards RET ligand while RET^{SHC+} or RET^{Y1062F} cells did not display increased migration compared to mock transfected cells (Fig. 1E). In order to confirm that this represents directional migration, ligands were supplied only to the upper compartments (Fig. S2E), or to both the upper and lower compartment (Fig. S2F) producing a uniform concentration of ligands surrounding the cells. These conditions did not lead to any significant increase of cells migrating through the membrane into the lower compartment. To confirm a direct dependence of FRS2 for RET induced

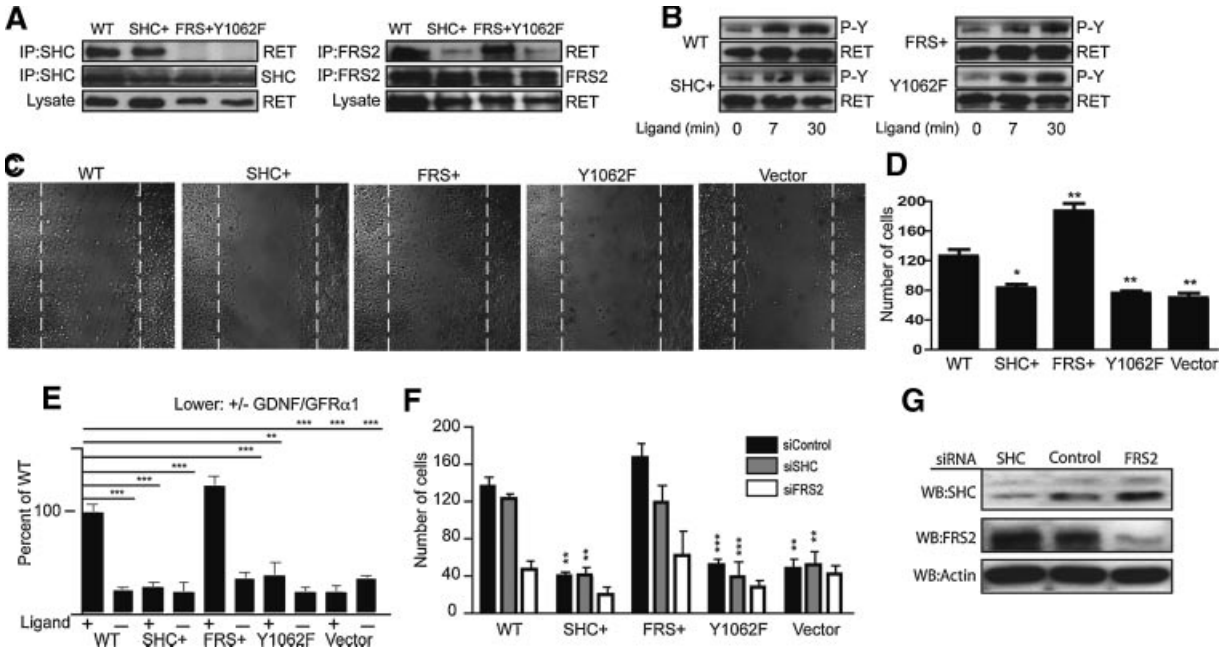

Fig. 1. FRS2-dependent cellular migration by RET. **A**: Cos7 cells expressing RET mutants were immunoprecipitated for endogenous SHC or FRS2 adaptors and immunoblotted for RET showing selective adaptor binding in mutants. **B**: All RET mutants display similar phosphorylation levels after ligand application. **C**: Photomicrographs of Cos7 cells expressing RET mutants as indicated grown to confluence on glass slides. A vertical tear was made in the cell layer using a medical caliper and cell migration was examined in the tear. RET ligand was applied to starvation medium. **D**: Quantification of (C). Migration into the wound area along a 1.2 mm stretch was after 6 h quantified as an average of three random fields (n = 3). One-way ANOVA, $^{**}P < 0.01$, $^{*}P < 0.05$ compared to WT. **E**: SK-N-MC cells expressing RET mutants or empty vector were seeded on top of a ChemoTX 12 μm porous membrane with ligand in the lower compartment. Cells migrating through the membrane pores to the lower compartment after 8h were detached and stained with crystal violet. Absorbance was measured in a spectrophotometer and RET^{WT} values were set to 100%. Ret ligand was supplied above/below the membrane as indicated (n = 4, for vector condition n = 2). One-way ANOVA, $^{***}P < 0.001$. **F**: Quantification of SK-N-MC cells, expressing RET mutants or empty vector and siRNA as indicated, in transwell chemotaxis migration assays towards ligands (n = 3). Two-way ANOVA compared to the corresponding WT condition, $^{***}P < 0.001$. $^{**}P < 0.01$. **G**: Immunoblot towards SHC or FRS2 in siRNA transfected SK-N-MC cells as indicated.

migratory signaling we silenced endogenous FRS2 gene expression using siRNA. Two independent siRNAs against FRS2 were tested with similar effect (Fig. S2G) both sequences were then used in combination for future assays. Reduction of FRS2 protein levels by 67% at 48 h (Fig. 1G) led to reduced migration in RET^{WT} and RET^{FRS+} cells with less than 40% of cells migrating towards ligand as compared to control siRNA transfected cells. siRNA against FRS2 resulted in a non-statistically significant small reduction of migration also in RET^{SHC+} and RET^{Y1062F} as compared to Ret^{WT} cells perhaps due to effects of FRS2 on general cell motility [Hadari et al., 2001]. siRNA against SHC (62% reduction of SHC protein at 48 h; Fig. 1G) had little effect on migration (Fig. 1F). These data suggest that recruitment of FRS2, but not SHC, to tyrosine 1062 can direct RET signaling to elicit directional cell motility.

FRS2 Signaling via SRC Family Kinases

The non-receptor tyrosine kinase SRC family of proteins is required for cell migration mediated by a number of RTKs [for review see Bromann et al., 2004] including the PDGF [Sieg et al., 2000] and epidermal growth factor (EGF) receptors [Brunton et al., 1997], and inhibition of SRC results in loss of migration. We investigated the activation of SRC in RET mediated migratory signaling via its recruitment of adaptors. We performed an in vitro kinase assay to test whether the SRC kinase activity was affected by the adaptor selective RET mutants. Endogenous SRC was precipitated from ligand stimulated Cos7 cells expressing RET mutants. The precipitate was allowed to phosphorylate an immobilized substrate. At 5 min RET^{WT} displayed a slightly higher level of phosphorylated substrate, as measured by spectrophotometry, compared to RET^{FRS+}, RET^{SHC+} and

RET^{Y1062F}. After 30 min of ligand stimuli the SRC kinase activity was approximately 1.5 and 2 times higher in RET^{WT} and RET^{FRS+}, respectively, compared to RET^{SHC+} and RET^{Y1062F} showing that SRC kinase activity is more profound in the RET versions that recruit FRS2 and mediate migration (Fig. 2A).

SRC Is Required for RET-Induced Directed Migration

SRC activity can be inhibited by the pharmacological kinase inhibitor PP2 [Hanke et al., 1996]. PP2 treatment of cells prevented directed migration of RET^{WT} and RET^{FRS+} expressing cells towards RET ligand in the chemotaxis assay (Fig. 2B,C). RET^{SHC+} and RET^{Y1062F} did not show any significant change in migration following treatment with PP2 from the already low levels without inhibitor (Fig. 2B,C). Similar results were obtained also in kidney MDCK cells (data not shown). We have previously shown that mitogen activated protein kinases (MAPK) are robustly activated by RET^{FRS+} [Lundgren et al., 2006]. Blocking this signaling pathway with the pharmacological inhibitor U0126 reduced the number of migrating cells to half that without inhibitor in RET^{WT} and RET^{FRS+} expressing cells (Fig. 2C). The reduction in migration was slightly more pronounced when interrupting the SRC family kinases with PP2 than the MAPK pathway (Fig. 2C). Thus, while both pathways seem required for full effect of RET mediated chemotaxis, SRC exerts a more pronounced effect. Although developed as a specific SRC inhibitor PP2 has recently been shown to inhibit other kinases at various concentrations, among them the constitutively active MEN2a form of RET [Carlomagno et al., 2003]. To corroborate the biochemical data from pharmacological inhibition of SRC in RET mediated migration we performed additional experiments using another SRC inhibitor, SU6656, recently shown to inhibit SRC with great specificity at a reported IC_{50} between 20 and 280 nM [Blake et al., 2000]. When SRC was blocked with SU6656 in the chemotaxis assay cell migration towards RET ligands was also markedly reduced (Fig. 2D). Total tyrosine phosphorylation was examined in lysates of cells after starvation for 12 h and subsequent RET ligand stimulation for 30 min. Cells were treated with no inhibitor, PP2 or SU6656 before RET ligand stimulation. The pattern of tyrosine phosphorylation was similar between the two inhibitors, but PP2 displayed a more extensive inhibition of tyrosine phoshorylation of several non-identified phosphotyrosine proteins as seen on membranes immunoblotted against total phospho-tyrosine (Fig. 2E). Phosphorylation of SRC and p42,44 ERK MAPK was examined following PP2 and SU6556 treatment. Both PP2 and SU6556 efficiently blocked phosphorylation of SRC while PP2 prevented phosphorylation of p42, 44 ERK MAPK to a greater extent than SU6556, presumably reflecting non-specific effects of the PP2 compound (Fig. 2F,G).

We next took a genetic approach to confirm a SRC-dependent cell migration by RET. For this purpose mouse embryo fibroblasts (MEFs) derived from SRC-YES-FYN null mice (MEF^{SYF-}) were used [Klinghoffer et al., 1999]. MEF^{SYF-}cells were co-transfected with RET mutants and SRC or an empty vector and the requirement for SRC in RET-induced motility was examined in the wound healing assay. Cells were starved and after subsequent application of RET ligand the number of cells migrating into the wound was quantified. After 9 h of ligand stimuli RET^{WT} and RET^{FRS+} expressing cells co-transfected with SRC had already migrated into the area while little migration was seen in RET^{SHC+} and RET^{Y1062F} expressing cells (Fig. 2H,I). MEF^{SYF-} cells transfected with SRC but not RET showed a small increase in migration compared to RET^{SHC+} and RET^{Y1062F}, perhaps due to low levels of endogenous RET expression (Fig. 2H,I). In MEF^{SYF-} lacking SRC neither condition showed any significant migration at 9 h of RET ligand stimuli (Fig. 2H,I). RET^{WT} and RET^{FRS+} induced migration in MEF^{SYF-} cells in the presence of SRC was ligand-dependent (Fig. 2H,I). Taken together, these results show that SRC is required for RET mediated migration.

SRC Recruitment to Tyrosine-981 Is Required for RET Induced Migration

RET binds to SRC primarily via its intracellular tyrosine-981 (Y981) [Encinas et al., 2004]. We constructed RET receptors with amino acid Y981 exchanged for a phenylalanine (RET^{Y981F}). Immunoblotting for activated SRC confirmed that RET mediated SRC activation was reduced in the RET^{Y981F} mutant (Fig. 3A). We also found that RET^{Y981F} was poor at generating directed migration in the chemotaxis

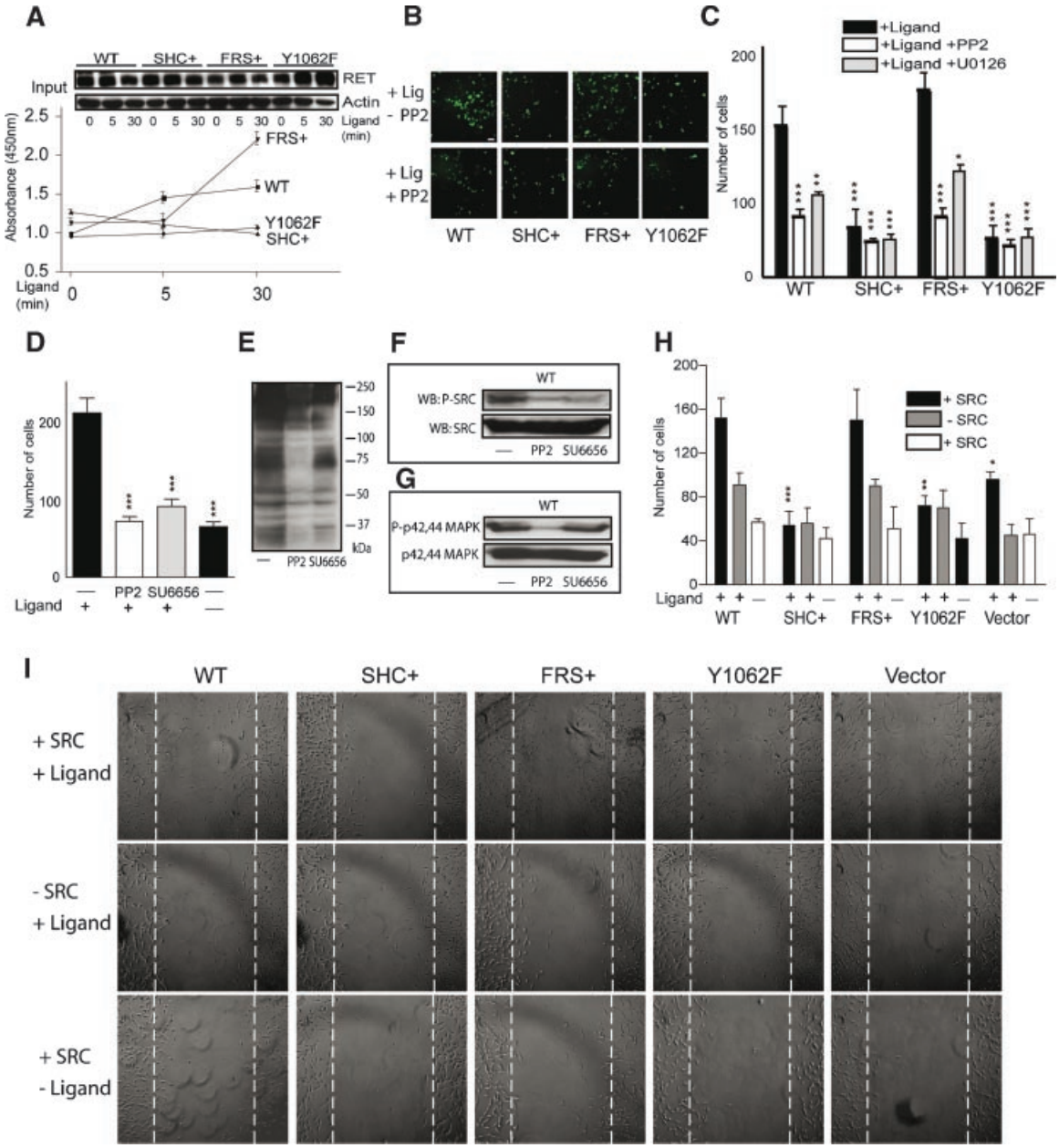

Fig. 2. A SRC and MAPK-dependent RET-induced migration. **A**: In-vitro kinase assay for SRC. Cos7 cells expressing RET mutants were stimulated as indicated. SRC was precipitated from cells and the kinase activity was measured by the absorbance of tetramethylebenzidine substrate reaction with horse radish peroxidase (HRP) conjugated phospho-tyrosine antibodies directed against phosphorylated substrate (n = 2). (Insert) Immunoblot for RET expression and endogenous actin from cell lysates used for the in-vitro kinase assay to control for equal loading in the assay. **B**: SK-N-MC cells were co-transfected with RET mutants and EGFP. After 24 h cells were seeded in transwell chambers. RET ligands were supplied underneath the membrane to assay for directed migration with or without the SRC inhibitor PP2, as indicated. After 10 h, images of EGFP fluorescence were captured from the underside of the membrane. **C**: Quantification of experiments in (B) and in the presence of inhibitors for MAPK (U0126) (n = 4). **D**: Same experiments as in (B) in the presence of SRC inhibitors PP2 or SU6656 (n = 3). In (C) and (D) One-way ANOVA compared to WT without inhibitor $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. **E–G**: Representative immunoblots of ligand stimulated (30 min) SK-N-MC cells after 10 h of starvation for detection of phospho-tyrosine (n = 3) (E), phospho-SRC and total SRC (F), phospho-p42,44 MAPK and total p42,44 MAPK (G) in the presence of the two SRC inhibitors. **H**: Quantification of MEF^{SYF-} cells lacking SRC family kinases transfected with the different RET mutants with or without transfection of SRC expression plasmids and subjected to the wound healing assay. After 9 h the number of cells migrating into the wound area along a 1 mm horizontal length was quantified as the average per field out of five fields (n = 3). RET ligands were supplied as indicated. Two-way ANOVA compared to the corresponding WT condition, $***P < 0.001$, $**P < 0.01$, $*P < 0.05$. **I**: Photomicrographic images of cells in experiment in (H).

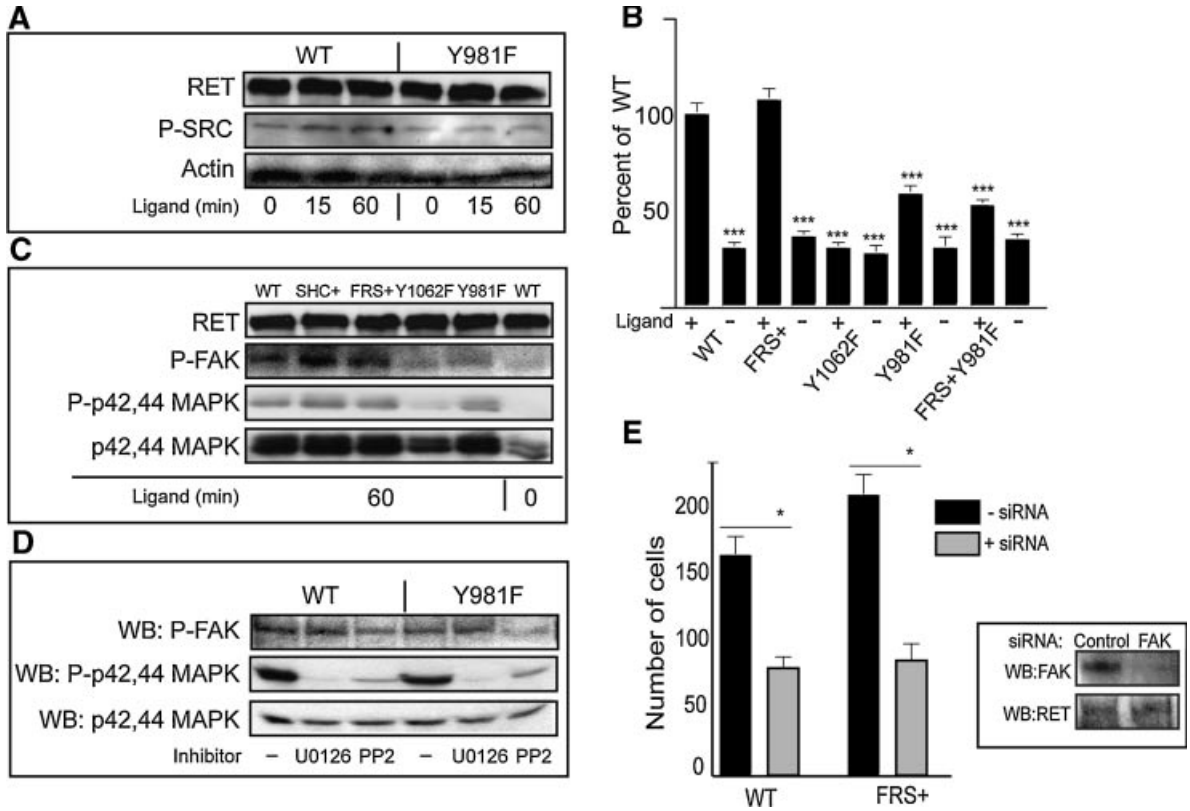

Fig. 3. RET recruitment of SRC by FRS2 occurs via RET Y981 and result in FAK activation and cell migration. **A**: Representative immunoblot for phospho-SRC (P-SRC) after indicated duration of ligand stimulation (n = 4). **B**: SK-N-MC cells expressing RET mutants were analyzed in ChemoTX assays after 8 h with RET ligands supplied below the membrane. RET^{WT} values were set to 100% (n = 3). One-way ANOVA compared to WT with ligand ***$P < 0.001$. **C**,**D**: Representative immunoblots (n = 3, respectively) towards phosphorylated FAK in the autoactivation loop (P-FAK) and phospho-MAPK (P-p42,44 MAPK) with (or without) inhibitors, as indicated. **E**: Transwell chemotaxis assay for migration of SK-N-MC cells as in (B) with siRNA against FAK. RET^{WT} values without siRNA were set to 100%. Insert shows efficiency of siRNA mediated FAK protein knockdown by immunoblot against FAK 50 h after transfection. (n = 3) One-way ANOVA *$P < 0.05$.

assay, as compared to RET^{WT} (Fig. 3B). Immunoblotting towards P-ERK MAPK did not show any overtly reduced levels of phosphorylation of p42, p44 ERK MAPK in RET^{Y981F} cells as compared to RET^{WT} cells (Fig. 3C). These data show that both Y981 and Y1062 are needed and suggest that the two tyrosines cooperate for full directional migration by RET.

Dependence on FAK for RET-Mediated Migration

In cells where SRC functions to activate cytoskeletal rearrangements it is dependent upon the FAK protein [Sieg et al., 2000]. Active SRC binds to phosphorylated FAK and kinase active SRC has previously been shown to further phosphorylate FAK [Lietha et al., 2007] which have been suggested to promote FA turnover and migration [Webb et al., 2004]. By immunoblotting we found that RET induces phosphorylation of FAK in SK-N-MC cells (Fig. 3C). We next investigated whether RET induced FAK activation depended on SRC. Immunoblotting towards FAK revealed a small statistically non-significant reduction of P-FAK in RET^{Y981F} cells compared to RET^{WT} cells (Fig. 3D). Blocking SRC signaling pharmacologically in RET activated cells, however, resulted in a marked reduction of P-FAK and P-p42, p44 MAPK levels (Fig. 3D) and the effect was greater in RET receptors containing the Y981F mutation. Hence, the residual activity in the RET^{Y981F}, as compared to cells in which SRC is pharmacologically inhibited, appears to be due to the ability of RET to recruit SRC also via sites other than tyrosine 981 although with lower efficiency [Encinas et al., 2004].

The finding that MAPK is required for a full migrational response, and that phosphorylation of FAK depends on SRC, led us to further investigate how FAK activation relates to

MAPK signaling. We performed immunoblots against P-p42, p44 MAPK while pharmacologically inhibiting SRC activity, so that SRC mediated phosphorylation of FAK is reduced. We found that P-p42, p44 MAPK levels induced by RET was reduced to levels corresponding to the reduction of FAK activation. However, the level of P-FAK was not affected by blocking MAPK activity with U0126 (Fig. 3D). This suggests an activation of P-p42, p44 MAPK downstream of SRC and possibly also FAK, as FAK can activate MAPK by recruitment of growth factor receptor bound protein 2 (Grb2) [Schlaepfer et al., 1999]. To investigate if FAK is functionally important for RET mediated migration we silenced gene expression of FAK by RNA inhibition. In these experiments, siRNA was transfected into SK-N-MC cells which resulted in a reduction of protein expression by >80% after 48 h (Fig. 3E insert). Activation of RET^{WT} and RET^{FRS+} led to markedly reduced migration in cells containing a lower level of FAK protein, as compared to cells expressing normal FAK levels (Fig. 3E).

Localization of FAK to Sites of Cell Protrusions and Focal Adhesions in RET^{WT} and RET^{FRS+} Cells

FAK was phosphorylated also by RET^{SHC+} expressing cells (Fig. 3C) but unlike RET^{FRS+} cells this activation did not lead to migration (Fig. 1) and these cells displayed a morphology with less actin bundling and lamellipodial like structures (Fig. 4). This suggested that FAK activation is not sufficient for migration per se and opens the possibility that the intracellular distribution of FAK determines its contribution to cell migration. FAK is known to localize specifically to sites of receptor and integrin clustering in migrating cells where it affects FA turnover [Sieg et al., 2000]. We therefore examined the intracellular distribution of FAK in FRS2 binding and non-FRS2 binding RET mutants. In cells expressing stimulated RET^{WT} and RET^{FRS+} FAK showed clustering of the protein at actin FAs and at cell extensions, as expected for the FAK protein in migrating cells (Fig. 4 A–D, I–L, respectively). However, in RET^{SHC+} and RET^{Y1062F} cells, which display fewer actin bundles and cell outgrowths, clusters were fewer and uniformly spread within the cell rather than localized to cell edges (Fig. 4 E–H, M–P, respectively). Further, when immunoprecipitating for total-FAK in cell lysates and probing membranes for P-FAK both RET^{Y981F}, which has a reduced SRC activation, and RET^{Y1062F} displayed a reduced level of phosphorylation of the activation loop residues in FAK 30 min after ligand stimulation (Fig. 4Q). Immunoprecipitation experiments were performed in which the cells were subjected to CO. CO disrupts the membrane architecture by oxidizing membrane integral sterol compounds and for the PDGF receptor has been shown to uncouple the receptor from its substrates [Liu et al., 2000]. CO treatment led to a significant reduction in FAK phosphorylation (Fig. 4Q). Thus, it appears that important interactions takes place in cholesterol rich regions of the membrane, and oxidiation of cholesterol prevents molecular interactions that are necessary for RET to activate FAK. Subfractionation of 1% Triton X-100 lysed cells were performed as a method to investigate ligand activated (30 min) RET^{SHC+} versus RET^{FRS+} partitioning into detergent resistant membrane (DRM) fractions and supernatant (SUP) fraction respectively. Both RET mutants were found in DRM as well as SUP fractions in agreement with previous results using this fractionation method [Pierchala et al., 2006] (Fig. 4R). Immunoblotting against SHC or FRS2 revealed that the former was present with no significant preponderance for either fraction whereas FRS2 was heavily enriched with 74% and 78% in the DRM for RET^{SHC+} and RET^{FRS+}, respectively ($P < 0.01$ Student's t-test; Fig. 4R). This suggest that Ret may interact with FRS2 predominantly in membrane ordered regions, while this is not necessarily the case with SHC.

A FRS2 Mediated Concentration of RET to Discrete Membrane Foci

We hypothesized that the focal activation of FAK seen in the case of RET receptors that recruit FRS2 might also correspond to a relocation of the RET receptor itself. The FRS2 adaptor protein has a N-terminal myristoylation moiety and exclusively localizes to lipid rafts/organized lipid structures in the cytofacial membrane [Ridyard and Robbins, 2003]. We constructed expression constructs with green fluorescent protein (GFP) fused to the intracellular carboxy (C)-terminus of RET. This RET-GFP fusion protein allowed us to directly examine subcellular localization of RET when engaging different PTB adaptors. Cells expressing the GFP-RET constructs were ligand

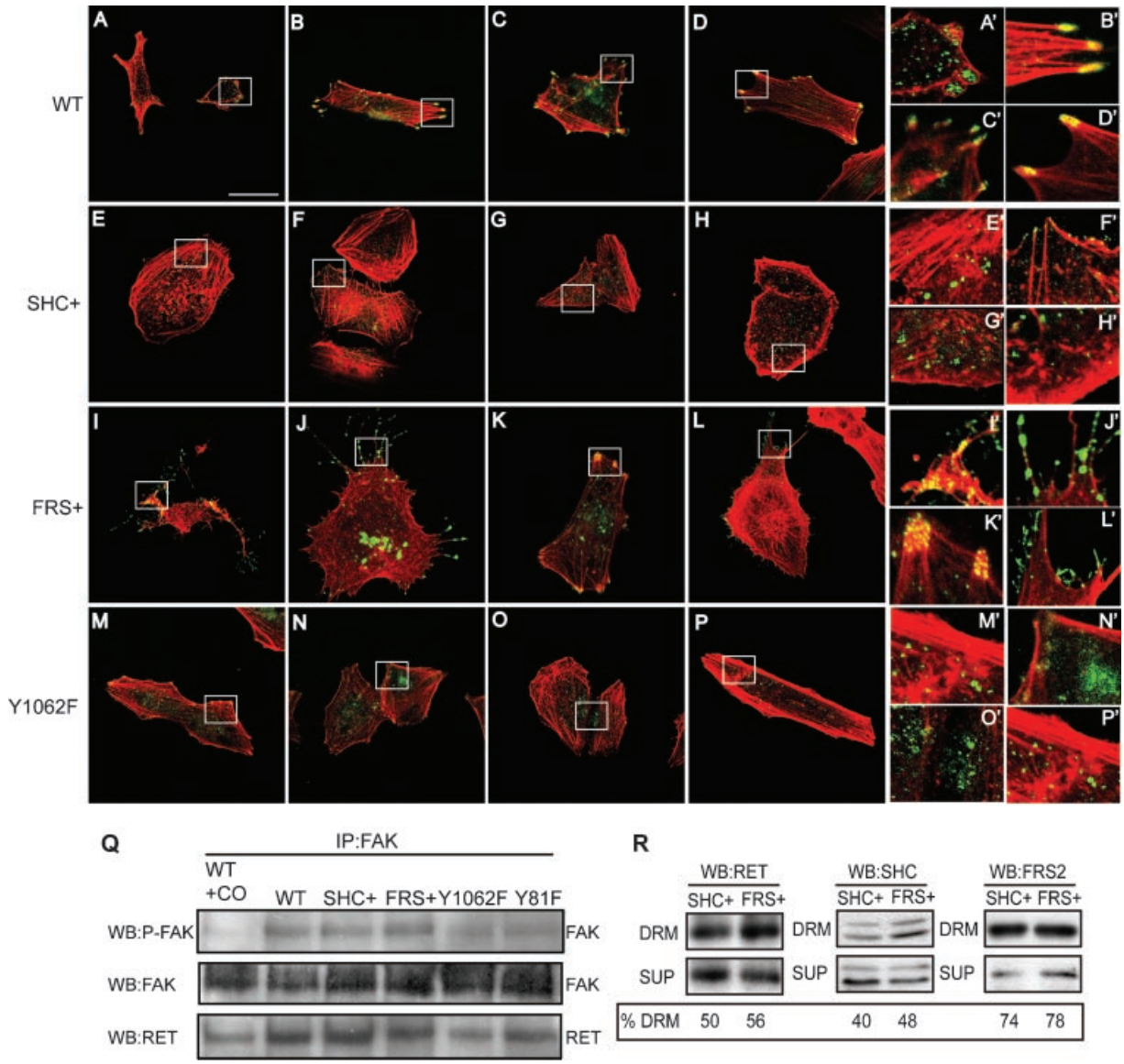

Fig. 4. A FRS2-dependent activation of FAK result in its distribution to FAs. **A–P**: Immunocytochemistry of neuronal SK-N-MC cells expressing RET mutants as indicated. Cells were starved for 12 h and RET ligands applied for 30 min before fixation. Actin was visualized by fluorescent conjugated phalloidin (red) and FAK by immunohistochemistry (green), colocalization (yellow). **A′–P′** shows enlargements of the corresponding areas in A–P indicated by white boxes. Note abundant appearance of FAs in RET^{WT} and RET^{FRS2+} but not RET^{SHC+} or RET^{Y1062F} expressing cells. **Q**: Immunoprecipitation for total-FAK and immunoblotting against phosphorylated FAK, total FAK and RET, as indicated. CO+ indicates a condition in which RET^{WT} expressing cells were treated with cholesterol oxidase prior to ligand stimulation. **R**: DRM and SUP fractionations of SK-N-MC transfected with RET mutants as indicated. Western blot against RET or FRS2 or SHC. Scale bar = 25 μm.

activated for 30 min, counterstained with fluorescently conjugated phalloidin and examined using a confocal microscope. The distribution of RET was distinctly different between migratory competent cells expressing RET^{WT} or RET^{FRS+} and the non-migratory RET^{SHC+} and RET^{Y1062F}. In RET^{WT} or RET^{FRS+}, RET receptors were clustered in foci of membrane protrusions (Fig. 5A–C, I–K). In RET^{SHC+} and RET^{Y1062F} the receptor was distributed uniformly throughout the cell soma and only occasional receptor clusters were seen (Fig. 5E–G, M–O). The distribution of clusters was quantified by measuring clustered RET-GFP fusion proteins on digital images at a predetermined threshold setting (Fig. 5D,H,L,P; supplementary movie 1 (SM1) and Fig. S2 A–D shows a 3D reconstruction of a cell expressing GFP-

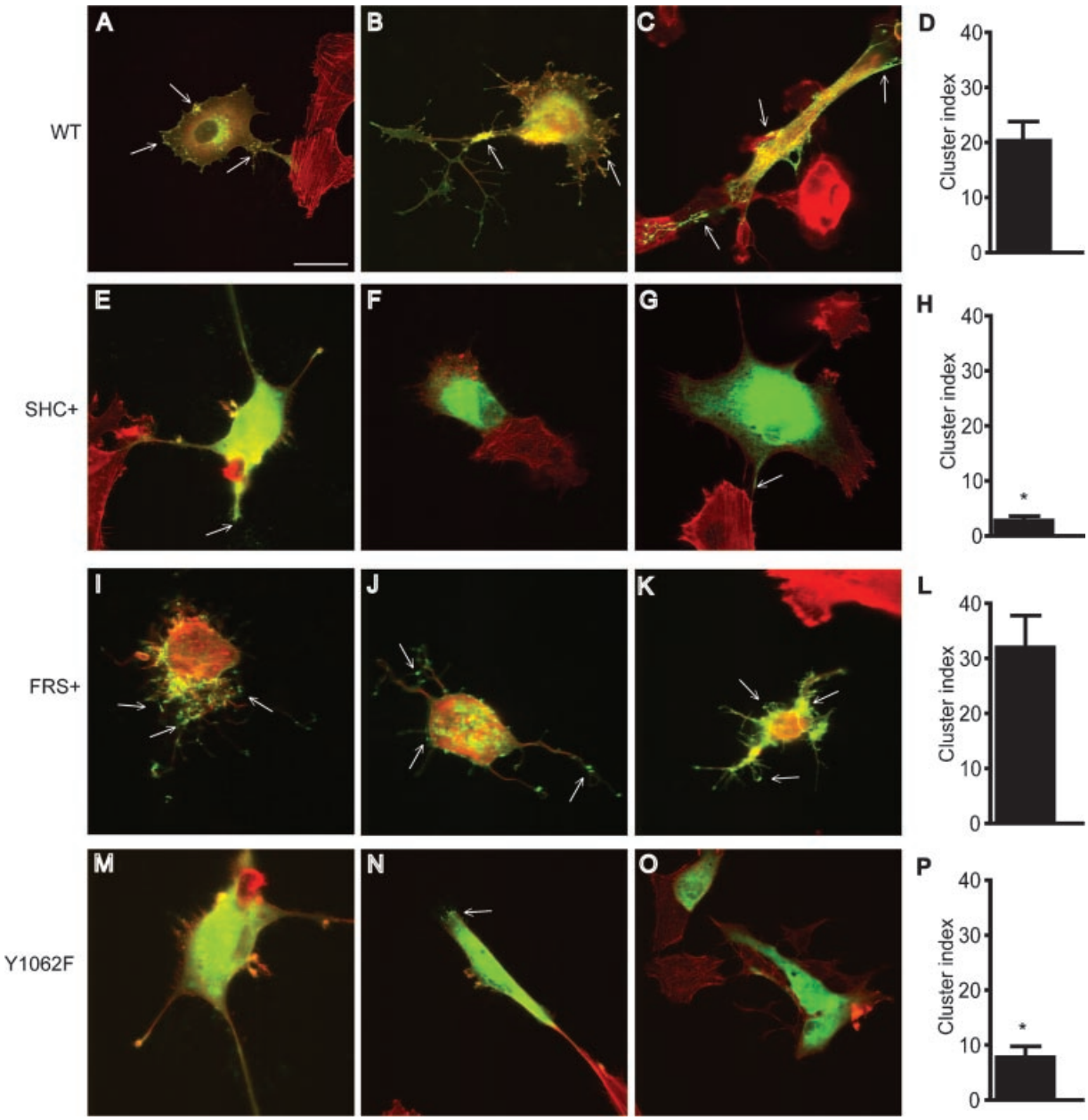

Fig. 5. A subcellular re-distribution of RET to lamellipodia and regions containing focal adhesions by FRS2 recruitment. **A**–**C**, **E**–**G**, **I**–**K**, **M**–**O**: Fluorescence images of representative neuronal SK-NM-C cells expressing RET-GFP fusion protein containing the different RET mutations, as indicated. Green fluorescence therefore indicates the cellular localization of RET receptors. Cells were starved for 12 h and RET ligand applied for 30 min before fixation. Cells were stained for actin using fluorescent conjugated phalloidin (red). Arrows point at some of the sites of RET clusters, often localized to regions of membrane ruffling and/or cell protrusions in RET^{WT} and RET^{FRS2+} expressing cells. **D**,**H**,**L**,**P**: Quantification of RET clustering in cells expressing the different RET variants. Average cluster index was determined from 25 cells per condition per independent experiment (n = 4). Scale bar = 25 μm. One-way ANOVA, $*P < 0.05$ compared to WT.

RET^{FRS+}). We next investigated localization of RET in cells by live imaging. Cells grown on glass slides were imaged after ligand addition and the distribution of fluorescent RET was captured in serial pictures. Representative movie SM 2 displays RET protein clustered at cell edges as the cell body changes shape in GFP-RET^{FRS+} expressing cells, while GFP-RET^{SHC+} (SM3) lacked this clustering effect.

DISCUSSION

Correct positioning of cells in the nervous system is achieved mainly by oriented migra-

tion of neuronal progenitors from their site of generation to their adult location. Attractant molecules interacting with receptors expressed on cells, as exemplified by RET and RET ligands, are important for these processes. However, much remains to be known on the actual molecular signaling leading to cytoskeletal rearrangment and cellular movement after receptor engagement. We have obtained evidence that PTB adaptors function to regulate RET stimulated migration and propose that one necessary role of PTB adaptor activity is to produce a concentration of RET receptors into clusters on the cell membrane. Activation of the EGF receptor leads to accumulation of the receptors in lipid rafts, followed by SRC family kinase activation. EGFR activation also leads to a clustering of activated EGFR on the membrane that depends on the activity of SRC family kinases [Kasai et al., 2005]. We find that the FRS2 PTB adaptor promotes receptor clustering of RET to a higher degree than SHC and at locations where the cell clearly exhibits cytoskeletal actin rearrangments, morphologically consistent with sites of FAs. Functionally, we find that cells expressing RET that can bind FRS2 migrate towards RET ligands whereas mutants binding SHC or where the PTB adaptor binding tyrosine 1062 is nullified does not. Further, siRNA against FRS2 inhibited migration in both RET^{WT} and RET^{FRS+} cells. Our results therefore suggest that PTB adaptors provide a means for the relocation of RET receptors into membrane clusters function, which expands their activity as signaling scaffolds.

Recruitment of FRS2 that is exclusively localized to lipid rafts leads to a specific signaling response to FGF2 signaling [Ridyard and Robbins, 2003]. Also RET seems to signal differently depending on its membrane compartment. In the absence of ligand, RET is localized outside of lipid rafts in non-ordered membrane regions [Tansey et al., 2000; Paratcha et al., 2001]. Following ligand activation, RET can translocate into lipid rafts which leads to a specific downstream signaling, including the association of FRS2 and the activation of SRC family kinases [Tansey et al., 2000; Encinas et al., 2001; Paratcha et al., 2001]. The localization of RET to lipid rafts potentiates RET signaling and lead to cell survival and cellular differentiation [Tansey et al., 2000; Pierchala et al., 2006]. However the roles of different adaptors, coupled to distinct downstream signaling pathways, in relation to RET clustering and its different biological functions have not been addressed. We have shown that FRS2 recruitment to RET upon ligand activation does not promote cell survival [Lundgren et al., 2006] and in the present study find that cellular redistribution of RET into membrane foci leads to sustained activation of SRC family kinases, that specifically participate in organizing a signaling complex required for RET-mediated cell migration. The present study does not provide direct evidence for lipid raft association of RET that is specific for the different PTB-adaptors. However, our biochemical results from disruption of cholesterol in the membrane, the predominant presence of Frs2 in DRM and previous studies reporting different downstream signaling of Ret in different membrane compartments [Saarma, 2001; Pierchala et al., 2006] warrants further investigation on the importance of lipid rafts in RET functions downstream of SHC and FRS2.

In order to associate cytoskeletal proteins with both the extracellular growth substratum and receptors responding to external ligands, these molecules must interact at precise subcellular positions. This creates a dimension of complexity since the local cytoplasmic context must be different between the leading edge where lamellipodia form and other parts of the cell. Activated FAK is localized specifically to membrane sites containing FAs. FAK participates in signaling elicited by several RTKs [Sieg et al., 2000; Shono et al., 2001]. By associating with RTKs and with integrin receptors, FAK has been suggested to recruit other molecules to the site of this interaction upon receptor activation, forming a signaling complex that transmits signals from the extracellular matrix to the cell cytoskeleton [Sieg et al., 2000; Dunty et al., 2004; Mitra et al., 2005]. Thus, FAK functions to bridge integrins with the RTK and regulate turnover of FAs while SRC proteins catalyze activation of FAK [Salazar and Rozengurt, 1999; Sieg et al., 2000]. Our biochemical results show that RET acts via SRC and FAK activation for migration and furthermore suggests that RET and FAK associate in a protein complex since FAK co-precipitates with RET. SRC interaction with RET is mainly mediated by interactions with RET Y981 [Encinas et al., 2004] and FAK in turn, interacts with SRC by its autophosphorylation site tyrosine-397. SRC can then further phosphorylate FAK

in a second step on the autoactivation loop on tyrosines-576/577, which leads to full activation. Autophosphorylation of FAK is temporally related to integrin receptor clustering in the cell membrane but can also be activated by the EGFR [Sieg et al., 2000] and as we report in this study, by RET. Furthermore, the different subcellular localization of FAK in RET^{WT} and RET^{FRS+} as compared to cells expressing RET^{SHC+} and RET^{Y1062F} that are non-migratory, suggest that a change in turnover of FA complexes contributes to the RET mediated migration. Consistently, $FAK^{-/-}$ cells display a rounded morphology with increased FAs and FAK may, thus, promote the actual turnover of FAs [Ilic et al., 1995].

Our results show that ligand stimulation of RET leads to FAK activation but that this is not exclusive to the association of FRS2 to RET, as it also occurs in RET receptors recruiting SHC. However, the localization of the activated FAK is distinctly different since FAK is found diffusely localized in the cells expressing RET^{SHC+}, consistent with the morphology of these cells that display less actin bundling and rather resembles a dysregulated Ras-related homolog A (RhoA) phenotype that previously has been shown to have reduced motile capacity [Ehrenreiter et al., 2005]. Consequently, FAK activation by itself is insufficient for migration, indicating that the difference in subcellular distribution of active FAK is one distinguishing functional feature, an idea previously expressed but not investigated experimentally [Sieg et al., 2000]. It should be noted, however, that differential phosphorylation of the numerous tyrosines and serines on FAK by RET recruitment of FRS2 versus SHC cannot be excluded to be participating in the migrational activity, as FAK phospho-activation is incompletely understood [Cox et al., 2006].

RTKs usually possess several tyrosine-based signaling motifs, and in RET, Y981 recruits SRC while Y1062 recruits PTB adaptor proteins [Jain et al., 2006]. We show here that the recruitment of two different RET binding proteins to two distinct intracellular residues collaborate to execute a biological function (Fig. S1). Based on our results, SRC interaction and FRS2 interaction with intracellular Y981 and Y1062, respectively, is needed. It is possible that other proteins than SRC also interact with Y981 and are affected by the mutation of this residue. The sequence surrounding Y981 (pYRLM) is closest to the motif recognized by phosphatidylinositol 3′-kinase (PI3K). This molecule, however, does not interact directly with RET [Encinas et al., 2004]. Pharmacological inhibition of PI3K/AKT, which is not activated by FRS2 [Kurokawa et al., 2001], as well as p42,44 MAPK ERK and protein kinase A (PKA) have been shown to reduce RET elicited motility to varying extents [Natarajan et al., 2002; Asai et al., 2006]. In vivo it is therefore likely that several pathways and residues on RET may contribute to migration, likely with some degree of redundancy in the event of abrogation of single residues. Nevertheless, our results show that FRS2 signaling from Y1062 is required and plays a critical role for directed migration by RET in several cell types. The results presented here are consistent with a model that FRS2 recruitment to Y1062 leads to a membrane relocation of the receptor causing its clustering in membrane foci. Upon such relocation, Y981 of RET interacts with SRC family kinases leading to activation of FAK locally at FAs, which is required for migration. Activation of MAPKs, which we in addition to the FAK pathway find to be necessary for migration, arise by a SRC-dependent as well as a SRC-independent signaling, the latter presumably via a direct activation by FRS2 by the well described recruitment of Grb2 and Son of sevenless guanine nucleotide exchange factor (SOS) [Melillo et al., 2001].

In conclusion we have found that PTB adaptors differentially affect RET receptor subcellular location and subsequent signaling, and that this provides a means for segregating different functional outcomes of RET activation. We propose that FRS2 recruitment to RET lead to the activation of two distinct signaling pathways, one via FRS2 and another via SRC family kinases, both of which are necessary for neuronal cell migration.

ACKNOWLEDGMENTS

This work was supported by the Swedish Cancer Society, the Swedish Medical Research Council and the Swedish foundation for strategic research (CEDB/DBRM grant) to PE and the Karolinska Institute MD-PhD program to TKL. We are grateful to Dr. Per Uhlén and to technical staff at ZEISS Europe for technical assistance and valuable input on experiments.

REFERENCES

Arighi E, Borrello MG, Sariola H. 2005. RET tyrosine kinase signaling in development and cancer. Cytokine Growth Factor Rev 16:441–467.

Asai N, Fukuda T, Wu Z, Enomoto A, Pachnis V, Takahashi M, Costantini F. 2006. Targeted mutation of serine 697 in the Ret tyrosine kinase causes migration defect of enteric neural crest cells. Development 133:4507–4516.

Ayala R, Shu T, Tsai LH. 2007. Trekking across the Brain: The Journey of Neuronal Migration. Cell 128:29–43.

Barker RA. 2006. Continuing trials of GDNF in Parkinson's disease. Lancet Neurol 5:285–286.

Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, Courtneidge SA. 2000. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. Mol Cell Biol 20:9018–9027.

Bromann PA, Korkaya H, Courtneidge SA. 2004. The interplay between Src family kinases and receptor tyrosine kinases. Oncogene 23:7957–7968.

Brunton VG, Ozanne BW, Paraskeva C, Frame MC. 1997. A role for epidermal growth factor receptor, c-Src and focal adhesion kinase in an in vitro model for the progression of colon cancer. Oncogene 14:283–293.

Carlomagno F, Vitagliano D, Guida T, Basolo F, Castellone MD, Melillo RM, Fusco A, Santoro M. 2003. Efficient inhibition of RET/papillary thyroid carcinoma oncogenic kinases by 4-amino-5-(4-chloro-phenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2). J Clin Endocrinol Metab 88:1897–1902.

Cerezo-Guisado MI, Alvarez-Barrientos A, Argent R, Garcia-Marin LJ, Bragado MJ, Lorenzo MJ. 2007. c-Jun N-terminal protein kinase signalling pathway mediates lovastatin-induced rat brain neuroblast apoptosis. Biochim Biophys Acta 1771:164–176.

Chang JH, Gill S, Settleman J, Parsons SJ. 1995. c-Src regulates the simultaneous rearrangement of actin cytoskeleton, p190RhoGAP, and p120RasGAP following epidermal growth factor stimulation. J Cell Biol 130:355–368.

Cox BD, Natarajan M, Stettner MR, Gladson CL. 2006. New concepts regarding focal adhesion kinase promotion of cell migration and proliferation. J Cell Biochem 99:35–52.

Dunty JM, Gabarra-Niecko V, King ML, Ceccarelli DF, Eck MJ, Schaller MD. 2004. FERM domain interaction promotes FAK signaling. Mol Cell Biol 24:5353–5368.

Ehrenreiter K, Piazzolla D, Velamoor V, Sobczak I, Small JV, Takeda J, Leung T, Baccarini M. 2005. Raf-1 regulates Rho signaling and cell migration. J Cell Biol 168:955–964.

Encinas M, Tansey MG, Tsui-Pierchala BA, Comella JX, Milbrandt J, Johnson EM, Jr. 2001. c-Src is required for glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway. J Neurosci 21:1464–1472.

Encinas M, Crowder RJ, Milbrandt J, Johnson EM, Jr. 2004. Tyrosine 981, a novel ret autophosphorylation site, binds c-Src to mediate neuronal survival. J Biol Chem 279:18262–18269.

Enomoto H, Crawford PA, Gorodinsky A, Heuckeroth RO, Johnson EM, Jr., Milbrandt J. 2001. RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development 128: 3963–3974.

Fukuda T, Kiuchi K, Takahashi M. 2002. Novel mechanism of regulation of Rac activity and lamellipodia formation by RET tyrosine kinase. J Biol Chem 277:19114–19121.

Gupton SL, Waterman-Storer CM. 2006. Spatiotemporal feedback between actomyosin and focal-adhesion systems optimizes rapid cell migration. Cell 125:1361–1374.

Hadari YR, Gotoh N, Kouhara H, Lax I, Schlessinger J. 2001. Critical role for the docking-protein FRS2 alpha in FGF receptor-mediated signal transduction pathways. Proc Natl Acad Sci USA 98:8578–8583.

Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem 271:695–701.

Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. Nature 377:539–544.

Jacobson K, Mouritsen OG, Anderson RG. 2007. Lipid rafts: At a crossroad between cell biology and physics. Nat Cell Biol 9:7–14.

Jain S, Encinas M, Johnson EM, Jr., Milbrandt J. 2006. Critical and distinct roles for key RET tyrosine docking sites in renal development. Genes Dev 20:321–333.

Jijiwa M, Fukuda T, Kawai K, Nakamura A, Kurokawa K, Murakumo Y, Ichihara M, Takahashi M. 2004. A targeting mutation of tyrosine 1062 in Ret causes a marked decrease of enteric neurons and renal hypoplasia. Mol Cell Biol 24:8026–8036.

Kasai A, Shima T, Okada M. 2005. Role of Src family tyrosine kinases in the down-regulation of epidermal growth factor signaling in PC12 cells. Genes Cells 10:1175–1187.

Kim DH, Behlke MA, Rose SD, Chang MS, Choi S, Rossi JJ. 2005. Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. Nat Biotechnol 23:222–226.

Klinghoffer RA, Sachsenmaier C, Cooper JA, Soriano P. 1999. Src family kinases are required for integrin but not PDGFR signal transduction. EMBO J 18:2459–2471.

Koegl M, Zlatkine P, Ley SC, Courtneidge SA, Magee AI. 1994. Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif. Biochem J 303(Pt 3):749–753.

Kramer ER, Aron L, Ramakers GM, Seitz S, Zhuang X, Beyer K, Smidt MP, Klein R. 2007. Absence of ret signaling in mice causes progressive and late degeneration of the nigrostriatal system. PLoS Biol 5:e39.

Kurokawa K, Iwashita T, Murakami H, Hayashi H, Kawai K, Takahashi M. 2001. Identification of SNT/FRS2 docking site on RET receptor tyrosine kinase and its role for signal transduction. Oncogene 20:1929–1938.

Ledda F, Paratcha G, Ibanez CF. 2002. Target-derived GFRalpha1 as an attractive guidance signal for developing sensory and sympathetic axons via activation of Cdk5. Neuron 36:387–401.

Liang X, Nazarian A, Erdjument-Bromage H, Bornmann W, Tempst P, Resh MD. 2001. Heterogeneous fatty acylation of Src family kinases with polyunsaturated

fatty acids regulates raft localization and signal transduction. J Biol Chem 276:30987–30994.
Lietha D, Cai X, Ceccarelli DF, Li Y, Schaller MD, Eck MJ. 2007. Structural basis for the autoinhibition of focal adhesion kinase. Cell 129:1177–1187.
Liu P, Wang P, Michaely P, Zhu M, Anderson RG. 2000. Presence of oxidized cholesterol in caveolae uncouples active platelet-derived growth factor receptors from tyrosine kinase substrates. J Biol Chem 275:31648–31654.
Lundgren TK, Scott RP, Smith M, Pawson T, Ernfors P. 2006. Engineering the recruitment of phosphotyrosine binding domain-containing adaptor proteins reveals distinct roles for RET receptor-mediated cell survival. J Biol Chem 281:29886–29896.
Madhani HD. 2001. Accounting for specificity in receptor tyrosine kinase signaling. Cell 106:9–11.
Melillo RM, Santoro M, Ong SH, Billaud M, Fusco A, Hadari YR, Schlessinger J, Lax I. 2001. Docking protein FRS2 links the protein tyrosine kinase RET and its oncogenic forms with the mitogen-activated protein kinase signaling cascade. Mol Cell Biol 21:4177–4187.
Mitra SK, Hanson DA, Schlaepfer DD. 2005. Focal adhesion kinase: In command and control of cell motility. Nat Rev Mol Cell Biol 6:56–68.
Murakumo Y, Jijiwa M, Asai N, Ichihara M, Takahashi M. 2006. RET and neuroendocrine tumors. Pituitary 9:179–192.
Natarajan D, Marcos-Gutierrez C, Pachnis V, de Graaff E. 2002. Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development 129:5151–5160.
Newgreen D, Young HM. 2002. Enteric nervous system: Development and developmental disturbances—Part 2. Pediatr Dev Pathol 5:329–349.
Paratcha G, Ledda F, Baars L, Coulpier M, Besset V, Anders J, Scott R, Ibanez CF. 2001. Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. Neuron 29:171–184.
Paratcha G, Ibanez CF, Ledda F. 2006. GDNF is a chemoattractant factor for neuronal precursor cells in the rostral migratory stream. Mol Cell Neurosci 31:505–514.
Pawson T, Scott JD. 1997. Signaling through scaffold, anchoring, and adaptor proteins. Science 278:2075–2080.
Pierchala BA, Milbrandt J, Johnson EM, Jr. 2006. Glial cell line-derived neurotrophic factor-dependent recruitment of Ret into lipid rafts enhances signaling by partitioning Ret from proteasome-dependent degradation. J Neurosci 26:2777–2787.
Pierchala BA, Tsui CC, Milbrandt J, Johnson EM. 2007. NGF augments the autophosphorylation of Ret via inhibition of ubiquitin-dependent degradation. J Neurochem 100:1169–1176.
Ponti A, Machacek M, Gupton SL, Waterman-Storer CM, Danuser G. 2004. Two distinct actin networks drive the protrusion of migrating cells. Science 305:1782–1786.
Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. 2003. Cell migration: Integrating signals from front to back. Science 302:1704–1709.
Ridyard MS, Robbins SM. 2003. Fibroblast growth factor-2-induced signaling through lipid raft-associated fibroblast growth factor receptor substrate 2 (FRS2). J Biol Chem 278:13803–13809.
Saarma M. 2001. GDNF recruits the signaling crew into lipid rafts. Trends Neurosci 24:427–429.
Salazar EP, Rozengurt E. 1999. Bombesin and platelet-derived growth factor induce association of endogenous focal adhesion kinase with Src in intact Swiss 3T3 cells. J Biol Chem 274:28371–28378.
Schlaepfer DD, Hauck CR, Sieg DJ. 1999. Signaling through focal adhesion kinase. Prog Biophys Mol Biol 71:435–478.
Sefton BM, Trowbridge IS, Cooper JA, Scolnick EM. 1982. The transforming proteins of Rous sarcoma virus, Harvey sarcoma virus and Abelson virus contain tightly bound lipid. Cell 31:465–474.
Shakya R, Watanabe T, Costantini F. 2005. The role of GDNF/Ret signaling in ureteric bud cell fate and branching morphogenesis. Dev Cell 8:65–74.
Shono T, Kanetake H, Kanda S. 2001. The role of mitogen-activated protein kinase activation within focal adhesions in chemotaxis toward FGF-2 by murine brain capillary endothelial cells. Exp Cell Res 264:275–283.
Sieg DJ, Hauck CR, Ilic D, Klingbeil CK, Schaefer E, Damsky CH, Schlaepfer DD. 2000. FAK integrates growth-factor and integrin signals to promote cell migration. Nat Cell Biol 2:249–256.
Smith MJ, Hardy WR, Murphy JM, Jones N, Pawson T. 2006. Screening for PTB domain binding partners and ligand specificity using proteome-derived NPXY peptide arrays. Mol Cell Biol 26:8461–8474.
Tang MJ, Worley D, Sanicola M, Dressler GR. 1998. The RET-glial cell-derived neurotrophic factor (GDNF) pathway stimulates migration and chemoattraction of epithelial cells. J Cell Biol 142:1337–1345.
Tansey MG, Baloh RH, Milbrandt J, Johnson EM, Jr. 2000. GFRalpha-mediated localization of RET to lipid rafts is required for effective downstream signaling, differentiation, and neuronal survival. Neuron 25:611–623.
Thomas SM, Brugge JS. 1997. Cellular functions regulated by Src family kinases. Annu Rev Cell Dev Biol 13:513–609.
Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 6:154–161.
Wong A, Bogni S, Kotka P, de Graaff E, D'Agati V, Costantini F, Pachnis V. 2005. Phosphotyrosine 1062 is critical for the in vivo activity of the Ret9 receptor tyrosine kinase isoform. Mol Cell Biol 25:9661–9673.
Wozniak MA, Modzelewska K, Kwong L, Keely PJ. 2004. Focal adhesion regulation of cell behavior. Biochim Biophys Acta 1692:103–119.
Young HM, Bergner AJ, Anderson RB, Enomoto H, Milbrandt J, Newgreen DF, Whitington PM. 2004. Dynamics of neural crest-derived cell migration in the embryonic mouse gut. Dev Biol 270:455–473.