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Fam65b Is a New Transcriptional Target of FOXO1 That Regulates RhoA Signaling for T Lymphocyte Migration

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Forkhead box O (FOXO) transcription factors favor both T cell quiescence and trafficking through their control of the expression of genes involved in cell cycle progression, adhesion, and homing. In this article, we report that the product of the fam65b gene is a new transcriptional target of FOXO1 that regulates RhoA activity. We show that family with sequence similarity 65 member b (Fam65b) binds the small GTPase RhoA via a noncanonical domain and represses its activity by decreasing its GTP loading. As a consequence, Fam65b negatively regulates chemokine-induced responses, such as adhesion, morphological polarization, and migration. These results show the existence of a new functional link between FOXO1 and RhoA pathways, through which the FOXO1 target Fam65b tonically dampens chemokine-induced migration by repressing RhoA activity. The Journal of Immunology, 2013, 190: 748–755.

Using a large-scale study of the genes that are specifically controlled by Forkhead box O (FOXO1) in human T cells, we showed previously that FOXO1 regulates a much larger set of genes than previously expected. In addition to controlling a specific category of genes involved in T lymphocyte quiescence and survival, FOXO1 controls expression of the CD62L and CCR7 homing receptors. These results have been largely confirmed in murine systems (reviewed in Ref. 4). However, no putative transcriptional targets of FOXO1 involved in the control of signals transduced downstream of these homing receptors have been identified.

In this article, we describe the function of a new gene controlled by FOXO1 called fam65b that fulfills such a function. In resting T cells, we report that family with sequence similarity 65 member b (Fam65b) negatively regulates adhesion, polarization, and migration. Mechanistically, we show that Fam65b represses these responses by inhibiting RhoA activity, a GTPase particularly important for cell migration (5). This shows the existence of a novel and unsuspected link between FOXO1 and RhoA pathways. Taken together, our results demonstrate that Fam65b is a target gene of FOXO1 that regulates the triggering threshold of RhoA-dependent chemokine responses.

Materials and Methods

In silico analysis

The following publicly accessed databases were used: Basic Local Alignment Search Tool, UniGene, GeneCards, GeneAtlas, Clustal, Pfam, InterProScan, PSORT II Prediction, Panther, and PROSITE. Accession numbers are: fam65b (UGID:1775160) (http://www.ncbi.nlm.nih.gov/UniGene/cluster?Org=Hs&CID=559459); Fam65b isoform 1 [Fam65b (1), 140 kDa, NP_055557.2]; and Fam65b isoform 2 [Fam65b(2), 85 kDa, NP_056948.2] (http://www.ncbi.nlm.nih.gov.gate2.inist.fr/protein?term=fam65b).

Quantitative RT-PCR

Total RNA was prepared using an RNeasy mini kit (QIAGEN). cDNA was produced with the Advantage RT-for-PCR kit (Clontech Laboratories) using 1 μg total RNA and random hexamer priming in a final volume of 20 μl. Real-time quantitative PCR was performed using the LightCycler FastStart DNA Master plus SYBR Green kit (Roche Diagnostics). Genes of interest were detected using primers that had been designed with Oligo6...
software (Molecular Biology Insights) and optimized to generate a single
amplon of 80–130 nt. The sequences of the primers used in qRT-PCR
experiments are as follows: ppia (F): 5′-GGTGACTTACCTCAGCAGCA-
TAAGT-3′, ppia (R): 5′-ACAAGATGTCCGAGGCACTCCTAT-3′; fam65b (F):
5′-GCAGGAGTTTAACTCCAGCAGC-3′, fam65b (R): 5′-CCCTCAGGTTGAC-
CTTGTGGG-3′; iso1(F): 5′-GGTCCCTTCTCATCAAGT-3′; iso1 (R):
5′-GGTCTCTGCTGCTATATAAAAG-3′; and iso2(F): 5′-GGCCAG-
GAATGCTACAAC-3′; iso2(R): 5′-GaAGGCAGCTTTGAGCAGCA-3′.

Luciferase assays
The FOXO1 enhancer in the iso1 (130 650-131 815) and iso2 promoter
region (164 220-165 360) was amplified from human genomic DNA using the following set of primers: iso1-KpnI: 5′-ATACTAGTGACC-
ATGTTCCCTTTCGGCTAATGTCTCA-3′ and iso1-BglII: 5′-ATGTAG-
TAGATCTGAGCCGCTTTCTGTACTGAGCCG-3′; and iso2-KpnI: 5′-ATACATGTGACCTCAAAATGGTACACGAAGACAC-3′ and iso2-BglII: 5′-ATGATAGATCTGCTAAGTCTGAGCCG-3′. DNA was amplified for 30 cycles (94°C 30 s, 68°C 30 s) in 2% buffer with 1.5 mM MgCl2, 0.25 mM 3′-deoxyribonucleoside 5′-triphosphates, 0.5 μM each primer, 100 ng genomic DNA, and 5 U Platinum PfX (Invitrogen) in
a total volume of 50 μl. The amplified sample was digested with KpnI and
BglII and introduced into the pcGL3 vector (Promega) opened by the same
enzymes. Jurkat T Ag cells (5 × 105/well) were cotransfected with firefly
luciferase reporter construct (5 μg), CMV–Renilla luciferase reporter con-
struct (0.1 μg), and FOXO1(3A)-GFP. Twenty-four hours post-transfection,
cells were lysed in passive cell lysis buffer (500 μl), and luciferase activity
was assayed using the Dual-Luciferase Reporter assay system (Promega),
as per the manufacturer’s instructions.

Constructs
The pEGFP-C3-RhoAN1 construct was provided by M.R. Phillips (New
York University School of Medicine, New York, NY). The FOXO1(3A)-
GFP and FOXO1(3A, H215R)-GFP plasmids were described previously (3,6).
Lentiviral vectors TRIPzE encoding GFP or FOXO1(3A)-GFP were described previously (3). Fam65b(2) was amplified via PCR using a V5-
tagged Fam65b vector (7) as a template; the PCR fragment was then in-
troduced into the pEGFP-N1 vector (Clontech). Fam65b(1) was amplified via PCR using T lymphocyte cDNA as a template and similarly introduced into pEGFP-N1. V5-tagged Fam65b(2) truncated mutants were described previously (7).

Chromatin immunoprecipitation assays
JTag cells infected with lentiviral vectors encoding GFP or FOXO1(3A)-
GFP were used 3 days later for some chromatin immunoprecipitation
experiments performed with the Chromatin Immunoprecipitation (ChIP)
Assay Kit (Millipore), according to the manufacturer’s instructions. Anti-
GFP and control irrelevant rabbit IgG were purchased from Abcam. DNA
was extracted using a phenol/chloroform method. PCR reactions were
conducted using the AmpliTaq Gold kit with Gene Amp, using 30 cycles (94°C 30 s, 58°C 1 min, 72°C 1.5 min) and the indicated primers (Sup-
plemental Fig. 2B, 2C). Migration of PCR products was performed with
2.5% agarose gels.

Yeast two-hybrid screen
Yeast two-hybrid screening was performed by HybriGenics (Paris, France),
using full-length Fam65b(2) as bait to screen a random-primed human
CD4+ CD8+ thymocyte cDNA library.

Cells
Human peripheral blood T lymphocytes (PBTs) were purified from the
blood of healthy donors, as described (8). Jurkat T Ag and 293T cells were
cultivated in complete RPMI 1640 medium.

DNA and RNA interference transfections
293T cells were transfected with Lipofectamine 2000 (Invitrogen),
according to the manufacturer’s instructions. A total of 2 × 105 Jurkat
T Ag cells were nucleofected with 5 μg DNA using the Amaxa system
(Lonza; kit V, program X-001). A total of 5 × 105 PBTs per cuvette was
nucleofected with 10 μg DNA of the indicated construct using Amaxa and
the U14 program. For RNA interference (RNAi) experiments, 2 × 105
PBTs per cuvette was nucleofected with 2 μl a 100-μM solution of GFP-
SMARTpool RNAi (Dharmacon) directed against human Fam65b or
nontargeting sequences as a control. Cells were rested for 10 min in RPMI
1640 at 37°C and then supplemented with complete RPMI 1640 medium
containing human AB serum and 5 U/ml IL-7. Cells were then tested for
functional experiments 3 d after nucleofection when the level of Fam65b
downknock was maximal.

Biochemistry
Protein expression levels of Fam65b were analyzed by Western blot, as
described (8). Both Fam65b isoforms were revealed by immunoblotting
with anti-Fam65b (Abnova). Other blotting Abs were anti-RhoA (Cyto-
skeleton) and anti-β-actin (Sigma), followed by goat anti-mouse-HRP
(Bio-Rad) incubation and ECL revelation.

For pull-down assays, beads containing GST-tagged RhoA (Cyto-
skeleton) were incubated with 1 mM GDP or 0.2 mM GTPyS in the presence of
15 mM EDTA at 30°C for 15 min and then locked in the GDP- or GTPyS-
bound conformation by supplementing with 60 mM MgCl2. They were then added to the cleared lysate of Fam65b-GFP–expressing 293T cells in
the following buffer: 100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Nonidet P-10, 0.1% glycerol, and 2 mM MgCl2 in the presence of complete
protease inhibitors. For pull-down assays on PBTs, 50 × 106 cells were
stimulated with 200 ng/ml CCL19 for different times, and the lysates were
similarly submitted to a pull-down assay using 5 μg GST or GST-RhoA
coated on glutathione beads. Alternatively, His-RhoA or His-RhoAL63
proteins (Cytoskeleton) were loaded on LiquiChip Penta-His beads (QIAGEN), according to the manufacturer’s instructions, and added to
GST-Fam65b(2) recombinant protein (Abnova) in PBS containing 1% BSA. All samples were then agitated for 1 h, washed, and processed as described above. Membranes were blotted with anti-GFP (Living Colors;
Molecular Probes), anti-V5 (Invitrogen), anti-His (Invitrogen), anti-Fam65b
(Abnova), or anti-GST (Amersham) Abs. Goat anti-mouse or anti-rabbit
Abs (Bio-Rad) conjugated to HRP were then used, and membranes were
revealed by ECL.

RhoA-activation assay
RhoA-GTP levels were measured using the G-LISA RhoA-activation assay
(Cytoskeleton). PBTs, stimulated or not with CCL19 (200 ng/ml) for 30 s,
were lysed for 30 min with occasional stirring in the following lysis buffer in
the presence of complete protease inhibitors: 50 mM HEPES, 1% Triton-
X100, 0.5% deoxycholate, 0.05% SDS, 500 mM NaCl, 10 mM MgCl2, 2
mM EGTA, and 20 mM Benzanidine. Protein concentrations were quan-
tified according to the manufacturer’s recommendations. Equal amounts of
lysates were added in triplicate to a 96-well plate coated with the RhoA-
binding domain of rhotekin and incubated at 4°C for 1 h. Wells containing
only some lysis buffer were used as blank samples. After washing, the
amount of RhoA-GTP bound to each well was revealed by an anti-RhoA
Ab, followed by a secondary HRP-labeled Ab and detection of HRP. Sig-
als were measured with a microplate spectrophotometer by quantifying
absorbance at 490 nm.

Nucleotide-exchange kinetics assay
Nucleotide-exchange activity on 2 μM RhoA was measured with or
without 2 μM Dlb’s Big Sister (Dbs) DH/PH GEF domain using a Rho-
GEF exchange assay kit (Cytoskeleton), according to the manufacturer
instructions, with the exception that 6His-RhoA was produced in our labor-
atory (9). Fluorescence measurements were performed at 25°C in a 384-
well plate using a FlexStation 3 (Molecular Devices) with excitation and
emission wavelengths of 360 and 440 nm, respectively. The exchange re-
action was initiated by the addition of Dbs, with or without 0.6 μM re-
combinant Fam65b (Abnova), and monitored for 30 min. The observed
rate constants (kobs) were calculated by fitting the fluorescence changes to
a single exponential, using Prism 5 software (GraphPad Software).

Flow cytometry
PBTs were stimulated at 37°C with 100 ng/ml CCL19 for different times,
fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1%
saponin, and incubated with phalloidin-tetramethylrhodamine isothio-
cyanate (Sigma). The F-actin content was then measured in each sample by
flow cytometry using a FACScan (Becton Dickinson). JTag cells nucleo-
fected with GFP, FOXO1-GFP, FOXO1(3A)-GFP, or FOXO1(3A, H215R)-
GFP were similarly processed for flow cytometry using an anti-Fam65b Ab
(Santa Cruz).

Immunocytochemistry
JTag cells or PBTs, which were stimulated or not with 100 ng/ml CCL19 for
different times, were fixed with 4% paraformaldehyde for 10 min, per-
meabilized with 0.1% Triton-X100, and incubated with a combination of
phalloidin–Alexa Fluor 350 (Invitrogen), anti-moesin (C-15; Santa Cruz),
and anti-Fam65b (Ab Research or Santa Cruz). Because we confirmed that
paraformaldehyde fixation does not work for RhoA staining (10), anti-
RhoA (26CA, Santa Cruz) immunofluorescence was performed using a 10% trichloroacetic acid fixation method together with p-ERM (Cell Signaling Technology) staining to localize the uropod. Unfortunately, Fam65b staining does not work with trichloroacetic acid fixation, which precludes the possibility of performing a RhoA-Fam65b costaining. DAPI or Hoechst stainings (blue) were used in some cases to stain the nucleus. Primary Abs were revealed by biotin-, FITC-, or Texas Red–conjugated anti-rabbit, anti-mouse, or anti-goat IgG Abs (Jackson ImmunoResearch). Streptavidin–Alexa Fluor 568 (Invitrogen) was used to reveal secondary biotinylated Abs. T lymphocytes were allowed to sediment and were mounted on glass coverslips using FluorSave Reagent (Calbiochem) and imaged by confocal microscopy.

**Adhesion assays**

For under-flow adhesion assays, control (CT) or Fam65b knocked-down (KD) T cells were resuspended at 10⁶/ml in standard adhesion buffer (PBS, 1 mM CaCl₂, 1 mM MgCl₂, 10% FCS [pH 7.2]). Cellular adhesive interactions were studied in underflow conditions with the BioFlux 200 system (Fluxion Biosciences). A 48-well plate microfluidics was first coated overnight at room temperature with human E-selectin (5 μg/ml). Before use, microfluidic channels were washed with PBS and coated with 2 μM CCL19 in PBS for 3 h at room temperature, and the assay was done at a wall shear stress force of 2 dyne/cm². After extensive washing of channels with adhesion buffer, the behavior of interacting lymphocytes was recorded on digital drive with a fast CCD videocamera (25 frames/sec, capable of 1/2 sublime 20-ms recording) and analyzed subframe by subframe.

Single areas of 0.2 mm² were recorded for ≥120 s. Interactions ≥20 ms were considered significant and scored. Lymphocytes that remained firmly adhesive for ≥1 s were considered fully arrested. Cells arrested for ≥1 s or ≥10 s were scored.

In vivo lymphocyte arrest on blood vessel endothelial cells was studied by intravital microscopy, as described (11). Briefly, CT or Fam65b KD T cells (in DMEM without sodium bicarbonate supplemented with 20 mM HEPES, 5% FCS [pH 7.1]) were labeled with either 5-chloromethylfluorescein diacetate (1 min at 37°C) or 5-(and-6)-(di(4-chloromethyl)benzoyl)aminotetramethylrhodamine) (3 min at 37°C). A total of 10⁵ labeled cells was injected i.v. in the tail vein of C57BL/6J mice. In situ videomicroscopic analyses were carried out on Peyer’s patches’ HEVs. Experiments were recorded on digital videotape with a high sensitive fast SIT videocamera (25 frames/sec). Cell behavior was analyzed over a period of 20–30 min, starting 2 min after i.v. injection. T lymphocytes that remained firmly arrested for ≥10 s were scored.

**Polarization**

T cell-polarization assays and the analysis of the four categories of morphological changes elicited by CCL19 stimulation were performed as described (12).

**Migration**

Nucleofected PBTs were placed on the upper chamber of a 5-μm-diameter Transwell (Nunc). Different concentrations of CCL19 were put in the lower chamber, and the cells were allowed to migrate for 3 h. Lymphocytes that reached the lower chamber were harvested and put in a FACScan tube.

![FIGURE 1. Fam65b expression is controlled by FOXO1. (A) Jurkat cells transduced with GFP, FOXO1(3A)-GFP, or FOXO1(3A, H215R)-GFP were analyzed 48 h later for fam65b isoform 1 or 2 transcript levels by qRT-PCR. Data are from three independent experiments. (B) Jurkat cells were cotransfected with a construct encoding FOXO1(3A)-GFP together with a luciferase reporter plasmid containing the putative FOXO1 enhancer in fam65b isoform 1 or 2 promoters and an internal Renilla luciferase reporter construct driven by the CMV promoter. Twenty-four hours posttransfection, cells were lysed, and firefly and Renilla luciferase activities were measured. Data are shown as means of two independent experiments conducted in triplicate. (C) Chromatin proteins in Jurkat cells infected with GFP (control) or FOXO1(3A)-GFP were cross-linked to DNA and immunoprecipitated using anti-GFP or nonimmune rabbit IgG control. Eluted DNAs were diluted for PCR, and amplified DNA fragments using specific primers (Supplemental Fig. 2) were resolved on gel. (D) Flow cytometry analysis of Fam65b expression in Jurkat cells transfected with plasmids encoding GFP, FOXO1(3A)-GFP, FOXO1(3A)-GFP, or FOXO1(3A,H215R)-GFP. Left and middle panels, Examples of dot plots showing the GFP expression levels as a function of Fam65b expression. Top right panel, Overlays of Fam65b expression gated in cells expressing GFP (red line), FOXO1(3A)-GFP (orange line), active FOXO1(3A)-GFP (green line), and active FOXO1(3A,H215R)-GFP (blue line). Bottom right panel, Mean fluorescence intensities of Fam65b expression in the GFP⁺ gate of the different transfectants (means ± SE from six independent experiments). (E) Subcellular localization of the different FOXO1 mutants in Jurkat cells. The following stainings are shown: GFP (green), Hoechst (blue) and Fam65b (red). Original magnification ×63. Examples of representative cells are shown.](http://www.jimmunol.org/)

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with an equal amount of Flow-Check Fluospheres (Beckman Coulter). The number of migrating cells, relative to the number of beads, was analyzed by flow cytometry.

Statistics
Statistically significant differences between groups were assessed with an unpaired Student t test calculated using KaleidaGraph.

Results
Fam65b is induced by FOXO1
We initially identified fam65b as a major response gene of FOXO1 by microarray experiments in human T lymphocytes (3). Fam65b, also called c6orf32, has two paralogs (Fam65a and Fam65c); all three molecules are well conserved from zebrafish to humans (Supplemental Fig. 1A), especially in their N-terminal regions. In humans, this gene is located on chromosome 6 and encodes two mRNA isoforms that give rise to two proteins (Supplemental Fig. 1B). A UniGene search for the distribution of the transcripts indicated that fam65b mRNA levels are particularly high in blood cells and adult tissues of hematopoietic origin, such as the secondary lymphoid organs (Supplemental Fig. 1C).

As presented in Supplemental Fig. 2, the fam65b gene encodes two mRNA isoforms. To further characterize the regulation of Fam65b expression by FOXO1, we designed PCR primers amplifying each isoform and followed their expression in the Jurkat T cell line expressing a constitutively active nuclear form of FOXO1 [FOXO1(3A)]. As shown in Fig. 1A and as measured by qRT-PCR, Jurkat cells expressing FOXO1(3A) exhibited a 4.5 ± 0.1-fold and 6.5 ± 3-fold (mean ± SEM) increase in Fam65b isoform 1 and isoform 2 transcript levels, respectively. The FOXO1(3A,H215R) mutant, which exhibits impaired DNA binding, only induced a marginal 2 ± 0.1-fold increase in isoform 1 transcript levels and a 1.75 ± 0.35-fold increase in isoform 2 transcript levels.

The two fam65b transcripts have their own promoters, which were identified using the Ensembl database (Supplemental Fig. 2A). To determine whether FOXO1 directly controlled fam65b gene transcription, we searched for an evolutionarily conserved FOXO1 binding site in the two fam65b promoters using the Genomatix program. We found one putative FOXO1 site within the 500 bp of the isoform 1 promoter region and four within isoform 2 that were conserved between mice and humans (Supplemental Fig. 2B, 2C). To further demonstrate that FOXO1 regulates Fam65b expression, luciferase reporter plasmids of fam65b promoters using the FOXO1 binding site in the two fam65b promoters were cotransfected with FOXO1(3A) vector into Jurkat cells. The results showed that the luciferase activity of isoform 1 promoter (3.5 ± 0.4) and isoform 2 promoter (8.9 ± 0.9) was significantly increased by FOXO1(3A) (Fig. 1B).

To investigate further whether FOXO1 directly binds these DNA elements, we used Jurkat cells transduced with FOXO1(3A) fused with GFP or GFP alone to perform chromatin immunoprecipitation experiments. For the promoter of isoform 1, a genomic fragment containing the FOXO1 site, but not an irrelevant sequence, is selectively enriched with anti-GFP Ab only in FOXO1(3A)-transduced cells (Fig. 1C). For the isoform 2 promoter, only the most proximal 5′ fragment is detected with anti-GFP in FOXO1(3A)-transduced cells, demonstrating that only this site is active in T lymphocytes for regulating isoform 2 expression by FOXO1.

These findings demonstrate that the two isoforms of Fam65b are direct FOXO1 targets in T cells.

We next aimed at determining whether FOXO1 also increases Fam65b protein levels by transfecting Jurkat cells with different FOXO1-GFP constructs [FOXO1-GFP, FOXO1(A3)-GFP, FOXO1(3A,H215R)] or GFP alone. Fam65b levels were then measured by flow cytometry in the different cell populations discriminated by the expression of GFP. The results show that the constitutively active form of FOXO1 induces Fam65b expression, whereas the wild-type form of FOXO1 fails to trigger this expression (Fig. 1D). This is in accordance with FOXO1 complete exclusion of the nucleus in this cellular model (Fig. 1E) as a result of the fact that Jurkat cells, which lack expression of the lipid phosphatase PTEN, have a tonic inactivation of wild-type FOXO1, even when it is overexpressed (3). As expected, the FOXO1(3A,H215R) molecule was poorly active compared with the fully active FOXO1(A3) mutant (Fig. 1D), despite its nuclear localization (Fig. 1E). Moreover, we show that Fam65b induced by active FOXO1 exhibits a cytotoxic distribution (Fig. 1E).

Altogether, these results demonstrate that FOXO1 directly controls fam65b transcription and the expression of this protein in T lymphocytes.

Fam65b negatively regulates adhesion, polarization, and migration upon chemokine stimulation
In unstimulated T cells, we and other investigators showed that FOXO1 controls the expression of CCR7, which binds the homoeostatic chemokines CCL19 and CCL21 (3, 13). CCR7 is crucial for LN homing (14) or during intranodal motility (15–17). Because we demonstrate in this study that FOXO1 also controls Fam65b expression in resting T lymphocytes, we next tested whether Fam65b plays a role in CCL19 responses.

Using an RNAi approach (Supplemental Fig. 3A), we first tested whether Fam65b plays a role in T cell adhesion upon CCL19 stimulation. Adhesion under flow upon chemokine stimulation elicits a rapid inside-out mechanism of integrin activation that supports a quick cell arrest that might be followed by additional mechanisms to stabilize adhesion (18). Under-flow adhesion assays were conducted for CT and Fam65b KD T lymphocytes visualized in microfluidic channels coated with CCL19, E-selectin,
and ICAM-1. The behavior of CT and KD T cells is quantified in Fig. 2A. KD T cells have a lower tendency to exhibit rolling (Fig. 2A, left panel) and, reciprocally, show an increased propensity to adhere briefly (Fig. 2A, middle panel) or more stably (Fig. 2A, right panel). We next aimed at evaluating the adhesive role of Fam65b in the complexity of an in vivo situation under physiological shear stress forces. We used a validated xenобiotic setting that consists of imaging the microcirculation of human T cells in the Peyer’s patches’ HEVs of anesthetized mice by intravital microscopy (11). Under these conditions, Fam65b depletion increases the percentage of stably arrested T lymphocytes (Fig. 2B). Therefore, Fam65b negatively regulates T lymphocyte adhesion, both in vitro and in vivo.

We then analyzed the capacity of Fam65b KD cells to polarize morphologically upon CCL19 stimulation, as described (12). Interestingly, even without any chemokine stimulation, Fam65b KD cells tend to spontaneously polarize slightly more than CT cells (Fig. 2C). After CCL19 stimulation, twice as many KD cells progressed toward the fully polarized stage compared with CT cells (Fig. 2C).

Because polarity establishment is considered a prerequisite for optimal migration (19, 20), we next aimed at testing a role for Fam65b in T cell migration. We first observed that, in the absence of chemokine stimulation, KD cells are more prone to migrate spontaneously (Fig. 2D). This phenomenon was more amplified at suboptimal CCL19 concentrations, because the sole Fam65b depletion was able to turn immobile cells into lymphocytes efficiently migrating four times above the baseline level (Fig. 2D, middle panel), most likely as a result of the higher fraction of polarized cells.

Altogether, these results show that Fam65b negatively regulates the threshold for T cell adhesion, polarization, and migration.

Fam65b is a new partner of RhoA

In our initial attempt to delineate the signaling pathways regulated by Fam65b, several independent observations pointed toward the RhoA GTPase. A remarkable aspect is that the T cell functions reported above, in which Fam65b has an inhibitory role, were reported to depend on RhoA activity, whereas actin polymerization in primary T lymphocytes is controlled neither by RhoA nor by Fam65b (Supplemental Fig. 3B) (21–25). An in silico approach using Panther classification system for phylogenetically related proteins identified Fam65b as a molecule related to the RhoA partner PKN (26, 27) (PTHR15829). Finally, a yeast two-hybrid screen set up for identifying Fam65b partners identified the small GTPase RhoA as a possible candidate for Fam65b binding.

To confirm this interaction, we performed a series of pull-down assays. As shown in Fig. 3A and 3B, both Fam65b isoforms strongly interact with RhoA in an inactive GDP-bound form, as well as an active GTP-bound form. Titering down the amount of RhoA-GDP or RhoA-GTP in this assay maintained similar binding to Fam65b (Fig. 3C), irrespective of the type of nucleotide.
RhoA+Dbs+Fam65b (was put in a FlexStation to read the evolution of RhoA GTP loading by fluorescence (Fig. 5A). The active RhoA-GTP content was measured for each condition in triplicate by the G-LISA method. Data from three independent experiments are shown.

FIGURE 4. The role of Fam65b in T cell migration depends on RhoA binding. (A) PBTs transfected with GFP or GFP-RhoAN19 were tested, as described previously, in a Transwell assay. The percentages of migratory T cells were normalized to the GFP-transfected population in each experiment set to 100. Data are from two independent experiments. *p = 0.039. (B) PBTs transfected with empty vector (EV), full-length (FL) Fam65b(2), or Nt-Fam65b(2)Δ113 (Δ113) were similarly tested in a migration assay. Data obtained for each condition were normalized to the migration index obtained in EV-transfected T cells set to 100. Data are from eight donors. *p = 0.02, **p = 0.005.

loaded. Association of Fam65b with wild-type RhoA or the constitutively active mutant RhoAL63 was also detected purely in vitro with recombinant proteins, demonstrating a direct association between Fam65b and RhoA (Fig. 3D).

We next attempted to identify the Fam65b region responsible for RhoA binding. A series of truncated Fam65b mutants was tested in a pull-down assay. N-terminal deletion of the first 54 aa maintained the ability of this mutant to bind RhoA (Fig. 3E). However, removal from amino acid 113 and beyond completely abrogated RhoA interaction. Conversely, deletion of the last 101 aa did not affect binding to RhoA. We conclude that Fam65b binds RhoA through the 54–113 region of Fam65b.

In addition, this interaction between RhoA and endogenous Fam65b was detected in the lysates of primary human T cells (Fig. 3F). Importantly, although a strong association was observed in unstimulated T lymphocytes, it was transiently decreased upon CCL19 stimulation. This result indicates that CCL19 signaling frees RhoA from Fam65b binding.

Fam65b inhibition of migration depends on RhoA binding

We next aimed at determining whether the Fam65b–RhoA interaction could account for the effect of Fam65b on T cell migration.

We first checked whether RhoA controls T cell migration as described for many other cell types (5). This was verified in our system for T cell migration elicited by CCL19 signaling using a dominant-negative mutant of RhoA. The inhibition of RhoA activity elicited a strong inhibition of T cell migration (Fig. 4A). Overexpression of full-length Fam65b also inhibited T cell migration, whereas the Δ113 mutant, which does not bind RhoA, did not (Fig. 4B). This result confirms the inhibitory effect of Fam65b on chemokine-induced migration and unveils the requirement for the RhoA-binding region of the protein for this inhibition.

Fam65b inhibits RhoA activity

Thus, the functional effects of Fam65b on migration could arise from an inhibition of the activity of its partner RhoA. To further test this hypothesis, we directly measured the content of active GTP-bound RhoA in Fam65b-KD or CT cells. The results show that resting KD cells had a higher RhoA-GTP content, which did not increase substantially after chemokine stimulation, in contrast to CT T cells (Fig. 5A). Thus, Fam65b acts as a factor that tonically inhibits the RhoA pathway by decreasing the T cell RhoA-GTP content.

To delineate the molecular mechanism by which Fam65b inhibits RhoA activity, we also performed an in vitro nucleotide-exchange assay to measure the kinetics of RhoA GTP loading by the GEF domain of the RhoGEF Dbs in the presence of Fam65b. In a solution containing fluorescent GTP, we observed a slow and passive loading of GTP on RhoA that was largely independent of the presence of Fam65b (Fig. 5B, left panel). Addition of recombinant Dbs accelerated GTP loading as expected. However, the presence of Fam65b slowed down the exchange rate of Dbs on RhoA, as shown by a 5-fold reduction in the k_{obs} value (Fig. 5B, right panel).

Therefore, we conclude that Fam65b downmodulates the exchange reaction that GEFs perform on RhoA and, consequently, dampens the levels of active RhoA in T cells.

Discussion

In this article, we report that the transcription factor FOXO1 regulates cell growth and the expression of homing receptors, as previously demonstrated (3); it also controls an important signaling pathway involved in T cell migration. Indeed, we found that Fam65b, a phylogenetically well-conserved protein, is induced by FOXO1 and represses RhoA activity, providing a new

FIGURE 5. Fam65b inhibits RhoA activity. (A) PBTs transfected with CT or Fam65b (KD) RNAi were stimulated or not with 200 ng/ml CCL19 for 30 s. The active RhoA-GTP content was measured for each condition in triplicate by the G-LISA method. Data from three independent experiments are shown. (B) Recombinant RhoA and Fam65b were placed alone or in combination in wells of a 384-well plate. Recombinant Dbs was then added, and the plate was put in a FlexStation to read the evolution of RhoA GTP loading by fluorescence (left panel). Means ± SE of k_{obs} values for RhoA+Dbs (n = 8) or RhoA+Dbs+Fam65b (n = 6) (right panel). *p = 0.014, **p = 0.0317, ***p = 0.0075, n.s., Not significant.
and unexpected bridge between the FOXO1 and RhoA pathways to modulate T cell motility.

It is puzzling to note that FOXO1 seems to have opposite effects on motility, because it induces the expression of CD62L and CCR7, which favor homing, and the expression of Fam65b, which negatively regulates migration. It is not clear whether this paradox constitutes a way for FOXO1 to finely regulate motility. Alternatively, our in vivo data demonstrate a negative role for Fam65b in T cell adhesion to HEVs. This could allow FOXO1 to indirectly promote homing again by avoiding T cells to exhibit too strong adhesive properties that would maintain them otherwise stuck onto blood vessels. The use of a mouse model deficient for Fam65b should allow us to test this possibility.

We show that Fam65b markedly affects adhesion, polarization, and migration upon chemokine exposure, although it does so without exhibiting obvious changes in its subcellular localization (Supplemental Fig. 4A). This is in agreement with the fact that Fam65b can interact with RhoA, independently of the type of nucleotide it bears, and that the total pool of RhoA is distributed on both sides of a polarized T cell (Supplemental Fig. 4B) (10). We report in this article that Fam65b exerts a tonic inhibition on RhoA activity. Together with other necessary signaling pathways triggered upon chemokine stimulation, the depletion in Fam65b is likely to account for the advantage exhibited by the KD cells to polarize and migrate. Interestingly, we also show that the Fam65b–RhoA interaction decreases upon CCL19 stimulation, suggesting that the loss of Fam65b control on RhoA activity is likely to allow for RhoA activation under normal conditions. Fam65b is an unexpected regulator of RhoA. Indeed, it does not contain any known consensus domain found in typical RhoA interactants, such as GAP and GEF proteins. It also does not present any sequence similarity with GDI proteins. In addition, because effectors can discriminate between the GDP- and GTP-bound forms of Rho GTPases, Fam65b does not belong to this class of Rho partners. Interestingly, previous articles reported possible new mechanisms for RhoA regulation. The unrelated F11L (28) and Memo (29) proteins, which do not contain any canonical domain found in typical RhoA effectors can discriminate between the GDP- and GTP-bound forms of Rho GTPases, Fam65b does not belong to this class of Rho partners. Fam65b is an unexpected regulator of RhoA. Indeed, it does not contain any known consensus domain found in typical RhoA interactants, such as GAP and GEF proteins. It also does not present any sequence similarity with GDI proteins. In addition, because effectors can discriminate between the GDP- and GTP-bound forms of Rho GTPases, Fam65b does not belong to this class of Rho partners. Interestingly, previous articles reported possible new mechanisms for RhoA regulation. The unrelated F11L (28) and Memo (29) proteins, which do not contain any canonical domain found in typical RhoA effectors can discriminate between the GDP- and GTP-bound forms of Rho GTPases, Fam65b does not belong to this class of Rho partners. Interestingly, previous articles reported possible new mechanisms for RhoA regulation. The unrelated F11L (28) and Memo (29) proteins, which do not contain any canonical domain found in typical RhoA

and, therefore, represents a new target by which FOXO1 can regulate motile processes.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 3

A

IB:

Fam65b (1)  anti-Fam65b
Fam65b (2)  anti-RhoA
anti-β actin

B

Relative F-actin content

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Supplemental Figure 4

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Supplemental Figure Legends

Supplemental Fig. 1: *In silico* analysis of Fam65b.

(A) Phylogenetic tree for Fam65b. Fam65b (red) is conserved from Zebra fish to humans. Its two homologs (Fam65a in green; Fam65c in blue) are also expressed in the same species. (B) The two Fam65b isoforms expressed by alternative splicing of the same gene are schematically represented. The dashed segment in isoform 1 corresponds to a unique amino acid sequence encoded by an exon that is not found in isoform 2. (C) EST profile of *fam65b*. Relative distribution of *fam65b* transcripts in various adult human tissues or organs reported by UniGene. No transcript was found in the following: adrenal gland, bladder, bone, cervix, ear, esophagus, heart, larynx, mouth, nerve, ovary, parathyroid, salivary gland and umbilical cord.

Supplemental Fig. 2: Schematic representation of the initial locus of the human *fam65b* gene. (A) Positions of the two promoter regions, transcription initiation sites (arrows), exons and the two *fam65b* 5′ end transcripts detected. The names of the transcripts using Ensembl are ENST00000259698 and ENST00000378023 for iso1 and iso2, respectively. Sequences of promoters 1 (B) and 2 (C) are shown. Predicted FOXO1 sites are depicted with bold blue letters. Sequences of the primers used for the ChIP experiments are underlined.

Supplemental Fig. 3: Fam65b knocked-down (KD) T cells exhibit normal actin polymerization upon CCL19 stimulation.

(A) The efficiency of Fam65b KD in human PBT was checked by immunoblotting with anti-
Fam65b, anti-β actin and anti-RhoA. (B) Actin polymerization in CT or Fam65b KD T cells stimulated during different times with CCL19 (100 ng/ml) was measured. No statistical difference between CT and KD was observed.

**Supplemental Fig. 4: Fam65b is homogenously distributed in chemokine-polarized T cells.**

(A) PBT were unstimulated (time 0, top) or stimulated with 100 ng/ml CCL19 for 8 mins (bottom), fixed in PFA, and co-stained for moesin (red) and Fam65b (green). (B) PBT were stimulated with 100 ng/ml CCL19 for 8 mins, fixed with TCA and stained for RhoA (green), P-ERM (red) and DAPI (blue). The transmitted-light visible picture is shown in the left panel. Images are from three independent experiments.