Deorphanization of the human leukocyte tyrosine kinase (LTK) receptor by a signaling screen of the extracellular proteome

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There are many transmembrane receptor-like proteins whose ligands have not been identified. A strategy for finding ligands when little is known about their tissue source is to screen each extracellular protein individually expressed in an array format by using a sensitive functional readout. Taking this approach, we have screened a large collection (3,191 proteins) of extracellular proteins for their ability to activate signaling of an orphan receptor, leukocyte tyrosine kinase (LTK). Only two related secreted factors, FAM150A and FAM150B (family with sequence similarity 150 member A and member B), stimulated LTK phosphorylation. FAM150A binds LTK extracellular domain with high affinity ($K_D = 28$ pM). FAM150A stimulates LTK phosphorylation in a ligand-dependent manner. This strategy provides an efficient approach for identifying functional ligands for other orphan receptors.

leukocyte tyrosine kinase | extracellular protein | library screening | FAM150A | orphan receptor

Many biological processes and pathogenic conditions involve ligand/receptor signaling. There are numerous transmembrane receptor-like proteins whose ligands have not been identified. Most known ligand–receptor interactions were discovered by painstaking protein purification, genetic approaches, or sequence homology. Strategies using cDNA library expression—such as secretion trapping (1), mammalian expression cloning (2), yeast signaling display (3), and λ phage binding display (4)—have also identified ligands for orphan receptors. However, these approaches are limited by the unknown comprehensiveness of the cDNA library and by the fact that the libraries are biased toward high-abundance transcripts and not focused on the extracellular proteome. An alternative strategy is to screen secreted factors that are individually expressed in an array format in which there is no bias in expression of each protein based on the abundance of its cDNA. The advantages of this approach for deorphanization of receptors are that it is extremely sensitive and selective and that it covers the majority of the extracellular proteome. Although arrayed proteins have been used to identify receptor partners with low-affinity, high-avidity interactions (5–7), few studies have used a cell-signaling measurement to study "classic" high-affinity ligand–receptor interactions.

Leukocyte tyrosine kinase (LTK) is a receptor tyrosine kinase that was identified in 1988 (8). The extracellular domain (ECD) of LTK has no known domain structure except a glycine-rich region, leading to a proposal that LTK is not activated by a protein ligand. LTK has a close homolog named anaplastic lymphoma kinase (ALK) (9). The LTK pathway has been implicated in autoimmunity, neuronal development, and cancer. One study showed that gain-of-function polymorphism of Ltk has been associated with systemic lupus erythematosus (SLE) pathogenesis (10). Ltk is expressed throughout the adult hippocampus, and mouse knockout studies indicated that both Ltk and Alk are involved in adult neurogenesis with some functional redundancy (11). Loss-of-function studies showed that LTK may

have function in mammalian neuronal development. In addition, a high-throughput kinase expression study showed that LTK expression is increased in acute myeloid leukemia patients (12). It is likely that LTK signaling is involved in multiple biological and pathological processes. The identification of the ligand(s) for LTK should help decipher its function.

In this study, we have taken the strategy of screening most of the extracellular proteome by using a large collection of individually expressed secreted proteins, tested for their ability to activate signaling of an orphan receptor, LTK. We set up a signaling assay for LTK and screened the extracellular proteome (3,191 extracellular proteins) for its ligand(s). We identified two related ligands [family with sequence similarity 150 member A and member B (FAM150A and FAM150B)] that stimulate LTK phosphorylation. FAM150A binds LTK with high affinity (28 pM). The finding will further facilitate revealing the biological function of the LTK signaling pathway, both in physiological and pathological conditions. A similar strategy could be used to deorphanize ligands of other types of receptors.

Results

An Extracellular Proteome Signaling Screen Identified FAM150A and FAM150B as LTK Ligands. The lack of information regarding the cellular source of the LTK ligand has added to the difficulty in its identification. We have taken the approach of screening thousands

Significance

Secreted factors and their cell-surface receptors play important roles in the communication between cells in normal and pathological conditions. There are many transmembrane receptor-like proteins whose ligands have not been identified (also known as orphan receptors). Knowledge of the ligand should help in understanding the biological role of the receptor. We used a strategy of screening the extracellular proteome, one protein at a time, to identify ligands for such receptors. We discovered the ligands for the orphan receptor leukocyte tyrosine kinase. To our knowledge, this is the first case in which secreted factor ligands were identified for an orphan receptor with this technique. This approach is especially valuable when little is known about the ligand.

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of extracellular proteins derived from numerous human tissues for their ability to activate LTK. Using similar methods that have been described (13), we constructed full-length cDNAs encoding 3,191 extracellular proteins, including "classically secreted" proteins (1,795), soluble ECDs of type I transmembrane proteins (962), soluble ECDs of type ll transmembrane proteins (335), and soluble ECDs of glycosylphosphatidylinositol-anchored proteins (99). The proteins encoded by each cDNA were produced by described methods (13) and formatted for screening (see below).

To test each extracellular protein for its ability to activate LTK, we developed a quantitative signaling assay for LTK (Fig. 1A). We observed that HEK293 cells responded to signaling from the LTK intracellular domain because a chimeric epidermal growth factor (EGF) receptor–LTK receptor could signal when transfected into HEK293 cells (14). Therefore, a reporter cell line was generated by transfecting a full-length LTK, with an HA epitope tag (amino acid sequence: YPYDVPDYA) at the C terminus. The activation of the LTK receptor was analyzed by receptor immunoprecipitation (IP) with anti-HA antibody followed by receptor phosphorylation detection with an antiphosphotyrosine antibody in an ELISA format. The assay was designed to quantitate phosphorylation of the receptor on tyrosine residues in the kinase domain following activation. Individual cDNAs for each of 3,191 secreted proteins were transiently transfected in separate wells as described (13). Each well of expressed protein supernatant was added to the HA-tagged LTK-overexpressing cell line, and an LTK phosphorylation ELISA was performed. The initial screening of the library of secreted proteins identified proteins with possible activity on the LTK receptor. The potential hits were retested with freshly prepared proteins in the LTK phosphorylation assay. The results of the screening are shown in Fig. 1B. Two related proteins showed significant activity in the assay, FAM150A and FAM150B (Fig. 1B). A third protein, insulin-like growth factor 2 (IGF2),

showed some activity, but later experiments by Biacore analysis showed that IGF2 did not bind LTK (*SI Materials and Methods* and Fig. S1). FAM150A induced LTK phosphorylation by >30-fold relative to an unrelated control protein (heparin-binding EGF-like growth factor; HB-EGF), and FAM150B induced LTK phosphorylation by a little more than twofold relative to HB-EGF (Fig. 1C). FAM150A induced LTK phosphorylation in a dose-dependent manner. The EC_{50} for FAM150A-induced LTK phosphorylation is 1.96 nM (Fig. 1D).

FAM150 Family and the Expression Patterns of FAM150A, FAM150B, and LTK. FAM150A and FAM150B do not have similarity with any other proteins, except similarities between themselves (47.4% identical; Fig. 2A). The family members are conserved among mammals. The amino acid sequence of human FAM150A (hFAM150A) shares a sequence identity of 72.1% with that of the mouse ortholog. The hFAM150B shares 74% identity with mouse FAM150B (mFam150B). The expression patterns of FAM150A and FAM150B in various human tissues were analyzed by quantitative RT-PCR (qRT-PCR), using gene-specific primers. Both FAM150A and FAM150B are relatively widely expressed (Fig. 2 B and C). FAM150A has the highest relative level of expression in thyroid, as well as moderate expression in stomach, trachea, small intestine, prostate, and brain (Fig. 2B). FAM150B message has a high relative level of expression in adrenal gland and modest levels of expression in pancreas, testis, and uterus (Fig. 2C). The expression of Fam150a, determined by RT-PCR in mice, has been published [SEC511N1 (15)]. Fam150a is expressed in mouse brain, spleen, lung, intestine, and skin. Human LTK-specific primers were used to study its expression in various immune cell types, with the highest expression in plasmacytoid dendritic cells (pDCs) (Fig. 2D). This expression
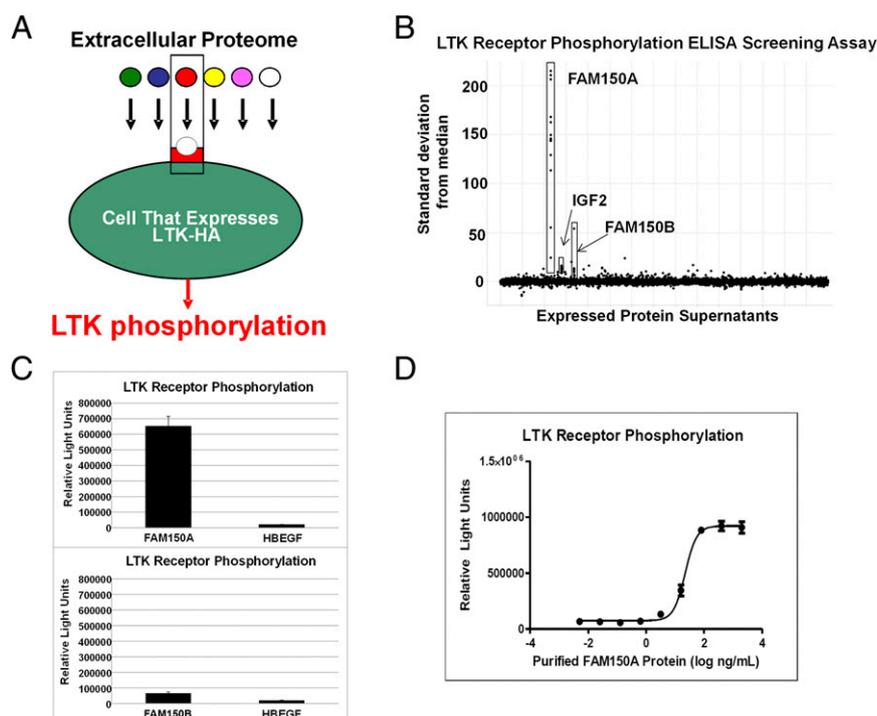
Fig. 1. Extracellular proteome signaling screening to identify LTK ligand(s). (A) Screening strategy: 293 cells stably transfected with LTK–HA were treated with the extracellular proteome library, one protein at a time. The readout is LTK phosphorylation by ELISA. (B) Relative LTK receptor phosphorylation is shown as the SD from the median chemiluminescent units within each assay plate (y axis) for each secreted protein (displayed on x axis). Each point within a boxed area is a technical replicate of individual wells of expressed protein from the same cDNA clone. (C) Relative activities of FAM150A and FAM150B. (D) Dose-dependent phosphorylation of LTK induced by purified FAM150A.

Fig. 2. Expression of FAM150 family (FAM150A and FAM150B) and LTK. (*A*) Homology between FAM150A and FAM150B. (*B*) FAM150A expression in a panel of human tissue RNA samples normalized to the housekeeping gene GUSB. Error bars indicate SD of duplicate data points. (*C*) FAM150B expression in the same tissue panel normalized to the housekeeping gene GUSB. Error bars indicate SD of duplicate data points. (*D*) LTK expression in human peripheral blood mononuclear cells (PBMCs). LTK RNA levels in each cell type were analyzed by qRT-PCR. Expression levels were normalized to 18S rRNA as indicated. Each RNA sample was assayed as a single value. Data shown are representative of at least two independent experiments with two different donors.

pattern is consistent with LTK expression data in the BioGPS database (16).

FAM150A Binds LTK-ECD with High Affinity. The binding affinity of hFAM150A to immobilized ECDs of human LTK and mouse Ltk was determined by Biacore analysis. FAM150A was produced by transient expression in 293 6E cells, followed by SP Sepharose HP column and Butyl Sepharose HP column purification. The purified FAM150A was a monomer, judged by its molecular weight. Multiple bands were present due to different glycosylation (Fig. 3*A*). ECDs of human LTK and mouse Ltk were produced as Fc fusion proteins. FAM150A bound to LTK-ECD-Fc with a dissociation

constant (K_D) of 28 pM (Fig. 3*B*). FAM150A also bound mouse Ltk-ECD-Fc, although with lower affinity ($K_D = 0.95$ nM).

FAM150A Stimulates LTK Phosphorylation. Purified FAM150A was used to stimulate 293 cells that overexpress the LTK–HA. Phosphorylation of LTK upon FAM150A stimulation is shown in Fig. 3*C*. There were other proteins phosphorylated, potentially LTK-associated proteins (Fig. 3*C*). We found that the neuroblastoma cell line SK-N-SH expresses full-length LTK. Western blot analysis of phosphorylated tyrosine of immunoprecipitated LTK showed that FAM150A simulates LTK phosphorylation (Fig. 3*D*). Crizotinib is a small-molecule tyrosine kinase inhibitor that has been shown to

	ka (1/Ms)	kd (1/s)	KD (nM)	Chi² (RU²)
Human LTK-MouseFc	1.0E+07	2.8E-04	0.028	0.39
Mouse LTK-HumanFc	8.5E+06	8.0E-03	0.95	0.44

Fig. 3. High-affinity binding of FAM150A to LTK–ECD-Fc and LTK stimulation by purified FAM150A. (*A*) Purification products of FAM150A. (*B*) Binding kinetics of FAM150A to human LTK–ECD-Fc and mouse Ltk–ECD-Fc fusion proteins by Biacore analysis. (*C*) Phosphorylation of LTK and downstream proteins after FAM150A stimulation in 293 cells overexpressing LTK–HA. (*D*) Phosphorylation of LTK and downstream proteins after FAM150A stimulation in SK-N-SH cells.

inhibit the kinase activity of ALK and c-Met by binding to their kinase domains. Because the LTK kinase domain is more homologous to ALK than c-Met, we reasoned that crizotinib should be able to inhibit LTK phosphorylation. Indeed, crizotinib inhibited the LTK receptor phosphorylation induced by FAM150A (Fig. 3D). To explore downstream signaling after FAM150A stimulation of LTK, we measured the downstream phosphorylation events after FAM150A stimulation. ERK1/2 was found to be phosphorylated upon FAM150A treatment, as shown in Fig. 3D.

Discussion

In this study, extracellular proteome signaling screening was used to identify a family of ligands for LTK, FAM150A and FAM150B. Although our data showed that FAM150A and FAM150B are expressed in various tissues, LTK expression is more restricted to certain cell types. Tissues in which FAM150A/FAM150B and LTK are highly expressed did not overlap. Because the expression pattern of the FAM150 ligands and LTK did not provide insight into their functional relationship, the extracellular proteome screening approach was uniquely suited to deorphanizing LTK. The biological role of the LTK pathway is not clear. The LTK gene has different isoforms and is expressed in various tissues (17–19). LTK is highly expressed in human pDCs (Fig. 2D and ref. 16). pDC is a cell type known to be crucial for SLE pathogenesis (20), and gain-of-function polymorphism of Ltk is implicated in SLE pathogenesis (10). The availability of a LTK ligand will aid the elucidation of LTK biology in SLE and potentially other conditions. Transgenic mice overexpressing Fam150a or Fam150b may provide some answers. Ltk is expressed throughout the adult hippocampus, and mouse knockout studies indicated that Ltk and Alk are involved in adult neurogenesis with some functional redundancy (11). Both hFAM150A (Fig. 2B) and mFam150a (15) are expressed in the brain. FAM150A, through LTK, may be involved in normal neuronal development, and aberrant expression may be involved in neurological disorders. Both thyroid (highest FAM150A expressing tissue; Fig. 2B) and adrenal gland (highest FAM150B expressing tissue; Fig. 2C) are endocrine tissues. This expression pattern may suggest that LTK has a functional role in endocrine pathways besides its roles in central nerve system and immunity or that LTK plays a role in autoimmune thyroid disease. The most homologous kinase receptor to LTK is ALK. ALK is a target for cancer therapy by a small-molecule kinase inhibitor (21, 22). Pleiotrophin and midkine have been proposed as its ligands (23, 24). Because of the homology between LTK and ALK, it is testable whether FAM150A or FAM150B will bind and activate ALK as a ligand. Identification of additional ALK ligand(s) would greatly facilitate further understanding of ALK biology.

To our knowledge, this is the first case in which secreted factor ligand(s) were identified for an orphan receptor, by using extracellular proteome signaling screening. Finally, with the availability of the extracellular proteome, this sensitive and selective strategy could be generalized to deorphanize other receptors. This approach is especially valuable when little is known about the biology or tissue source of the ligand.

Materials and Methods

Extracellular Proteome Signal Screening. Stable cell lines were generated in human HEK293 cells by transfection of the full-length LTK cDNA (codon-optimized sequence) with an HA tag at the C terminus in vector pcDNA5/FRT (Invitrogen). Transfected cells were generated by using the Flip-In plasmid system (Invitrogen) and selected for stable integration. Cells were grown and maintained at 37 °C in 5% CO_2 in medium comprising Eagle's Minimum Essential Medium (American Type Culture Collection), 10% (vol/vol) FBS (Corning Cellgro), 1% penicillin–streptomycin (Corning Cellgro), and 100 μg/mL hygromycin (Invitrogen). Supernatants enriched in FAM150A and FAM150B protein were generated by transfecting cDNAs into HEK293T cells in DMEM (Mediatech) supplemented with 5% (vol/vol) FBS (Mediatech no. 35-010-CV) with Fugene6 (Promega) following the manufacturer's recommended conditions for 40 h followed by a medium change into fresh medium for 48 h.

The supernatants from FAM150A- and FAM150B-transfected cells, as well as supernatant from a negative control protein, were collected and used to treat the LTK–HA-overexpressing cells.

Cells were plated at 50,000 LTK-293 cells per well in 175 μL of growth medium in a BD Biocoat Poly-D-Lysine Cellware 96-well plate (no. 356461) and incubated for 24 h at 37 °C in 5% CO_2. Serum starvation in minimum essential medium (MEM) (ATCC no. 30-2003) plus 0.1% FBS was performed for 24 h by removing the culture medium and replacing it with 175 μL of starvation medium. Cells were treated with the enriched protein expression supernatants by removing the starvation medium and replacing it with 100 μL of a 1:1 mix of expressed protein supernatant and fresh starvation medium. Cells were treated for 20 min in a 37 °C incubator. The cell lysates were generated by removing the medium completely and adding 75 μL of cold lysis buffer [10× Cell Lysis Buffer (Cell Signaling no. 9803) diluted to 1× in water] plus Roche complete mini EDTA-free protease inhibitor mixture (Roche 04-093-159-001) and Roche Phosstop phosphatase inhibitor (Roche 04-906-837-001) into each well. The lysates were frozen at −80 °C for later ELISA processing. The ELISA was performed by first coating ELISA plates [96-1/2 area wells, hi-bind, white, flat bottom ELISA Plates (E&K no. 78074; Greiner no. 675074)] with the capture antibody, a mouse monoclonal (HA.C5) to HA tag (Abcam ab18181). The monoclonal mouse anti-HA IgG Affinity Purified Antibody was diluted in PBS to 4.5 μg/mL, and 50 μL was dispensed per well. The plates were sealed and incubated overnight at 4 °C. Plates were washed three times with PBS 0.05%/Tween 20 (PBST) and then blocked with 180 μL per well of PBS plus 1% BSA buffer and incubated for 1 h at room temperature (RT). Plates were washed three times with PBST. For the capture step, lysates were thawed, and the samples were transferred to the ELISA half-well plates and incubated for 2 h at RT. Plates were washed six times with PBST. Fifty microliters per well of a 1:9,000 dilution of the antiphosphotyrosine horseradish peroxidase (HRP)-conjugated antibody (R&D, no. HAM1674) were added, and the plates were sealed and incubated for 1 h at RT. The plates were washed six times with PBST followed by the addition of 50 μL per well of luminescent substrate [SuperSignal ELISA Pico Chemiluminescent Substrate kit (Thermo no. 37069)], incubated for 2 min, and then read on an Envision instrument following the manufacturer's recommended settings. The primary screen of all 3,191 proteins was performed in duplicate. Hits were called and selected for an independent retest experiment if the SD from the assay plate median value was either >2.5 SDs for both replicates or an average of at least 5 SDs.

Gene Expression Analysis.

FAM150A and FAM150B expression. Human tissue RNA (Clontech) was reverse transcribed into cDNA following the manufacture's protocol (Qiagen). cDNA was diluted and distributed to parallel wells for qPCR using gene specific primers for FAM150A, FAM150B, or β-glucuronidase (GUSB) (Qiagen) and SYBR Green reagent (Qiagen). qPCR was performed following the manufacture's recommended protocol for a total of 40 cycles of 95 °C 15 s, 55 °C for 30 s, and 72 °C for 30 s Expression within each tissue was normalized to the relative expression of GUSB using the ΔΔCt method.
Human LTK expression. One-day-old buffy coats from normal human donors were obtained from the Stanford Blood Center, and peripheral blood mononuclear cells were isolated by Ficoll gradient centrifugation. Immune cells were further purified by using cell-type–specific MACS cell separation kits according to manufacturer's protocols (Miltenyi Biotec). For qRT-PCR, RNA was isolated from cells by using the RNeasy Plus Kit (Qiagen), and qRT-PCR was performed by using the QuantiFast Probe RT-PCR Plus Kit (Qiagen) and the TaqMan Gene Expression Assay for human LTK (Hs01587788_m1) from Applied Biosystems. Data were analyzed by using the ΔΔCt method and normalized to 18S rRNA levels.

Expression/Purification of FAM150A. FAM150A is produced by transient transfection of 3 L of 293-6E. Briefly, DNA was complexed with polyethylenimine (PolyPlus) in PBS and added to cells. At 24 h after transfection, TryptoneN1 (Organtechnie) was added. Supernatants were clarified on day 6 by centrifugation and filtered through 0.22-μm polyethersulfone. This material was applied to a 30-mL SP Sepharose HP (GE Healthcare Life Sciences) column equilibrated in 10 mM potassium phosphate buffer (pH 7.0) containing 30 mM NaCl. The column was washed with 10 volumes of equilibration buffer and eluted into 3-mL fractions at 2 mL/min over a linear gradient to 900 mM NaCl in equilibration buffer. Fractions containing FAM150A (as determined by Tris/tricine gel) were pooled and diluted with 0.7 volumes of 2 M ammonium sulfate to bring conductivity equal to 1.0 M ammonium sulfate in 10 mM potassium phosphate (pH 7.2). This diluted material was loaded onto a 5-mL Butyl HP (GE Healthcare Life Sciences) equilibrated in 10 mM potassium phosphate (pH 7.2) with 1.0 M ammonium sulfate. The column was

washed with 10 volumes of equilibration buffer, and bound protein was eluted by using a linear gradient to 0 mM ammonium sulfate in 10 mM potassium phosphate (pH 7.2) over 20 column volumes, collecting 1.5 mL per fraction at a rate of 1 mL/min. Fractions were pooled by analysis of Tris/tricine gel and dialyzed against 30 volumes of PBS. Following dialysis, protein was aliquotted and frozen at −80 °C for subsequent analysis.

LTK ECD-Fc fusion proteins were expressed transiently in 293-6E cells as described above. Day 6 harvest supernatants were clarified by centrifugation and filtration before loading onto a 5-mL HiTrap Protein A HP (GE Healthcare Life Sciences) column equilibrated in PBS with 500 mM NaCl. The column was washed with equilibration buffer and eluted by using a linear gradient to 100 mM glycine (pH 2.7), with 500 mM NaCl over 20 column volume collecting 1.5 mL per fraction at 2 mL/min. Elution fractions are collected into 150 μL of 1.0 M Tris (pH 8.0) to neutralize the pH. Elution fractions were pooled based on gel analysis; the pooled protein was dialyzed overnight against PBS, filter sterilized, and stored at −80 °C.

Purified Fam150A was deglycosylated and/or desialylated by using the Prozyme Deglycosylation Kit (Prozyme; catalog no. 80110) using recommended nondenaturing protocols therein. Briefly, 10 μL of 250 mM sodium phosphate (pH 7.0) was added to 50 μg of Fam150A protein. Then, 1 μL of Sialidase A and 1 μL of O-Glycanase were added, or 1 μL of Sialidase A was added to the protein/buffer solution. Water was added to bring the final volume to 100 μL. Samples were incubated at RT for 1 h, then at 4 °C for 48 h. A total of 8 μg of each reaction was mixed with 20 mM iodoacetamide, loaded on a Criterion Tricine Gel (Bio-Rad Laboratories), and run at 125 V for 90 min. Following electrophoresis, the gel was stained by using Coomassie Blue R250 dye and destained per standard protocols.

Determination of FAM150A Binding Constants for Human and Mouse LTK ECD-Fc Fusions. Binding kinetics of FAM150A to human and mouse LTK ECD-Fc fusion proteins were determined by using the Biacore T100 Surface Plasmon Resonance instrument (GE Healthcare Life Sciences). Purified LTK-Fc fusions were captured on a CM4 sensor chip immobilized with Protein A (Thermo Scientific). We used 10 mM Hepes-buffered saline (pH 7.4) with 0.05% Tween

20 (HBSP) (GE Healthcare Life Sciences) for dilution of all samples and as the running buffer for data collection. Capture levels of the ECD-Fcs were adjusted to 300–500 response units to ensure that binding values would be much greater than levels of nonspecific binding to the reference flow cell. FAM150A was injected at eight concentrations (100, 33.3, 11.1, 3.7, 1.2, 0.41, 0.13, 0.046, and 0 nM) for 120 s each. Dissociation was followed for an additional 120 or 600 s. The association and dissociation constants and affinity of FAM150A for LTK ECD-Fc fusions was calculated by using the Biacore T100 Evaluation software package using the 1:1 binding model with standard double referencing. BMPR1A ECD-Fc was included as a negative binding control, and no nonspecific binding of FAM150A was observed.

FAM150A Induce Phosphorylation of LTK. The 293 cells expressing LTK or SK-N-SH cells (ATCC) were seeded at 5×10^6 cells per well in six-well culture plates in DMEM with 10% FBS and grown overnight at 37 °C. The culture medium was replaced with starvation medium (DMEM, 0.1% FBS), and the cells were starved for 24 h at 37 °C. FAM150A (200 ng/mL), with or without 1 μM kinase inhibitor crizotinib (catalog no. S1068, Selleckchem), was then added to the cells for 20 min. At the end of the incubation, cells were washed with cold PBS, and 250 μL of cell lysis buffer (catalog no. 9803S, Cell Signaling Technology) containing protease inhibitor mixture (catalog no. P8340; Sigma-Aldrich) and phosphatase inhibitor mixture 2 (catalog no. 5726, Sigma-Aldrich) was added to each well. Cell lysate was immunoprecipitated with a sheep anti-human LTK affinity purified polyclonal antibody (R&D Systems) overnight, and the immunoprecipitate was separated on a reducing SDS/PAGE gel. Tyrosine phosphorylation was detected by blotting with a mouse antiphosphotyrosine monoclonal antibody conjugated to HRP (R&D Systems), and the signal was developed according to the manufacturer's instructions. Whole-cell lysate was run on a separate reducing SDS/PAGE gel and probed for ERK1/2 phosphorylation by using an anti–phospho-p44/42 MAPK (ERK1/2) (Thr-202/Tyr-204) antibody (Cell Signaling Technology). B-actin was detected as a loading control by using an anti–β-actin antibody conjugated to HRP (Abcam).

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