IL13Rα2 signaling in colorectal cancer metastasis

**IL-13 receptor α2 signaling requires a scaffold protein, FAM120A, to activate the FAK and PI3K pathways in colon cancer metastasis**

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ABSTRACT

IL-13 signaling through its receptor IL13Rα2 plays a critical role in colon cancer invasion and liver metastasis, but the mechanistic features of this process are obscure. In this study, we identified a scaffold protein, FAM120A (C9ORF10), as a signaling partner in this process. FAM120A was overexpressed in human colon cancer cell lines and 55% of human colon cancer specimens. IL13Rα2-FAM120A co-immunoprecipitation experiments revealed further signaling network associations that could regulate the activity of IL13Rα2, including FAK, SRC, PI3K, G-protein coupled receptors and TRAIL receptors. In addition, FAM120A associated with kinesins and motor proteins involved in cargo movement along microtubules. IL13Rα2-triggered activation of the FAK and PI3K/AKT/mTOR pathways was mediated by FAM120A, which also recruited PI3K and functioned as a scaffold protein to enable phosphorylation and activation of PI3K by Src family kinases. FAM120A silencing abolished IL-13-induced cell migration, invasion and survival. Finally, antibody blockade of IL13Rα2 or FAM120A silencing precluded liver colonization in nude mice or metastasis. In conclusion, we identified FAM120A in the IL-13/IL13Rα2 signaling pathway as a key mediator of invasion and liver metastasis in colon cancer.
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INTRODUCTION

Interleukin 13 (IL-13) has been associated to different pathological conditions (asthma, autoimmune diseases, ulcerative colitis and others) (1). More recently, we demonstrated that IL-13 signaling through interleukin-13 receptor subunit alpha-2 (IL13Rα2) plays a critical role in colon cancer invasion and liver metastasis (2). We described the overexpression of IL13Rα2 in 66% of tumor samples from colon cancer patients, which was associated to late stages of progression (metastasis in lymph nodes or liver) and poor outcome of colorectal cancer patients (2). IL13Rα2 is overexpressed in a variety of human tumor types, such as colon, glioblastoma, renal cell carcinoma, pancreatic, melanoma, head and neck, mesothelioma, and ovarian, where it has been proposed as biomarker and potential therapeutic target (2-10). In glioblastoma multiforme, IL13Rα2 expression occurs in 75% of tumors and is associated with poor prognosis (3). A similar situation occurs for head and neck squamous cell carcinoma (8) and ovarian cancer (9).

IL-13 binding to IL13Rα2 induced a significant increase in cell adhesion, migration and invasion capacity of colorectal cancer cells (2). Cell signaling was independent of type 2 IL-4 receptor and IL13Rα1 receptor as KM12 cells did not express these alternative IL-13 receptors on the cell surface (2). Moreover, silencing of IL13Rα2 increased mice survival and provoked a clear reduction in liver colonization in mouse xenograft models (2). Although initially was considered a decoy receptor (11), there are multiple evidences that IL13Rα2 is functional and induces the activation of several pathways and the transcription factor AP-1, inducing the expression of TGFβ (2, 12, 13). IL13Rα2 cytoplasmic domain is very short, consisting of only 14 amino acids residues, which does not contain any recognizable motif and makes difficult its interaction with other proteins. Nevertheless, this receptor participates in signal transduction, triggering the activation of several
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signaling proteins, such as PI3K and Src kinases (2, 12, 13). Little was known about the signaling mechanisms of IL-13 through IL13Rα2 in metastasis and cancer progression.

Here, we have identified the molecular partners of IL13Rα2 and the mechanisms of signal transduction by using immunoprecipitation experiments combined with a proteomic approach. We identified the adaptor FAM120A as the scaffold protein necessary for IL13Rα2-mediated signaling. FAM120A, also known as c9orf10 or OSSA (oxidative stress-associated Src activator), was required for the activation and recruitment of FAK, Src, PI3K and most of the proteins involved in IL13Rα2 signaling, providing an overall picture of IL-13 signaling in colorectal cancer cells and its relevance in liver metastasis. FAM120A and/or IL13Rα2 targeting abolished liver colonization in a mouse model.
MATERIALS AND METHODS

Cell culture and reagents.

KM12C and KM12SM human colon cancer cells were obtained from I. Fidler’s lab (MD Anderson Cancer Center. Houston, TX, USA). These cell lines were authenticated by short tandem repeat analysis. Other cell lines were obtained directly from the American Type Culture Collection (ATCC). These cell lines were passaged fewer than 6 months after purchase for all the experiments. All cell lines were cultured in DMEM (Gibco-Life Technologies) containing 10% fetal calf serum (Sigma-Aldrich) and antibiotics at 37ºC in a 5% CO2-humidified atmosphere.

IL-13 was used at 10 ng/ml and purchased from PeproTech. PP2 (used at 3 µM), PP3 (3 µM) and UO126 (5 µM) inhibitors were from Calbiochem. LY294002 (25 µM) was from Sigma-Aldrich and FAK inhibitor 14 (10 µM) from Santa Cruz Biotechnology. All antibodies used in this article are listed in Supplementary Table S1.

siRNAs transfections

For transient transfections, siRNAs targeting specifically FAM120A (SASI-Hs01-00149752, Sigma-Aldrich) or control siRNAs were transfected with JetPrime (Polyplus Transfection) according to manufacturer’s instructions.

Western blot and immunoprecipitation assays.

Cells were detached, washed and lysed with protease and phosphatase inhibitors in lysis buffer (1% Igepal, 100 mM NaCl, 2 mM MgCl2, 10% Glycerol in 50 mM Tris-HCl). Protein extracts were separated in SDS-PAGE, transferred to nitrocellulose membranes, which were incubated with primary antibodies (Supplementary Table S1) followed by incubation with either HRP-anti-mouse IgG (Thermo Scientific) or HRP-anti-rabbit IgG.
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(Sigma-Aldrich). Specific reactive proteins were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

For immunoprecipitation, cells were lysed and 500 μg of cell lysate were incubated with the indicated antibodies. The immunocomplex was captured by adding 100 μL of Protein G-sepharose beads (Sigma-Aldrich). After washing, samples were resuspended in 2x Laemmli buffer, boiled for 5 min, centrifuged and subsequently loaded onto SDS-PAGE gels, which were analyzed by western blot. As a control, we incubated the lysates with an unrelated immunoglobulin G coupled to Sepharose beads to discard unspecific proteins.

**Mass spectrometry of immunoprecipitated proteins**

For proteomic analysis, 10 mg of cell lysates were immunoprecipitated as before, and the proteins were loaded in SDS–PAGE, which were divided in three slices for in-gel digestion with trypsin. Peptides were trapped onto a C18-A1 2 cm precolumn (Thermo Scientific) and separated on a Biosphere C18 column (NanoSeparations) using a flow rate of 250 nl/min in a 100 min gradient from 0–95% Buffer B (0.1% formic acid in acetonitrile) on a nanoEasy HPLC coupled to a nanoelectrospray ion source (Proxeon). Mass spectra were acquired in a linear ion trap quadrupole (LTQ) Orbitrap Velos in data-dependent mode with an automatic switch between mass spectrometry and MS/MS scans using a top 15 method. Full scans were acquired in the Orbitrap with a mass range of 400 to 1200 Th, a target value of 10^6 ions and a resolution of 3 x 10^4 (at m/z 400). The 15 most intense ions were submitted to collision induced dissociation in the LTQ using normalized collision energy of 35% and a target value of 10^4 ions. Dynamic exclusion was enabled with a repeat count of one and exclusion duration of 30 s. Mass spectra were searched using SEQUEST search engine with Proteome Discoverer (Thermo Scientific) against the Uniprot Database. Search parameters included precursor and fragment mass tolerances of 10 p.p.m. and 0.8 Da,
respectively, a maximum of two missed cleavages allowed, a fixed modification of carbamidomethyl cysteine and a variable modification of methionine oxidation. Identified peptides were validated using Percolator algorithm with a q-value threshold ≥ 0.01.

**Immunohistochemistry analysis**

Paraffin samples were obtained from 119 patients diagnosed and treated of colorectal adenocarcinoma between 2001 and 2014 in Fundación Jiménez Díaz (Madrid). Clinico-pathological data are shown in supplementary Table S2. Each sample was deparaffinated for antigen retrieval using citrate sodium buffer at pH 6.0 for 20 min and subsequent incubation with the primary antibody against FAM120A. The reaction was revealed using DAB as chromogen and hematoxylin for counterstaining. In all cases, sections from normal colonic mucosa distant from the tumor site were used as negative controls.

**Wound Healing.**

Cells were cultured to confluence in Matrigel-coated plates (0.4 µg/mm²). A 1 mm-wide wound was done across the monolayer. Cells were incubated in serum-free medium containing IL-13, inhibitors and antibodies. Pictures were taken either immediately (0 h) or after 48 h in culture at 37°C after the injury. Migration was quantified as percentage of wound closure.

**Invasion assays.**

For Matrigel invasion assays, 6 x 10⁴ KM12C or KM12SM cells were resuspended in invasion medium (serum-free DMEM containing 0.4% BSA) and loaded onto 8 µm pore-size filters coated with 35 µl of Matrigel (1:3 dilution) (BD Biosciences) in Transwell plates (Costar) in presence of inhibitors or antibodies. The lower compartment of the invasion chamber was filled with medium containing IL-13 or with medium alone. After 22 h at 37°C, non-invading cells were removed from the upper surface of the filter, and cells
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that migrated through the filter were fixed with 4% paraformaldehyde (Sigma-Aldrich), stained with crystal violet and counted under a microscope.

Survival assays.

Cells were incubated with 1 mM H₂O₂ for 16 h without serum in the presence or absence of IL-13, antibodies or inhibitors. Cells were detached and incubated with FITC labeled-Annexin V (Miltenyi Biotec Inc.) and propidium iodide according to manufacturer’s instructions, and analyzed by cytofluorometry (Coulter Epics XL).

Flow cytometry.

Cells were detached with 2 mM EDTA in PBS, incubated at 4 °C with primary antibodies (10 µg/mL) for 30 min, washed and incubated with Alexa 488 labeled-secondary antibodies (Dako). Fluorescence was analyzed in a Coulter Epics XL cytofluorometer.

Immunofluorescence

KM12SM cells were cultured onto Matrigel-coated coverslips, treated with or without IL-13 (10 ng/ml) for 5 min and fixed with 1% paraformaldehyde (Sigma-Aldrich) in PBS containing 0.1% Triton X-100. Cells were washed three times with PBS and incubated 30 min with primary antibodies (supplementary Table S1), washed again and incubated 25 min with secondary antibodies labeled with Alexa-468 or Alexa-568, and 4’,6-diamidino-2-phenylindole (Life Technologies). Then, samples were mounted with Mounting Fluorescence Medium (Dako) and images were captured using a TCS-SP5-AOBS confocal microscope (LEICA) with a 100x oil immersion objective.

Metastasis experiments in nude mice.

The Ethical Committee of the Consejo Superior de Investigaciones Científicas (Madrid, Spain) approved the protocols used for experimental work with mice. Swiss nude mice (Charles River) (n = 10 per condition) were inoculated in spleen with 10⁶ KM12SM cells in
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0.1 ml PBS. After 22 h, mice were subjected to removal of the spleen. Mice were daily inspected for signs of disease, such as abdominal distension, locomotive deficit, or tumor detectable by palpation. When signs were visible, mice were euthanized, subjected to necropsy and inspected for metastasis in liver.

For liver colonization assessment, mice were inoculated as before and euthanized at various times after cells inoculation (2, 8, 24 and 56 h). RNA was isolated from liver using Trizol (Invitrogen), retrotranscribed and 0.3 μg cDNA subjected to PCR with Taq DNA polymerase (Invitrogen) to amplify human GAPDH as previously described (14). As a control, a 20 cycles-amplification of murine β-actin was performed.

Statistical analyses.

Data were analyzed by one-way ANOVA followed by Tukey-Kramer multiple comparison test. The significance of the difference for survival curves was estimated with the log-rank test, whereas the differences in early recurrence were analysed by Chi-square test. In all analyses the minimum acceptable level of significance was $p < 0.05$. 
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RESULTS

Identification of protein partners for IL13Rα2

To characterize the protein interaction network of IL13Rα2, cell lysates of the highly metastatic cell line KM12SM were immunoprecipitated using anti-IL13Rα2 monoclonal antibody coupled to Sepharose beads. An irrelevant antibody was used as a control. Precipitated proteins were fractionated by SDS–PAGE followed by mass spectrometry analysis. We identified 158 proteins co-immunoprecipitated exclusively with anti-IL13Rα2 antibody, using Percolator with q-value ≥ 0.01 (Fig. 1A). Proteins within a location biologically inaccessible for a membrane protein (such as nuclear, mitochondrial and peroxisomal) or those proteins involved in irrelevant protein-binding functions as ribosomal proteins, chaperons and common background proteins (15) were removed from further analysis. At the end, 5 proteins (Supplementary Table S3) were selected as the best candidates for specific interaction with IL13Rα2. In addition, we identified other 22 proteins involved in vesicle trafficking from Golgi and endoplasmatic reticulum to cell membrane (Supplementary Table S4). One of the 5 proteins, FAM120A (Family with sequence similarity 120A), a scaffold protein, was related to the Src family kinases (16), which were already known to be involved in IL13Rα2 signaling (2). Amongst the other four identified proteins were EPHB4, a receptor tyrosine kinase that mediates ephrin signaling; CMTM6, an unknown transmembrane protein and PDZD8 and ACTB proteins, which are involved in cytoskeletal organization (Fig. 1B).

The association of FAM120A with IL13Rα2 was confirmed by co-immunoprecipitation in four human colorectal cancer cell lines and further detection by Western blot (Fig. 1C, Fig. 2D). Expression of FAM120A was demonstrated in a panel of nine human colorectal cancer cell lines. All of them were positive, except Caco2 (Fig. 1D).
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To study the clinical relevance of FAM120A, we carried out immunohistochemical analysis of 119 paired colorectal cancer patient samples. We observed cytoplasmic staining of FAM120A (ranging from low/moderate (FAM120A<sub>lo</sub>) to intense (FAM120A<sub>hi</sub>)) in 55% of tumor samples, whereas adjacent normal tissue samples showed a very weak expression (Fig. 1E). Patients with tumors expressing high levels of both, IL13Rα2 and FAM120A, showed a significantly increased risk of recurrence at 18 months (Fig. 1F) compared to the rest of patients. These results indicate a significant overexpression of FAM120A in colon cancer, and the association of such overexpression with a higher risk of early recurrence in combination with IL13Rα2.

**Identification of FAM120A protein networks**

To identify protein networks containing FAM120A, KM12SM lysates were subjected to immunoprecipitation using anti-FAM120A antibodies followed by mass spectrometry. After discard proteins co-immunoprecipitated with control beads and those placed in inaccessible place for a cytosolic protein, 222 proteins were found to interact with FAM120A (Fig. 2A). As before, we removed 131 proteins based on their protein-binding functions (i.e. ribosomal, chaperones, etc). From the remaining 91 proteins, 48 were involved in protein transport. This large number of proteins involved in vesicle trafficking, not commonly associated with cytosolic proteins, suggests an additional role for FAM120A in protein transport (Supplementary Table S5).

The remaining 43 proteins associated with FAM120A (Supplementary Table S6) were subjected to GO analysis (Fig. 2B). FAM120A interactors were involved in actin cytoskeleton dynamics, cell signaling, apoptosis and cell adhesion. According to STRING interaction analysis and data mining, FAM120A forms part of different protein complexes,
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such as focal adhesion complexes, G-protein coupled receptors and TNFRSF10B receptor (also known as TRAILR2) (Fig. 2C). A selection of the proteins coimmunoprecipitated with FAM120A, together with other proteins involved in signaling, cell adhesion, cell death and actin cytoskeleton dynamics (FAK, Src, HCK, RAC, RHOA, PI3K, AKT, RAF1, ERK1/2 and CASP8) were confirmed for association with FAM120A by immunoprecipitation and western blot (Fig. 2D). No association was found between the adaptor protein FAM120A and IL13Rα1or Ras, in agreement with data obtained by mass spectrometry.

To study subcellular localization of IL13Rα2 and FAM120A we carried out confocal microscopy analysis (Fig. 2E). IL13Rα2 showed an intracellular distribution, with some surface staining, whereas FAM120A staining was cytosolic and associated to membrane. In addition, we tested AKT as surrogate for signaling interaction. There was a clear co-localization of IL13Rα2 with FAM120A and AKT, showing a slight increase for AKT after 5 min of IL-13 treatment.

Together, these results confirm the co-localization of IL13Rα2 with FAM120A and other signaling molecules.

**IL-13 signaling through IL13Rα2 requires FAM120A**

We explored the kinetics of IL-13 signaling through IL13Rα2 in KM12SM cells. IL-13 induced the activation of mTOR, FAK, Src family kinases, AKT, and ERK1/2, along with a decrease of p53 levels (Fig. 3A). Then, we studied the interaction of cell signaling proteins with FAM120A upon treatment with IL-13 (Fig. 3B). FAK, PI3K, Src, Fyn, AKT and p53 showed a quick association with FAM120A at 5 min after treatment with IL-13 and further dissociation at 60 min. ERK1/2 MAP kinases showed also association to FAM120A after 5
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min, but remained associated for longer times. While RHOA decreased, RAC increased with time, supporting the high invasive capacity of these cells. In contrast, IL13Rα2 remained permanently associated with FAM120A.

To study the role of FAM120A in IL-13 signal transduction in colon cancer, KM12SM cells were FAM120A-silenced with specific siRNAs or treated with scrambled siRNAs (Fig 3C). Silencing caused a loss of mTOR, FAK and AKT activation after IL-13 treatment. In contrast, activation of Src family kinases and ERK1/2 were not affected by FAM120A silencing (Fig. 3D). To examine the effect of Src in IL-13 signaling, we used the PP2 inhibitor in KM12SM cells treated with IL-13. PP2 caused a marked inhibition of mTOR, AKT and ERK1/2, but not FAK (Fig. 3E). In contrast, PP3 a mock inhibitor, did not affect Src pathway activation. Together, these results indicate that IL-13 signaling requires Src activation for AKT/PI3K activation together with FAM120A. However, Src activation was FAM120A-independent.

FAM120A is required for IL-13-induced cell migration, invasion and survival.

To characterize FAM120A in cell invasion and metastasis induced by IL-13, we tested two metastatic cell lines, KM12SM and SW620. After FAM120A silencing, KM12SM or SW620 cells knocked-down for FAM120A or scrambled were treated with IL-13 alone or combined with Src, PI3K, FAK and MEK inhibitors. As a control, we performed the experiments in the presence of anti-IL13Rα2 or control antibodies competing with IL-13 for IL13Ra2 binding (17). IL-13 provoked a strong increase in the migratory and, in particular, invasive capacity of KM12SM and SW620 cells (Fig. 4A, B). This increase was abolished after FAM120A silencing. In SW620, the silencing of FAM120A inhibited also basal migration, indicating that the presence of this protein is required for cell motility.
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Treatment with Src, PI3K and, at a minor extent, FAK inhibitors caused a similar effect to FAM120A silencing and similar to anti-IL13Rα2 blocking. Regarding cell survival, IL-13 induced a significant increase in viable KM12SM cell number in control cells, but not in FAM120A-silenced cells. IL-13-mediated increase in cell survival was inhibited by all the inhibitors and, more strongly, by PI3K and FAK inhibitors (Fig. 4C). In SW620 cells, IL-13 also promoted cell survival; but this effect was more moderate, probably because SW620 cells had a strong basal resistance to oxidative stress. Collectively, these data indicate that FAM120A, Src, PI3K and FAK are critical mediators in IL-13-induced cell migration, invasion and survival.

FAM120A controls IL13Rα2 location in cell membrane.

We also investigated the role of FAM120A in protein trafficking and location. By flow cytometry, KM12SM and KM12C cells knocked-down for FAM120A displayed lower levels of IL13Rα2 in cell membrane (Fig. 5A). However, the total amount of IL13Rα2 was identical in FAM120A-silenced or control cells by western blot (Fig. 5B). These results suggest that FAM120A participates in the traffic and location of IL13Rα2 in cell surface and, therefore, in the functionality of this receptor. To provide further evidence, we verified the association of FAM120A with some of the identified proteins involved in protein traffic, such as MYH14, COPE or Rab1A/B GTPase by western blot (Fig. 5C). In addition, treatment of KM12SM cells with anti-IL13Rα2 antibody showed that anti-IL13Rα2 promoted IL13Rα2 degradation (Fig. 5D), probably by a mechanism of cell internalization mediated by lysosomes. This effect could contribute to the blocking capacities of the anti-IL13Rα2 antibody.
FAM120A plays a key role in liver metastasis of colorectal cancer

Finally, we studied the effect of FAM120A in cancer metastasis *in vivo*. FAM120A-silenced or control KM12SM cells, in the presence of anti-IL13Rα2 or control antibodies, were inoculated in the spleen of nude mice. Spleens were removed 24 h after inoculation, to prevent the formation of tumors in spleen. About 60% of mice inoculated with control cells and control antibody showed severe signs of disease and were euthanized (Fig. 6A). Subsequent necropsy revealed macroscopic metastasis in liver (Fig. 6B). In contrast, mice inoculated either with FAM120A knocked-down cells or treated with anti-IL13Rα2 antibodies did not develop disease, except for a mouse inoculated with FAM120A knocked-down cells, which developed a tumor in peritoneal cavity without liver metastasis (Fig. 6A). After 120 days post-inoculation, surviving mouse did not show macroscopic metastasis in liver (Fig. 6B).

In addition, time-assays were performed to analyze the behavior of tumor cells in the first three days after inoculation. For that purpose, mice were euthanized between 2 and 56 h after inoculation and RNA was isolated from the livers. After PCR amplification, human GAPDH, as a surrogate marker, was detected 2 h after inoculation in all livers at similar levels, independently of the treatment. This quick detection was probably due to fluid flow and cell travelling via the portal circulation to the liver within minutes. After 8 h, PCR amplification showed a marked reduction, except for control mice. Moreover, bands were barely discernible at 24 or 56 h after inoculation for cells transfected with FAM120A siRNAs or treated with anti-IL13Rα2 antibodies, except for control siRNAs and control antibodies (Fig. 6C). These results suggest that FAM120A and IL13Rα2 are required for IL-13-mediated engraftment or colonization of colorectal cancer cells, increasing tumor cell adhesion and survival in the liver after cell arrival.
**DISCUSSION**

In this report we describe a key role for FAM120A, also known as OSSA (Oxidative stress-associated Src activator) or C9orf10 (16), in the signal transduction of IL-13 through IL13Rα2 for the migration and invasion of colon cancer cells. FAM120A was identified as a scaffold protein that interacts with IL13Rα2 using mass spectrometry and coimmunoprecipitation experiments. FAM120A is an abundant cytosolic protein that showed association with, at least, three protein networks involved in focal adhesion, GPCR and TRAIL receptors. The expression of FAM120A was required for the IL-13-triggered Src and PI3K/AKT/mTOR signaling pathway activation (Fig. 7). Besides its scaffold activity, FAM120A was associated with proteins involved in motor proteins (kinesins) involved in cargo movement that could modulate the presence of IL13Rα2 on the cell surface or its secretion (Fig. 7). Finally, the blocking of IL13Rα2 with specific antibodies or the silencing of FAM120A strongly decreased metastatic growth and dissemination in colon cancer cells, providing two powerful independent strategies for metastasis control.

The binding of IL-13 triggers the recruitment of several molecules to the IL13Rα2-FAM120A complex, such as FAK, PI3K, AKT, Src, p53, ERK1/2, and changes in the small GTPases associated to the complex (RAC instead of RHOA). FAM120A recruits PI3K and works as a scaffold protein for PI3K and the Src family kinases (SFKs), enabling phosphorylation and activation of PI3K (16). Silencing of FAM120A impaired the activation of PI3K, AKT and mTOR in the metastatic cells. However, it did not alter the activation of SFKs as the activation levels, based on its phosphorylation status, are similar in FAM120A-silenced cells than in control siRNA transfected cells. The activation of AKT was dependent of Src kinase, confirming that FAM120A is the scaffold required for Src-mediated PI3K activation, as previously reported (16). Along this line, a protector role to
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guard cells from oxidative stress-induced apoptosis by activation of SFKs was given to FAM120A (16). In addition, FAM120A could recruit FAK into the IL13Rα2 complex to facilitate their mutual activation. Probably, transient FAK dimerization leads to autophosphorylation in Tyr397, which increases the catalytic activity of FAK (18, 19). The presence of β1 integrin together with FAK in the FAM120A network favors metastasis and cancer progression due to the activation of the integrin signaling pathway that promotes tumor growth (20, 21). Activation of both pathways by IL-13 in colon cancer cells required the presence of FAM120A.

Scaffold proteins modulate cell signaling by tethering individual pathway components together and isolating different pathways from one another. Scaffold proteins can establish connections between pathways in order to distribute signals (22). Furthermore, many scaffold proteins interact with kinesin proteins and are involved in cargo movement (23). In our proteomic analysis, we identified a large number of proteins involved in transport, including kinesin proteins (KIF). Knocking-down FAM120A decreased IL13Rα2 presence on cell surface, without altering total IL13Rα2 expression. Therefore, FAM120A would play a dual role: i) IL13Rα2 transport and location and ii) bringing together IL13Rα2 with FAK, PI3K and SFKs. Since FAM120A modulates IL13Rα2 expression on cell surface, FAM120A silencing would impair IL13Rα2 access to cell membrane, avoiding the interaction with IL-13 and precluding signaling pathway activation.

Regarding other protein networks, FAM120A was also associated to TNFRSF10B (a pro-apoptotic death receptor for TRAIL) and caspase 8. The binding of TRAIL to TNFRSF10B leads to caspase activation and initiation of apoptosis (24). Further investigation is required to clarify if IL13Rα2 contributes to resistance to apoptosis and
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survival by sequestering FAM120A from the TRAIL complex in addition to the activation of PI3K. On the other hand, TRAIL signaling promotes a proinflammatory pathway (25) that can establish a relationship with a similar IL-13 activity. Finally, FAM120A showed an association with GPR56, a G-protein coupled receptor for type III collagen, which activates the RHOA pathway (26). RHOA overexpression is usually associated with elevated lethality and aggressive proliferation (27, 28). Therefore, IL-13 might have different roles, through FAM120A, in the activation of different pathways that not only promote cell migration and invasion, but adhesion, survival and proliferation. These events facilitate the colonization by tumor cells and the subsequent formation of metastasis. The identification of IL13Rα2 signaling should help to understand the signaling events of IL-13 in other cancers, asthma, ulcerative colitis and other diseases.

In summary, FAM120A is a scaffold protein required for the proper IL13Rα2-trigger signaling, which leads to colon cancer cell activation and liver metastasis. FAM120A modulates IL13Rα2 location and transport between the cell membrane and intercellular compartments as well as the activation of different signaling pathways. Our results with a metastatic mouse model suggest that the silencing of FAM120A is as effective as the treatment with anti-IL13Rα2 blocking antibodies (17) in preventing liver colonization by colorectal cancer cells and the formation of macroscopic liver metastases. These results support the use of FAM120A as potential target for colon cancer therapy and reinforce the therapeutic value of IL13Rα2.
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LEGENDS TO FIGURES

Figure 1. FAM120A associates with IL13Rα2. A, classification of IL13Rα2-coimmunoprecipitated proteins detected by proteomics assays in KM12SM cells, according to their location (left) and function (right). B, protein interaction network for IL13Rα2. C, verification of FAM120A-IL13Rα2 interaction by immunoprecipitation and western blot. D, expression of FAM120A in the indicated human colorectal cancer cell lines by western blotting. E, by immunohistochemistry, 55% of colon cancer patients (n = 119) expressed FAM120A in tumor samples (25% showed low/moderate expression (FAM120Alo) and 30% high expression (FAM120Ahi)). IL13Rα2 was positive in 65% of patient samples (left). Representative images of FAM120A expression intensity in colorectal cancer samples and adjacent normal colon tissue of three patients obtained by immunohistochemistry (right). F, patients with tumors co-expressing IL13Rα2 and high levels of FAM120A showed an increased risk of recurrence at 18 months (*, p < 0.05).

Figure 2. Proteins coimmunoprecipitated with FAM120A. A, classification FAM120A-coimmunoprecipitated proteins detected by proteomics assays in KM12SM cells. B, function of proteins that interact with FAM120A. C, representation of FAM120A-coimmunoprecipitated proteins according to different functional clusters (cell protrusion, GPCR and death receptor). D, confirmation of FAM120A-coimmunoprecipitated proteins by immunoprecipitation and western blot. E, KM12SM cells treated or not with IL-13 were used to determine IL13Rα2, FAM120A and AKT localization by immunofluorescent confocal microscopy. IL13Rα2 showed membrane and intracellular expression with a clear co-localization with FAM120A and AKT.

Figure 3. Signaling triggered by IL-13 is partly controlled by FAM120A. A, KM12SM cells were exposed to IL-13 for the indicated times, lysed, and the extracts were resolved in
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SDS-PAGE and analyzed with the indicated antibodies by western blot. RHOGDI was used as loading control. B, western blot analysis showing the results of KM12SM cells treated with IL-13 for 0, 5 and 60 min and subjected to immunoprecipitation using anti-FAM120A or control antibodies. C, western blot analysis showing the results of KM12SM cells transfected with FAM120A or control siRNAs together with IL-13 for the indicated times. D, knocked down KM12SM cells or control cells were exposed to IL-13 for 48 h, lysed and analyzed as in (A). E, western blot analysis showing the results of cells treated with Src-inhibitors PP3 or PP2 and IL-13 for the indicated times.

**Figure 4. FAM120A controls IL-13-triggered cell migration, invasion and survival.** KM12SM and SW620 cells were transfected with control or FAM120A-targeting siRNAs and subjected to A, wound healing assays, B, cell invasion assays through Matrigel and C, survival to oxidative stress induced by H2O2. In addition, KM12SM cells were treated with anti-IL13Rα2 or control antibodies (5 μg/mL) and the indicated inhibitors. Cell migration/invasion/survival was significantly increased by addition of IL-13 (*, p < 0.05; **, p < 0.01; ***, p < 0.001) and decreased by treatment with the indicated inhibitors or antibodies (◊, p < 0.05; ◊◊, p < 0.01; ◊◊◊, p <0.001). D, western blot analysis showing the results of SW620 cells transfected with FAM120A or control siRNAs.

**Figure 5. FAM120A regulates IL13Rα2 expression levels in cell membrane.** A, Flow cytometry analysis showing the expression of IL13Rα2 in the surface of KM12C and KM12SM cells FAM120A-silenced or control. B, western blot was performed to detect the lack of changes in total IL13Rα2 in the same cells after FAM120A silencing. RHOGDI was used to assess total protein content. C, verification of FAM120A-interacting proteins involved in protein transport after immunoprecipitation and western blot. D, western blot analysis showing the results of KM12SM cells treated with anti-IL13Rα2 or control...
IL13Rα2 signaling in colorectal cancer metastasis antibody for the indicated times. A marked decrease of IL13Rα2 after antibody treatment was observed after 6 h.

**Figure 6. FAM120A and IL13Rα2 are required for liver homing and metastasis.** A, Kaplan-Meier survival results for nude mice inoculated in spleen with KM12SM cells previously transfected with FAM120A or control siRNAs and treated with anti-IL13Rα2 or control antibody at 5 μg/mL. Either silencing of FAM120A or treatment with anti-IL13Rα2 antibody significantly increased mice survival (*, p < 0.05; **, p < 0.01). B, percentage of mice with macroscopic metastasis in liver was significantly reduced in mice inoculated with FAM120A-silenced or anti-IL13Rα2-treated KM12SM cells (p < 0.01) (left). Images of livers of mice treated as in (A) showing metastasis (right). C, at the indicated times, RNA was isolated from livers of inoculated mice and subjected to RT-PCR assays to detect human GAPDH as surrogate of cell colonization. Murine β-actin was used as loading control.

**Figure 7. Dual role of FAM120A in IL13Rα2-driven metastasis of colorectal cancer cells.** FAM120A showed a dual role in IL13Rα2-driven metastasis. On the one hand, it promoted IL13Rα2 location in the cell membrane, and, then, after activation of the receptor by IL-13 binding, it recruited other cell signaling proteins to promote different biological events leading to liver colonization and survival.
**Figure 1**

A. Pie charts showing protein interactions with IL13Rα2.

B. Schematic representation of protein interactions with IL13Rα2.

C. Western blot analysis of FAM120A and IL13Rα2.

D. Western blot analysis of FAM120A and/RHOGDI in different cell lines.

E. Bar graph showing the percentage of patients with normal tissue and colorectal cancer.

F. Bar graph showing the percentage of patients with recurrence at 18 months.

(Additional details and annotations as per the original figure.)
Figure 2
**Figure 3**

(A) Western blots showing the expression of various proteins at different time points (0, 1, 2.5, 5, 10, 15, 30, 60 min) after IL-13 stimulation. The proteins include p-Ser2448-mTOR, mTOR, p-Tyr397-FAK, FAK, p-Tyr416-SFK, Src, HCK, FYN, p-Ser473-AKT, AKT, p-Ser15-P53, P53, p-Thr202-ERK1, p-Tyr204-ERK2, ERK1, ERK2, RHOGDI.

(B) Western blots showing the expression of proteins after IP: Control FAM120A and IP: Control FAM120A.

(C) Western blots showing the expression of FAM120A and RHOGDI after IP: Control FAM120A.

(D) Western blots showing the expression of various proteins (p-Ser2448-mTOR, mTOR, p-Tyr397-FAK, FAK, p-Tyr416-SFK, Src, FYN, p-Ser473-AKT, AKT, p-Ser15-P53, P53, p-Thr202-ERK1, p-Tyr204-ERK2, ERK1, ERK2, RHOGDI) at different time points (0, 5, 60 min) for Control siRNA and FAM120A siRNA.

(E) Western blots showing the expression of various proteins (p-Ser2448-mTOR, mTOR, p-Tyr397-FAK, FAK, p-Tyr416-SFK, Src, FYN, p-Ser473-AKT, AKT, p-Ser15-P53, P53, p-Thr202-ERK1, p-Tyr204-ERK2, ERK1, ERK2, RHOGDI) at different time points (0, 5, 60 min) for PP3 and PP2.
Figure 4

A. KM12SM

B. KM12SM

C. KM12SM

D. siRNA:

- Control
- FAM120A siRNA

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Figure 5
Figure 6

A. Percent survival over days after inoculation for different treatment groups: Control siRNA + Control antibody, FAM120A siRNA + Control antibody, Control siRNA + anti-IL13Rα2, FAM120A siRNA + anti-IL13Rα2.

B. Percentage of mice with macroscopic metastasis for different treatment groups: siRNA Control + Control antibody, siRNA FAM120A + Control antibody, siRNA Control + anti-IL13Rα2, siRNA FAM120A + anti-IL13Rα2.

C. Western blot analysis of liver samples showing expression levels of hGAPDH and β-actin for different time points (2 h, 8 h, 24 h, 56 h) and treatment groups: siRNA Control, siRNA FAM120A, siRNA Control, siRNA FAM120A.
Figure 7

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IL-13 receptor α2 signaling requires a scaffold protein, FAM120A, to activate the FAK and PI3K pathways in colon cancer metastasis

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