SUPPLEMENTARY FIGUREs

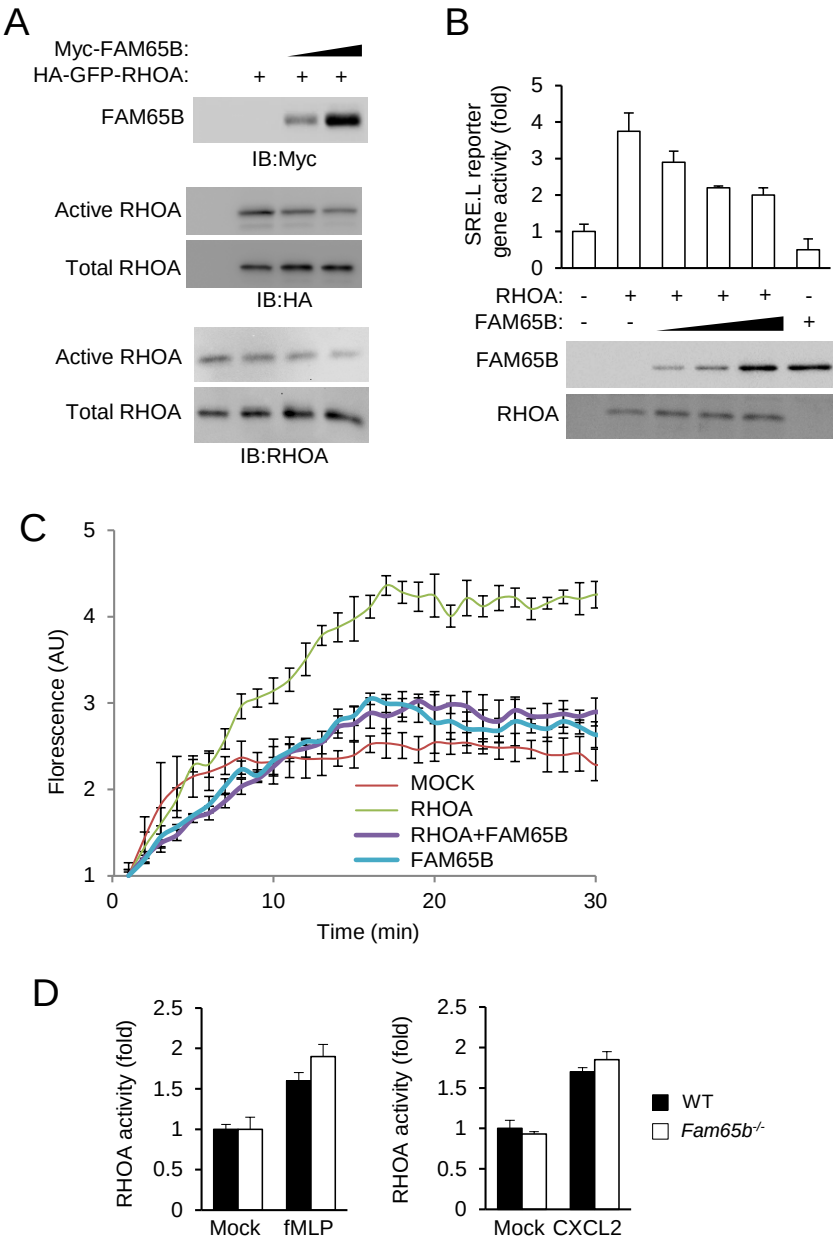

Figure S1. FAM65B inhibits RHOA. **A**. Expression of FAM65B in HEK293 cells suppresses the activity of coexpressed and endogenous RHOA. Active RHOA was assessed by the RBD pull down assay. **B**. Expression of FAM65B inhibits RHOA-induced activation of SER.L reporter gene in HEK293 cells. **C.** Recombinant GST-FAM65B protein inhibits GTP loading to recombinant RHOA. **D**. Effect of FAM65B-deficiency on RHOA activity in response to high concentrations of chemoattractants. Neutrophils were stimulated with fMLP (1 μM) or MIP2 (500 nM) for 3 min, followed by G-LISA assay. Error bars stand for standard derivations.

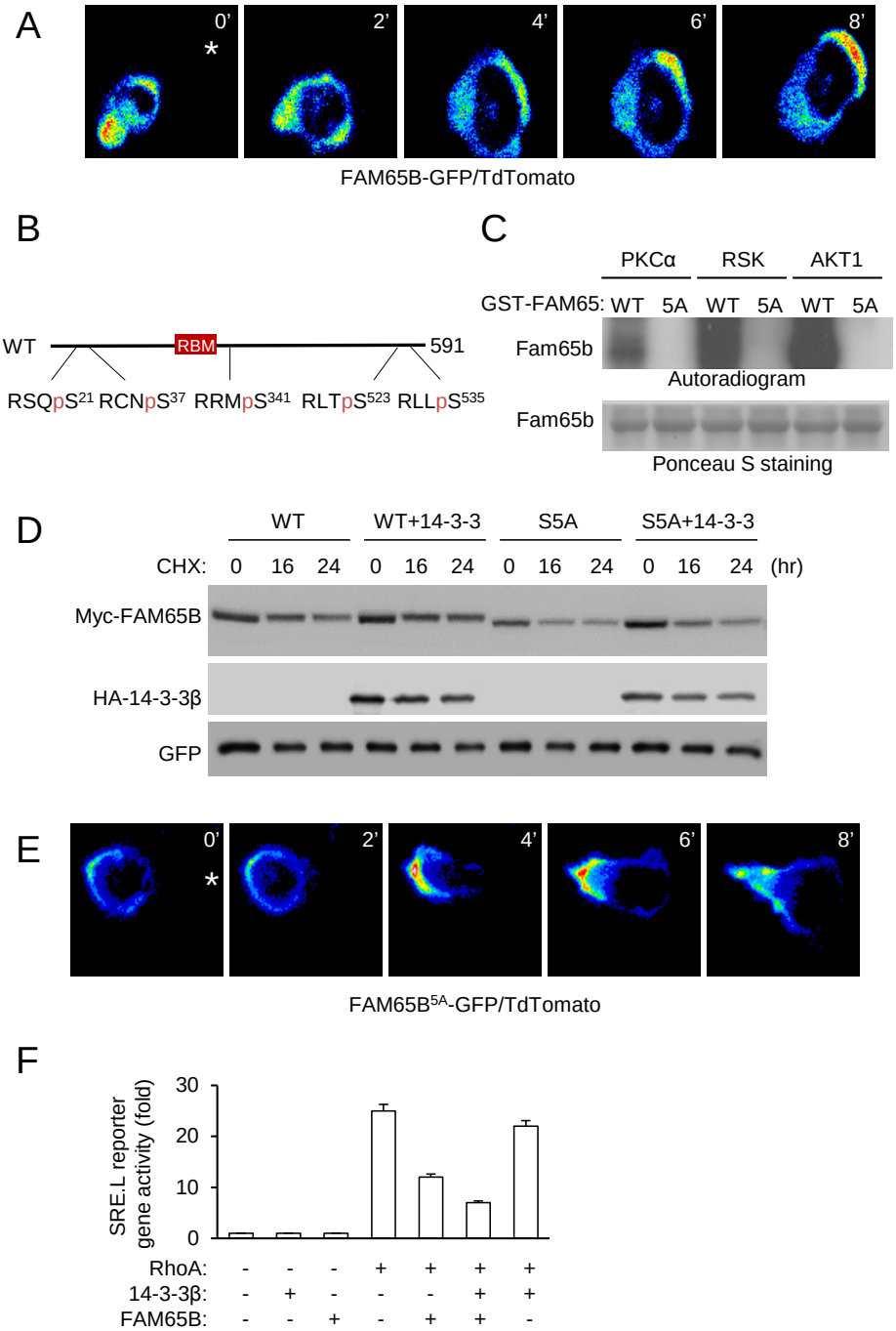

Figure S2. Supplementary data for Figure 3 and 4. A,E. Additional cells showing the localization of FAM65B-GFP (**A**) and FAM65B^{5A}-GFP (**E**). **B**. Phosphorylation sites on FAM65B identified by MS. **C**. In vitro phosphorylation of FAM65B. Recombinant GST-FAM65B proteins were subjected to phosphorylation by recombinant PKCα, RSK and AKT1 in an in vitro kinase assay using [^{32}P]γATP. **D**. Representative Western blotting images for Fig. 4C. **F**. The 14-3-3 protein does not decrease the inhibitory effect of FAM65B on RHOA-induced activation of the SRE.L reporter gene in HEK293 cells.

	RFP	Fam65b^{5A}
ADE (deg)	49±5.2	41±4.3
M (μm/min)	1.1±0.21	1.4±0.19

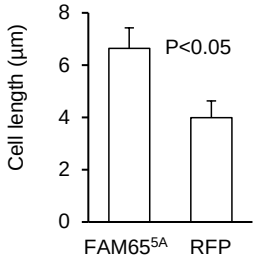

Figure S3. Effect of expression of FAM65B^{5A} mutant on neutrophil chemotaxis. Mouse neutrophils were transfected with RFP and FAM65B^{5A}-GFP, respectively, and mixed at 1:1 ratio followed by the chemotaxis assay in a Dunn chamber as described in Fig. 2F. Chemotactic parameters including ADE (average directional error)) and motility (motility) as well as cell body length are shown (Student's t-test, n>30).

Movie 1: A representative movie shows neutrophil migration in a Dunn chamber under an fMLP gradient. Equal numbers of WT (labelled with a red dye) and FAM65B-null (labelled with a green dye) neutrophils were mixed and subjected to the chemotaxis assay. The first image is the overlay of fluorescent and phase-contrast images to allow genotype identification. The rest of time-lapses images were taken under the phase contrast setting. The fMLP gradient is from right to left.

Movie 2

Movie 3

Movie 2 and 3: Localization of FAM65B-GFP and LifeAct-RFP in mouse neutrophils. Neutrophils expressing WT FAM65B-GFP (Movie 2) or FAM65B^{5A}-GFP (Movie 3) were stimulated by fMLP from a micropipette whose location is marked by the red dot in the first image.