

**Diacylglycerol kinase- $\epsilon$  is required for the formation  
of GPI-anchored CD14  
and the LPS-induced proinflammatory responses  
of macrophages**

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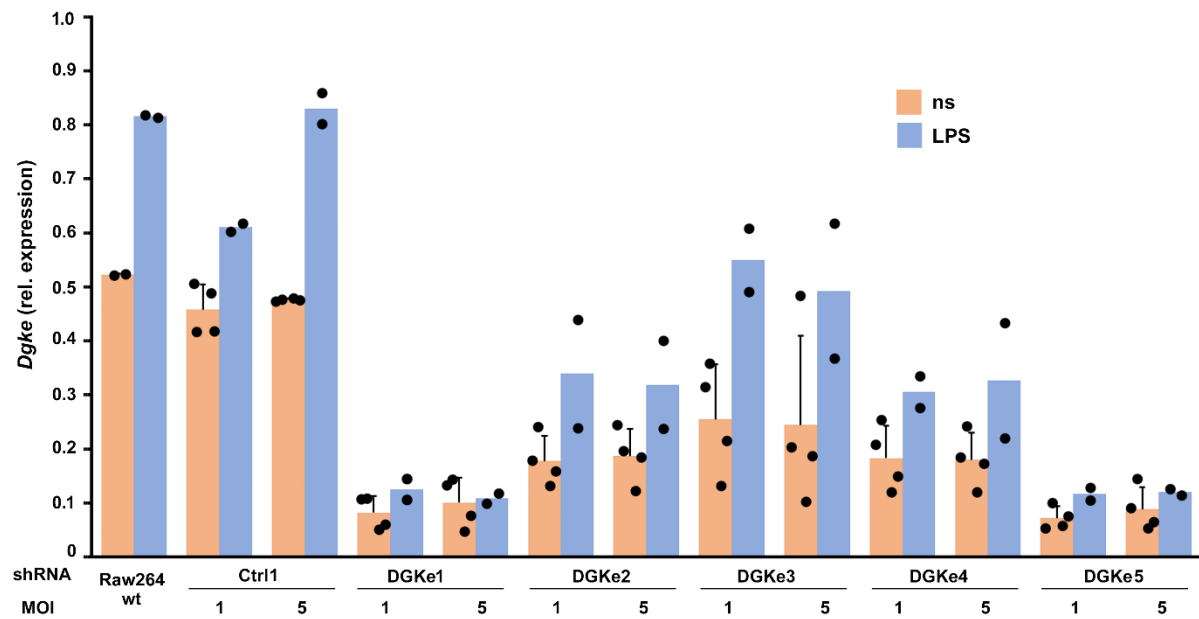
## Methods

### Construction of Raw264.7 cells with silenced and rescued expression of *Dgke*

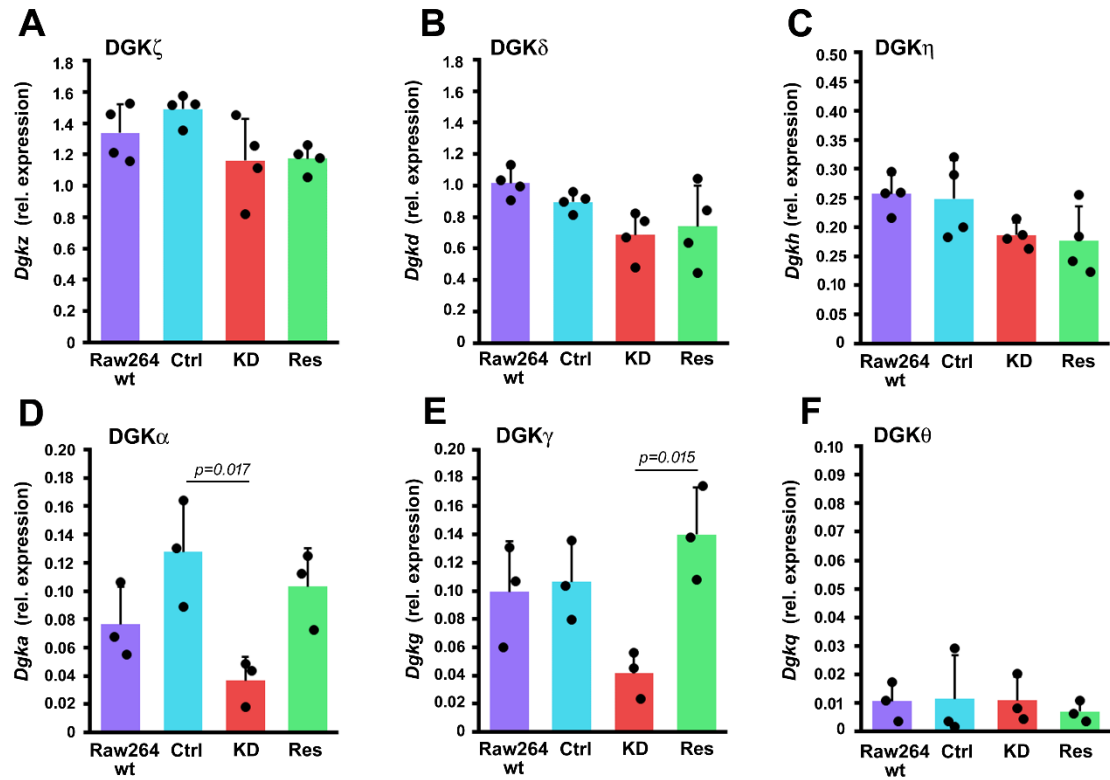
To obtain Raw264.7 cells depleted of DGK $\epsilon$ , they were transfected with lentiviral particles (at MOI = 1 or 5) containing five different *Dgke*-targeting shRNA (Merck, Supplementary Table 1). Cells infected with transduction particles bearing non-mammalian shRNA (at MOI = 1 or 5) (Merck, Supplementary Table 1) served as controls. The cells were cultured in the presence of 2  $\mu$ g/ml puromycin until the mortality of non-infected cells reached 100%, leaving only cells transfected with shRNA alive, essentially as described earlier (Traczyk *et al.*, 2022; Matveichuk *et al.*, 2024). To reintroduce DGK $\epsilon$ , cells transfected at MOI = 5 with shRNA No. 1 and selected as described above were transfected (at MOI = 5) with lentiviral particles bearing the DGK $\epsilon$  sequence with a double Myc tag added at the C-terminus, and conferring resistance to G418. The cDNA sequence was designed to contain eight point mutations in six codons, which did not change the amino acid sequence of DGK $\epsilon$  but protected its mRNA from degradation guided by the previously introduced shRNA (Supplementary Table 1). Cells were cultured in the presence of 0.3 mg/ml G418 and selected as in the first round of transfection to obtain DGK $\epsilon$ -Myc-rescued cells. In parallel, cells depleted of DGK $\epsilon$  with shRNA No. 1 and also control cells obtained after the first round of transfection and selection (MOI = 5) were subjected to a second round of transfection with lentiviral particles bearing the G418 resistance gene only (at MOI = 5) and selected to obtain the ultimate DGK $\epsilon$  knockdown cells (DGK $\epsilon$ -KD) and control (Ctrl) cells. Lentiviral particles bearing the pLenti-C-Myc-DGGK-IRES-Neo vector, with or without the DGK-Myc coding sequence, were custom-made by OriGene. The efficiency of *Dgke* silencing and its reversion was verified with RT-qPCR using primers specific to the mouse *Dgke* gene, with *Tbp* as a reference (Supplementary Table 2, see also Traczyk *et al.*, 2022).

### DGK $\epsilon$ activity assay. Cell lysis and preparation of micelle

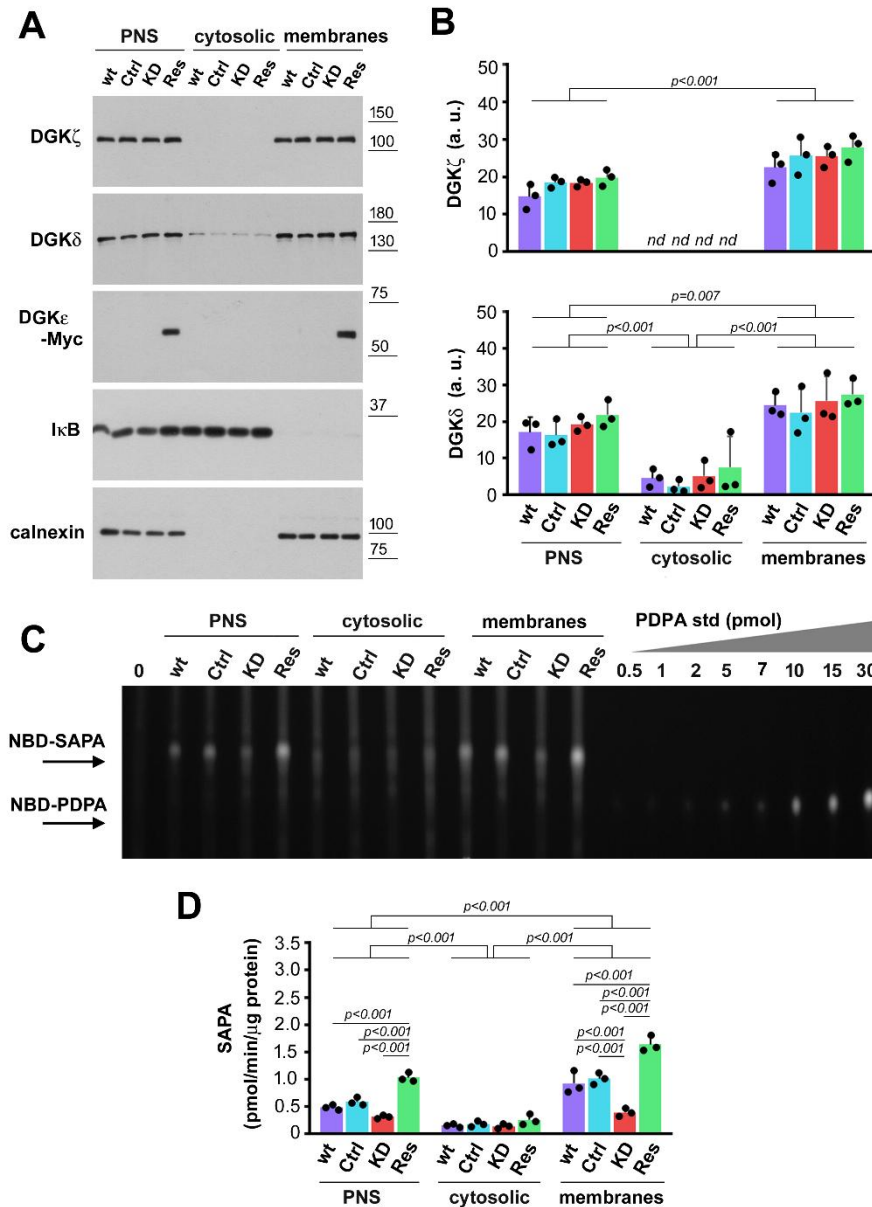
Raw264.7 cells ( $6 \times 10^5$  per sample) were lysed in 200  $\mu$ l of lysis buffer (1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 20  $\mu$ g/ml aprotinin, 20  $\mu$ g/ml leupeptin, 20 mM Tris-HCl, pH 7.4) and sonicated (Traczyk *et al.*, 2022). Subsequently, 50  $\mu$ l of the lysate containing 40 or 50  $\mu$ g of total protein was used for the activity assay with 50  $\mu$ l of micelle suspension, 100  $\mu$ l of H<sub>2</sub>O, and 0.5 mM ATP. The micelles were composed of NBD-SAG/SAG (at a 1:9 molar ratio, both from Cayman Chemical, cat. No. of SAG 10008650) and 1,2-dioleoyl-*sn*-glycero-phosphoserine (DOPS, Merck, cat. No. 840035C) at a final concentration of 1.45:2.03 mol% in 4 x reaction buffer containing 400 mM NaCl, 80 mM MgCl<sub>2</sub>, 4 mM EGTA, 4 mM DTT, 300 mM octyl- $\beta$ -glucoside, 200 mM MOPS, pH 7.2.



**Supplementary Fig. 1.** RT-qPCR analysis of the relative DGK $\epsilon$  mRNA level in Raw264.7 cells after the knockdown of *Dgke* with shRNA. To silence *Dgke* expression, commercially available lentiviral particles bearing five shRNA variants were used individually (DGKe1-5) at MOI = 1 or 5, followed by puromycin selection. In parallel, control shRNA (Ctrl1) was applied at MOI = 1 or 5. Wild-type Raw264.7 cells – Raw264 wt. Cells were left unstimulated (ns) or were stimulated with 100 ng/ml LPS for 4 h. DGK $\epsilon$  mRNA was quantified relative to TBP mRNA. Data shown are mean  $\pm$  SD from four biological replicates of unstimulated cells, except for WT, which includes two biological replicates. For LPS-stimulated cells, the mean of two biological replicates is shown.

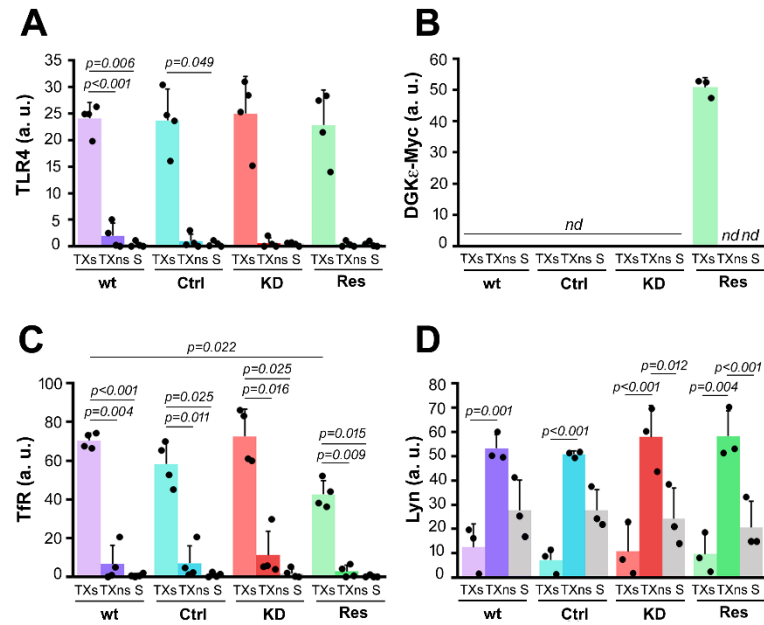


**Supplementary Fig. 2.** Relative expression levels of DGKs in Raw264.7 cells and their derivatives. Transcripts of *Dgkz* (A), *Dgkd* (B), *Dgkh* (C), *Dgka* (D), *Dgkg* (E), and *Dgkq* (F) were quantified relative to *Tbp*. Wild-type Raw264.7 cells – Raw264 wt. Data shown are mean  $\pm$  SD from four (A-C) or three (D-F) biological replicates. Each point represents one biological replicate. Significantly different values, as indicated by one-way ANOVA with Tukey's post hoc test, are marked.



**Supplementary Fig. 3.** SAG phosphorylation in cell fractions not treated with 1 M NaCl. Post-nuclear supernatants (PNS) of wild-type Raw264.7 (wt), Ctrl, DGK $\epsilon$ -KD, and DGK $\epsilon$ -Myc-rescued cell homogenates were fractionated into cytosolic and membrane fractions without the addition of 1 M NaCl. (A) Distribution of indicated proteins in the fractions determined by immunoblotting. Equal amounts of protein, 10  $\mu$ g, were loaded in each lane of the gel. Positions of molecular weight markers are shown on the right in kDa. (B) Abundance of DGK $\zeta$  (upper panel) and DGK $\delta$  (lower panel) in each fraction determined by densitometry of blots such as those shown in (A). Data shown are mean  $\pm$  SD. (C, D) Phosphorylation of SAG to SAPA in cell fractions determined using the fluorescence assay. (C) Representative TLC results revealing NBD-SAPA production. The reaction mixture contained 50  $\mu$ g of total protein; lipids from 1/5 of the reaction mixture were applied onto the plate. NBD-labeled lipids were separated by TLC together with 0.5–30 pmol NBD-PDPA used to draw a standard curve for each experiment. “0” – no homogenate added. (D) SAPA production based on densitometric analysis of SAPA and a calibration curve for NBD-PDPA. Data shown are mean  $\pm$  SD from three biological replicates. Each point represents one biological replicate. Because equal amounts of

protein were loaded on the gels and used for SAG phosphorylation, proteins in both the cytosolic and membrane fractions are enriched compared to the PNS. In (B, D) significantly different values, as indicated by two-way ANOVA with Tukey's post hoc test, are marked. *nd*, not detected; excluded from statistical analysis.



**Supplementary Fig. 4.** *Dgke* knockdown and rescue affect the abundance of mature forms of CD14. Cells were solubilized in 0.1% TX-100 and fractionated into TX-100 soluble (TXs), TX-100 insoluble (TXns), and SDS-soluble (S) fractions. Distribution of indicated proteins in the fractions determined by immunoblotting, as shown in Fig. 8 in the main text. (A-D) Abundance of TLR4 (A), DGK $\epsilon$ -Myc (B), transferrin receptor (TfR) (C), and Lyn (D) in each fraction determined by densitometry. Data shown are mean  $\pm$  SD from four (A, C) or three (B, D) biological replicates. Each point represents one biological replicate. In (A, C) significantly different values as indicated by Welch's ANOVA with Dunnett's T3 post hoc test are marked. In (D) the two-way ANOVA indicated no significant differences between cell variants, therefore differences between fractions in a given cell variant were analyzed with one-way ANOVA with Tukey's post hoc test. *nd*, not detected.

**Supplementary Table 1.** shRNA species used to silence *Dgke* and the *Dgke* sequence fragment with introduced silent point mutations to reverse *Dgke* silencing in rescue experiments

| shRNA species used to silence <i>Dgke</i>                                               |                                                                                   |                                          |                             |
|-----------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------|------------------------------------------|-----------------------------|
| Variant No.                                                                             | Nucleotide sequence 5'-3'                                                         | Merck catalog No.                        | Target sequence in the gene |
| 1                                                                                       | CCGGCCAAGCTACTTAACTTCTATTCTCGAGAA<br>TAGAAGTTAAGTAGCTTGGTTTTT                     | TRCN0000024744                           | nt 921-939                  |
| 2                                                                                       | CCGGGCTGACTTTGAAGTGCTCCATCTCGAGAT<br>GGAGCACTTCAAAGTCAGCTTTTT                     | TRCN0000024745                           | nt 1877-1898                |
| 3                                                                                       | CCGGGCCATTGATGAAATGAAGATTCTCGAGAA<br>TCTTCATTTCAATCAATGGCTTTTT                    | TRCN0000024748                           | nt 1221-1239                |
| 4                                                                                       | CCGGCGCCATTTCACTCCTAGTATTCTCGAGAA<br>TACTAGGAGTGAAATGGCGTTTTTG                    | TRCN0000274757                           | 3' UTR<br>nt 2119-2139      |
| 5                                                                                       | CCGGCCAAGCTACTTAACTTCTATTCTCGAGAA<br>TAGAAGTTAAGTAGCTTGGTTTTTG                    | TRCN0000274755                           | nt 921-939                  |
| control                                                                                 | CCGGCAACAAGATGAAGAGCACCAACTC GAGTT<br>GGTGCTCTTCATCTTGTTGTTTT                     | SHC002V                                  | ----                        |
| Eight point mutations (pm) introduced into <i>Dgke</i> to reverse <i>Dgke</i> silencing |                                                                                   |                                          |                             |
| <i>Dgke</i> WT<br><i>Dgke</i> -8 pm                                                     | 921 AAGCTACTTAACTTCTATT 939<br>921 AAGTTATCTTACAAGTATC 939<br>* * * * * * * * * * | self-designed,<br>produced by<br>OriGene |                             |

**Supplementary Table 2.** List of primers used for RT-qPCR analysis  
of *Dgka*, *d*, *e*, *g*, *h*, *k*, *q* and *z* expression

| Target gene | Direction | Nucleotide sequence 5'- 3' |
|-------------|-----------|----------------------------|
| <i>Dgka</i> | Forward   | CGGACCTACCACAGCCTAAC       |
|             | Reverse   | CCCACAGTCACACTCAGGAC       |
| <i>Dgkd</i> | Forward   | CCTCATTCACAGCATTCTG        |
|             | Reverse   | GCTACAGTTCAACCCCTC         |
| <i>Dgke</i> | Forward   | AGCCTAAACTGTGCGATTACA      |
|             | Reverse   | TTGGCGGGATGATGAGGTTTC      |
| <i>Dgkg</i> | Forward   | CAGACTTCCGTGTTCTTGCC       |
|             | Reverse   | CTTCGTAACCTCCTCCCCAG       |
| <i>Dgkh</i> | Forward   | AGGAAGTGTCACAGATGCGG       |
|             | Reverse   | CGTGGGTTGGCTTTGTTTCAG      |
| <i>Dgkk</i> | Forward   | AAGGGCTCAGAACTCCAGC        |
|             | Reverse   | CGTCGTCTCCGTCTCCCAGG       |
| <i>Dgkq</i> | Forward   | CTCCTGACTGCTGCCCTC         |
|             | Reverse   | CCATCTCCACCACACACCA        |
| <i>Dgkz</i> | Forward   | CGAGAAGCCAACCCAGAGA        |
|             | Reverse   | CCATTCCGTCACACACTACT       |

**Supplementary Table 3. Antibodies used in the study**

| Specificity of antibody              | Host                                 | Supplier                                     | Catalog No.   | Dilution        | Application |
|--------------------------------------|--------------------------------------|----------------------------------------------|---------------|-----------------|-------------|
| Primary antibodies                   |                                      |                                              |               |                 |             |
| actin                                | Mouse monoclonal IgG                 | BD Biosciences                               | #612656       | 1:15000         | IB          |
| CD14                                 | Rat monoclonal IgG                   | BD Biosciences                               | #553738       | 1:4000-5000     | IB          |
| CD14                                 | Rat monoclonal IgG2a-FITC            | eBioscience                                  | #11-0141-82   | 1:200           | FC          |
| F4/80                                | Rat IgG2a, $\kappa$ -Alexa Fluor 647 | BD Biosciences                               | #565854       | 1:300           | FC          |
| I $\kappa$ B $\alpha$                | Mouse monoclonal IgG                 | Cell Signaling Techn.                        | #4814         | 1:1000          | IB          |
| calnexin                             | Goat                                 | Abcam                                        | #ab192439     | 1:1000          | IB          |
| DGK $\zeta$                          | Rabbit                               | Abcam                                        | #ab239081     | 1:1000          | IB          |
| DGK $\delta$                         | Rabbit                               | Gift from Dr. Fumio Sakane, Ciba University* |               | 1:1000          | IB          |
| IRF3                                 | Rabbit monoclonal IgG                | Cell Signaling Techn.                        | #4302         | 1:1000          | IB          |
| Lyn                                  | Rabbit monoclonal IgG                | Cell Signaling Techn.                        | #2732         | 1:2000          | IB          |
| Myc-tag                              | Mouse                                | Cell Signaling Techn.                        | #2276         | 1:1000          | IB          |
| phospho-I $\kappa$ B $\alpha$        | Rabbit monoclonal IgG                | Cell Signaling Techn.                        | #2859         | 1:1000          | IB          |
| phospho-IRF3                         | Rabbit monoclonal IgG                | Cell Signaling Techn.                        | #4947         | 1:1000          | IB          |
| phospho-TBK1/NAK                     | Rabbit monoclonal IgG                | Cell Signaling Techn.                        | #5483         | 1:4000          | IB          |
| TLR4                                 | Rabbit monoclonal IgG                | Cell Signaling Techn.                        | #14358        | 1:1000          | IB          |
| TLR4                                 | Rat monoclonal IgG2a-PE              | BioLegend                                    | #145404       | 1:200           | FC          |
| TNF $\alpha$                         | Rabbit monoclonal IgG                | Cell Signaling Techn.                        | #11948        | 1:1000          | IB          |
| transferrin receptor                 | Mouse                                | Thermo Fisher                                | #13-6800      | 1:2000-4000     | IB          |
| uPAR                                 | Goat                                 | Bio-Techne                                   | #AF534        | 1:1000<br>1:300 | IB<br>FC    |
| Secondary antibodies/conjugated with |                                      |                                              |               |                 |             |
| goat IgG/HRP                         | Donkey                               | Rockland                                     | #605-703-125  | 1:3000-1:5000   | IB          |
| goat IgG/Alexa Fluor 647             | Donkey                               | Thermo Fisher                                | #A21447       | 1:1200          | FC          |
| mouse IgG/HRP                        | Goat                                 | Jackson ImmunoResearch                       | # 115-035-003 | 1:6000-30000    | IB          |
| rabbit IgG/HRP                       | Goat                                 | Rockland                                     | # 611-1302    | 1:8000          | IB          |
| rat IgG/HRP                          | Goat                                 | Merck                                        | # A9037       | 1:10000         | IB          |
| sheep IgG /HRP                       | Donkey                               | Jackson ImmunoResearch                       | # 713-035-003 | 1:6000          | IB          |

IB, immunoblotting; FC, flow cytometry

\*Sakane *et al.*, 1996, doi:10.1074/jbc.271.14.8394; Crotty *et al.*, 2006, doi: 10.1073/pnas.0604104103.