

## SHORT COMMUNICATION

# Mevalonate kinase deficiency leads to decreased prenylation of Rab GTPases

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Mevalonate kinase deficiency (MKD) is caused by mutations in a key enzyme of the mevalonate–cholesterol biosynthesis pathway, leading to recurrent autoinflammatory disease characterised by enhanced release of interleukin-1 $\beta$  (IL-1 $\beta$ ). It is currently believed that the inflammatory phenotype of MKD is triggered by temperature-sensitive loss of mevalonate kinase activity and reduced biosynthesis of isoprenoid lipids required for the prenylation of small GTPase proteins. However, previous studies have not clearly shown any change in protein prenylation in patient cells under normal conditions. With lymphoblast cell lines from two compound heterozygous MKD patients, we used a highly sensitive *in vitro* prenylation assay, together with quantitative mass spectrometry, to reveal a subtle accumulation of unprenylated Rab GTPases in cells cultured for 3 days or more at 40 °C compared with 37 °C. This included a 200% increase in unprenylated Rab7A, Rab14 and Rab1A. Inhibition of sterol regulatory element-binding protein (SREBP) activation by fatostatin led to more pronounced accumulation of unprenylated Rab proteins in MKD cells but not parent cells, suggesting that cultured MKD cells may partially overcome the loss of isoprenoid lipids by SREBP-mediated upregulation of enzymes required for isoprenoid biosynthesis. Furthermore, while inhibition of Rho/Rac/Rap prenylation promoted the release of IL-1 $\beta$ , specific inhibition of Rab prenylation by NE10790 had no effect in human peripheral blood mononuclear cells or human THP-1 monocytic cells. These studies demonstrate for the first time that mutations in mevalonate kinase can lead to a mild, temperature-induced defect in the prenylation of small GTPases, but that loss of prenylated Rab GTPases is not the cause of enhanced IL-1 $\beta$  release in MKD.

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## INTRODUCTION

Mevalonate kinase deficiency (MKD) is an autosomal recessive, autoinflammatory disorder caused by bi-allelic mutations in the *MVK* gene that encodes a critical enzyme in cholesterol and isoprenoid lipid biosynthesis.<sup>1,2</sup> With the severe form of MKD (mevalonic aciduria; OMIM 251170), patients typically have undetectable levels of mevalonate kinase (MVK) activity and display a variety of neurological and developmental abnormalities.<sup>3</sup> The less severe form (hyperimmunoglobulinemia D syndrome or HIDS, OMIM 260920) is characterised by 1–7% normal MVK activity<sup>1</sup> and the occurrence of frequent febrile, inflammatory episodes.<sup>4</sup> Most patients with MKD are compound heterozygous, commonly with V377I or H20N/P point mutations.<sup>4,5</sup>

The inflammatory phenotype of MKD appears to be caused principally by loss of synthesis of isoprenoid lipids downstream of MVK,<sup>6</sup> particularly geranylgeranyldiphosphate.<sup>7–9</sup> Geranylgeranyldiphosphate

is necessary for the post-translational prenylation of proteins of the Rho/Rac/Rap and Rab families of small GTPases. *In vitro* models of MKD involve treatment of cells with statins (HMG-CoA reductase inhibitor), nitrogen-containing bisphosphonates (inhibitors of farnesyl diphosphate synthase<sup>10</sup>) or specific inhibitors of geranylgeranyltransferase I such as GGTI-298<sup>11</sup> to mimic the block in protein prenylation that is assumed to occur in cells deficient in MVK. These inhibitors predispose cells to increased inflammasome activity and enhanced caspase-1-mediated cleavage of pro-interleukin (IL)-1 $\beta$ ,<sup>9,12–18</sup> a characteristic feature of MKD.

It has been proposed that mutations in *MVK* may lead to temperature-sensitive changes in folding or stability of the enzyme.<sup>5,19</sup> Hence, elevations in body temperature caused by exercise, stress or other triggers could lead to reduced MVK activity and decreased flux through the mevalonate pathway. Inhibition of Rho or Rac prenylation could lead to increased inflammasome activity via

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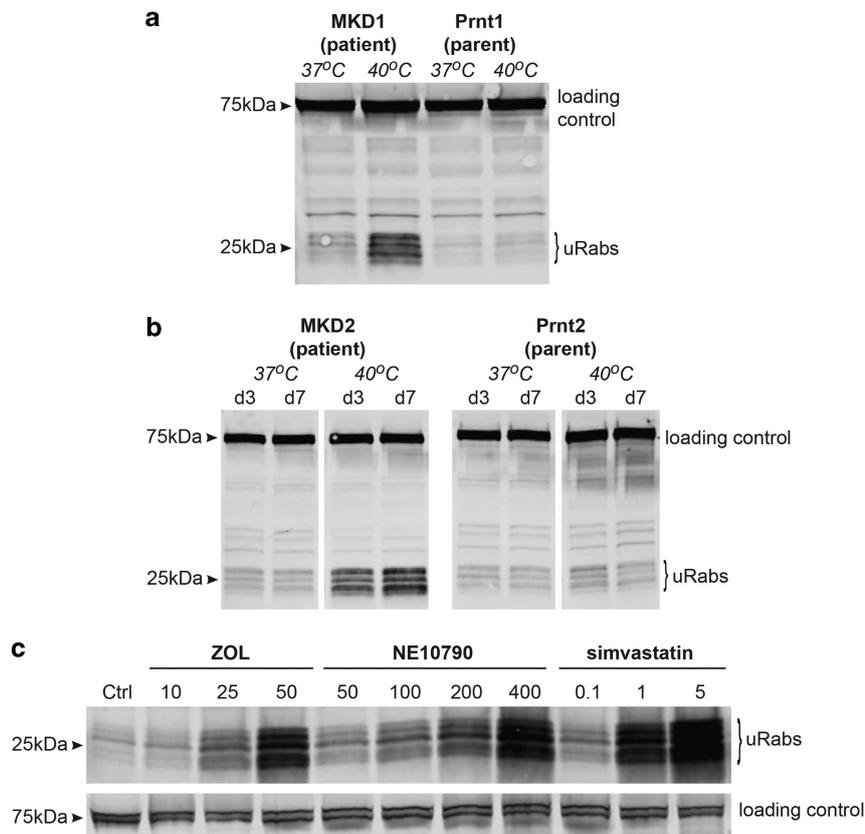
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**Figure 1** Increased temperature causes decreased Rab prenylation in MKD cell lines. (a) MKD1 (patient) cells, but not Prnt1 (parent) cells, have an accumulation of 21–27 kDa unprenylated Rab GTPases (uRabs) when cultured at 40 °C compared with 37 °C for 7 days. (b) MKD2 (patient) cells, but not Prnt2 (parent) cells, have an accumulation of 21–27 kDa unprenylated Rab GTPases (uRabs) when cultured at 40 °C compared with 37 °C for 3 days (d3) or 7 days (d7). (c) Culture of a control lymphoblast cell line at 37 °C for 48 h with 10–50  $\mu\text{M}$  zoledronic acid (ZOL), 50–400  $\mu\text{M}$  NE10790 or 0.1–5  $\mu\text{M}$  simvastatin causes a concentration-dependent increase in unprenylated Rab proteins. The doublet (or broad singlet) of 75 kDa endogenous biotinylated protein was used as a loading control in a–c. The data shown are representative of three independent experiments.

defects in autophagy.<sup>20,21</sup> However, numerous steps in autophagy are also regulated by Rab GTPases,<sup>22</sup> which are prenylated by geranylgeranyltransferase II (GGTase II or Rab GGTase). Despite proposed mechanistic models of enhanced IL-1 $\beta$  release involving decreased Rho/Rac prenylation,<sup>21,22</sup> previous studies have failed to demonstrate any intrinsic defect in protein prenylation in cells derived from MKD patients in the absence of statin treatment.<sup>23</sup> Furthermore, whether loss of Rab prenylation contributes to the inflammatory phenotype of MKD has not been examined. Here, we took a novel approach, utilising a highly sensitive *in vitro* prenylation assay,<sup>24</sup> to demonstrate for the first time that the prenylation of Rab proteins is altered in cell lines derived from MKD patients following mild heat stress. However, using NE10790 (a specific inhibitor of geranylgeranyltransferase II<sup>25–27</sup>) we found that the loss of Rab prenylation is not sufficient to enhance the release of IL-1 $\beta$  following lipopolysaccharide (LPS) stimulation of human monocytes.

## RESULTS AND DISCUSSION

In this study we specifically sought to answer the long-standing question whether protein prenylation is compromised in MKD cells. We identified two children with MKD. A 3-year-old daughter of unrelated parents (MKD1) was found to be compound heterozygous for missense *MVK* mutation V377I (c.1129G>A) and c.1058\_1060del deletion in exon 11 (a mutation that, to our knowledge, has not previously been reported). She presented with recurrent fever, spastic

cerebral palsy of unknown cause, failure to thrive, and elevated IgD and IgA levels (500 mg l<sup>-1</sup>, 2.74 g l<sup>-1</sup>, respectively). An 8-year-old boy (MKD2), born to non-consanguineous parents, presented by 12 months of age with 3–4 weekly episodes of periodic fevers lasting 3–4 days in association with abdominal pain, lethargy and arthralgia. His IgA and IgD levels were elevated (9.65 and 1.68 g l<sup>-1</sup>, respectively). He was confirmed to have compound heterozygous missense mutations in *MVK*; V377I and H20N (c.58C>A). His parents were confirmed heterozygous carriers.

Epstein-Barr Virus-transformed lymphoblastoid cell lines (EBV-LCLs) were generated using peripheral blood samples from the two MKD patients (MKD1 and MKD2) and from one heterozygous (V377I) parent of each child (Prnt1 and Prnt2, respectively).

### MKD patient cells show temperature-dependent accumulation of unprenylated Rab proteins

Previous studies have shown that fibroblasts from MKD patients, including those with V377I/H20P mutations, have an ~50% decrease in *MVK* activity after culturing for 24 h at 39 °C.<sup>19</sup> We used a highly sensitive *in vitro* prenylation assay with GGTase II<sup>24</sup> to determine whether mutations in *MVK* cause a temperature-dependent accumulation of unprenylated Rab GTPases in EBV-LCLs derived from MKD patients. Lysates from MKD1 and MKD2 cells (patients), and Prnt1 and Prnt2 cells (parents) grown under normal culture conditions (37 °C) showed very low levels of unprenylated Rab proteins



### Quantitative SILAC analysis reveals mild accumulation of unprenylated Rab7, Rab14 and Rab1 in MKD cells at 40 °C

To identify and quantify the unprenylated Rab proteins that accumulated in MKD cells at 40 °C versus 37 °C, we enriched the *in vitro*-prenylated (biotinylated) Rab proteins from lysates prepared from stable isotope labelling with amino acids in cell culture (SILAC)-labelled MKD2 EBV-LCLs cultured either at 37 or 40 °C for 7 days. Enriched, biotinylated Rab proteins were then identified by mass spectrometry<sup>24</sup> and the relative abundance of each was calculated as the fold change between cells cultured at 37 or 40 °C. Ten *in vitro*-prenylated Rab proteins were identified with a false discovery rate <1% (Table 1). After culture at 40 °C compared with 37 °C, there was an ~200% increase in unprenylated Rab7A, Rab14 and Rab1A, an ~100% increase in unprenylated Rab11A/B, Rab5B and Rab21, and an ~40% increase in Rab6A/B/39A, Rab1B/C and Rab2 A/B. Although the fold increase in the unprenylated form of these Rab proteins appears fairly small, the cumulative effect of a small increase in each of these unprenylated proteins, and probably other, less abundant Rab GTPases that could not be identified by mass spectrometry, is still evident in the corresponding protein blots of MKD2 cells in Figure 1b.

The increases in unprenylated Rab proteins in MKD2 cells that we identified by SILAC are considerably lower than those observed after treatment of macrophages with very low (nanomolar) concentrations of nitrogen-containing bisphosphonates.<sup>24</sup> This again raises the question whether treatment of cells in culture with high (micromolar) concentrations of nitrogen-containing bisphosphonates or statins truly reflects the more subtle changes in prenylation that occur in cultured MKD cells.

### Inhibition of Rab prenylation does not enhance IL-1 $\beta$ release by THP-1 monocytes or human PBMC

Human EBV-LCLs do not release IL-1 $\beta$ . Therefore, to determine whether lack of prenylation of Rab GTPases or Rho/Rac/Rap-family GTPases leads to enhanced IL-1 $\beta$  release, human THP-1 monocytic cells were treated with simvastatin (prevents prenylation of all small GTPases), GGTI-298 (prevents prenylation of Rho/Rac/Rap-family GTPases) or NE10790 (prevents prenylation of Rab GTPases)

(Figure 2a). THP-1 cells were pretreated for 24 h with concentrations of each inhibitor that had similar effects on Rab or Rap1A prenylation: 5  $\mu$ M simvastatin, 10  $\mu$ M GGTI-298 or 2 mM NE10790 (Figure 3a), before stimulation with 200 ng ml<sup>-1</sup> LPS for a further 6 h. These concentrations of inhibitors did not affect cell viability (which was consistently about 80% based on trypan blue exclusion) after 24 h treatment. Although exposure to simvastatin or GGTI-298 significantly enhanced the release of IL-1 $\beta$  caused by LPS stimulation, NE10790 had no effect (Figure 3b). IL-18 release (which, like IL-1 $\beta$ , is inflammasome-mediated) also increased with LPS stimulation, and, similar to IL-1 $\beta$ , LPS-mediated production of IL-18 was doubled by pretreating cells with simvastatin or GGTI-298, but not NE10790 (Supplementary Figure S2). Tumour necrosis factor $\alpha$  (TNF $\alpha$ ) was not detected in the absence of LPS. Treatment with LPS alone for 6 h caused considerable production of TNF $\alpha$  but neither simvastatin, GGTI-298 nor NE10790 showed any additional stimulatory effect on TNF $\alpha$  secretion (Supplementary Figure S2). This is consistent with a recent study demonstrating increased IL-1 $\beta$ , but not TNF $\alpha$ , production from MKD versus healthy control peripheral blood mononuclear cells (PBMC) following TLR4 ligation by LPS.<sup>30</sup> In cultures of PBMC from healthy controls, we found that treatment with 5  $\mu$ M simvastatin or 10  $\mu$ M GGTI-298 alone for 24 h caused the release of IL-1 $\beta$  even in the absence of LPS. However, similar to THP-1 cells, NE10790 had no effect despite comparable inhibition of Rab prenylation with simvastatin (Figures 3c and d).

In conclusion, our studies demonstrate for the first time that raised temperature causes a mild inhibition of protein prenylation and accumulation of unprenylated Rab GTPases in EBV-LCLs from MKD patients. However, loss of Rab prenylation alone does not lead to enhanced IL-1 $\beta$  release from monocytes following LPS treatment. In contrast, inhibition of prenylation of Rho/Rac/Rap GTPases is sufficient to predispose monocytes to excessive IL-1 $\beta$  release (Figure 3e); the exact mechanisms involved remain to be definitively proven and validated in MKD cells. Importantly, decreased MVK activity in cultured EBV-LCLs from MKD patients appears to be partially counteracted by increased activity of endogenous SREBP, therefore cultured MKD cell lines may not be a good model to study the functional consequences of human MVK mutations.

**Table 1** Quantitative SILAC analysis reveals increased accumulation of unprenylated Rab proteins in MKD2 cells cultured at 40 °C compared with 37 °C

Name	Relative abundance (%)	Ratio 40 °C/37 °C (H/L)	Ratio 40 °C/37 °C (L/H)
Rab11A/11B	8.7	2.1	1.8
Rab6A/6B/39A	23.4	1.6	1.4
Rab1A	0.8	ND	2.9
Rab1B/1C	12.6	1.6	1.5
Rab5C	4.6	1.4	0.8
Rab7A	10.0	3.0	2.7
Rab2A/2B	5.6	1.4	1.4
Rab5B	19.8	1.7	1.7
Rab14	1.0	2.7	ND
Rab21	13.5	2.4	1.3

Abbreviations: LC/MS, liquid chromatography-mass spectrometry; MKD2, mevalonate kinase deficiency; SILAC, stable isotope labelling with amino acids in cell culture. Cells labelled in 'heavy' (H) or 'light' (L) medium were cultured for 7 days at 40 °C or 37 °C, respectively. As an inverse labelling control, cells labelled in 'heavy' (H) or 'light' (L) medium were cultured for 7 days at 37 °C or 40 °C, respectively. Equal amounts of protein from cell lysates were mixed prior to *in vitro* prenylation, then biotinylated proteins were enriched with streptavidin beads and analysed by LC/MS. The amount of individual Rab proteins was expressed as a ratio of levels in cells at 40 or 37 °C (either H/L or L/H). For the 10 Rab proteins identified, the average relative abundance was calculated from the analysis of whole-cell lysates of H- or L-labelled cells cultured at 37 °C. Rab1A and Rab14 could not be detected (ND) in one of the pair of H- or L-labelled samples.

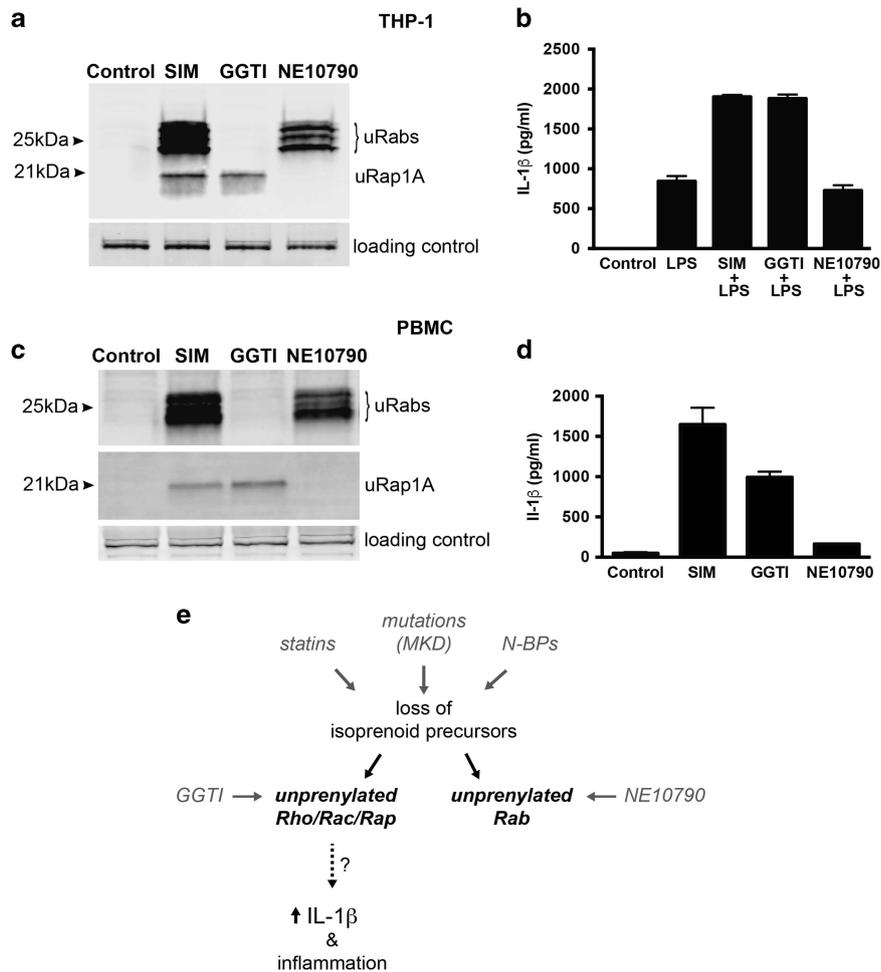
## METHODS

### Cells and reagents

With Human Research Ethics Committee (HREC) approval, informed consent was obtained prior to obtaining fresh blood samples from the patients and parents. Isolated PBMC were transformed with EBV using standard approaches. EBV-LCLs, THP-1 cells (mycoplasma-free, obtained from ATCC, Manassas, VA, USA) or human PBMC isolated from buffy coat preparations of healthy blood donors were cultured at 37 or 40 °C in RPMI with 10% foetal calf serum. Fatostatin (Tocris, Avonmouth, Bristol, UK), zoledronic acid (a gift from Dr Jonathan Green) and NE10790 (a gift from Dr Hal Ebetino) were dissolved as concentrated stocks in phosphate-buffered saline. GGTI-298 (Sigma, St Louis, MO, USA) was dissolved in dimethyl sulfoxide. Reagents for the prenylation assay were kindly provided by Professor Kirill Alexandrov and Dr Zakir Tnimov (University of Queensland).

### Quantitative PCR analysis

MKD1 and Prnt1 cells ( $5 \times 10^5$  cells per ml) were cultured at 40 °C and treated for 2 days with 10  $\mu$ M fatostatin or dimethyl sulfoxide vehicle before collection for RNA extraction (ISOLATE II RNA Mini Kit, Bioline) and cDNA synthesis (Tetro cDNA Synthesis Kit, Bioline, Alexandria, NSW, Australia) according to the manufacturer's



**Figure 3** Inhibition of Rab prenylation by NE10790 does not enhance IL-1 $\beta$  release from THP-1 monocytes. Unprenylated Rab GTPases (uRabs) or unprenylated Rap1A (uRap1A) were detected in THP-1 lysates (a) or human PBMC lysates (c) after treatment for 24 h with 5  $\mu$ M simvastatin (SIM), 10  $\mu$ M GGTI-298 or 2 mM NE10790. Endogenous 75 kDa biotinylated protein was used as a loading control. The data shown are representative of three independent experiments. (b) Pretreatment of THP-1 cells for 24 h with 5  $\mu$ M SIM or 10  $\mu$ M GGTI-298 enhanced the release of mature IL-1 $\beta$  after stimulation with 200 ng ml<sup>-1</sup> LPS for 6 h, whereas pretreatment with 2 mM NE10790 had no effect. (d) Treatment of human PBMC for 24 h with 5  $\mu$ M SIM or 10  $\mu$ M GGTI-298 caused the release of mature IL-1 $\beta$ , whereas 2 mM NE10790 had no effect. ELISA values in b, d are the mean  $\pm$  s.d. of triplicate samples and are representative of at least two independent experiments. (e) Disruption of the mevalonate pathway, by pharmacological inhibition or mutations in MVK, results in reduced synthesis of isoprenoid lipid precursors essential for protein prenylation. However, it is the loss of prenylation of GTPases modified by GGTase I (e.g., Rho, Rac and Rap), and not the loss of prenylated Rab GTPases (prenylated by GGTase II), that causes excessive IL-1 $\beta$  production.

instructions. RT-PCR was performed on a ABI Quantstudio7 (Scoresby, VIC, Australia) with specific Taqman primers for HMG-CoA reductase, MVK, FPP and beta-2-microglobulin (B2M) (Thermo Fisher Scientific Scoresby, VIC, Australia, Hs00168352\_m1, Hs00176077\_m1, Hs01560316\_g1 and Hs99999907 respectively). Comparative values of expression (dd-CT) were calculated using beta-2-microglobulin as a housekeeping gene, and presented as relative to the vehicle control.

#### In vitro prenylation assay

Cells were washed twice with phosphate-buffered saline and lysed by sonication in prenylation buffer (50 mM HEPES, pH 7.2, 50 mM NaCl, 2 mM MgCl<sub>2</sub>, 100  $\mu$ M GDP, 1 $\times$  Roche complete EDTA-free protease inhibitor cocktail, Basel, Switzerland). Protein was quantified using a BCA assay (Pierce, Scoresby, VIC, Australia). Dithiothreitol (DTT) was added to 50  $\mu$ g cell lysate to a final concentration of 2 mM, with final concentrations of 2  $\mu$ M Rab GGTase, 2  $\mu$ M recombinant *Danio rerio* Rab escort protein-1, 0.5  $\mu$ M Biotin-geranyl diphosphate (B-GPP) in a total volume of  $\sim$ 50  $\mu$ l and the reactions incubated for 5 h at

room temperature. *In vitro*-prenylated (that is, biotinylated) Rab proteins were detected on polyvinylidene difluoride blots using streptavidin-680RD (LiCOR, Lincoln, NE, USA).<sup>24</sup> We also consistently detected a narrow doublet (often appearing as a broad singlet) of endogenous biotinylated 75 kDa proteins, which were used as a sample loading control. Blots were also analysed for unprenylated Rap1A using goat anti-Rap1A (sc-1482).<sup>24</sup>

#### SILAC and mass spectrometry

For quantitative Mass Spectrometry analysis, MKD2 cells were labelled using SILAC for at least 10 cell doublings, as previously described.<sup>24</sup> Cells (10<sup>6</sup>) were seeded into T25 flasks with 8 ml of SILAC medium (Sigma) in duplicate. 'Light'-labelled cells were cultured at 37  $^{\circ}$ C for 7 days, whereas 'heavy'-labelled cells were cultured at 40  $^{\circ}$ C for 7 days. The culture medium was supplemented once with 4 ml of fresh medium. Labelling conditions were reversed in a replicate experiment. Cells were washed three times with phosphate-buffered saline and lysed by sonication in prenylation buffer. Protein lysate (840  $\mu$ g) was mixed in equal proportions from 'light'- and 'heavy'-labelled cells

and the *in vitro* prenylation assay performed as described above. *In vitro*-prenylated proteins were isolated using streptavidin beads, then the bound proteins were analysed on an Orbitrap Velos Pro mass spectrometer (Thermo Scientific, Scoresby, VIC, Australia).<sup>24</sup> For individual protein quantitation, normalised ratios were calculated from the MaxQuant data analysis software (Max Planck Institute of Biochemistry, Martinsried, Germany) for cells cultured at 37 and 40 °C.

### IL-1 $\beta$ , IL-18 and TNF- $\alpha$ ELISA

THP-1 cells or human PBMC from healthy donors ( $2 \times 10^5$  cells in 200  $\mu$ l in a 96-well plate) were treated with 5  $\mu$ M simvastatin, 10  $\mu$ M GGTI-298 or 2 mM NE10790 for 24 h. THP-1 cells were additionally stimulated with 200 ng ml<sup>-1</sup> LPS for a further 6 h. Conditioned media were collected and analysed by ELISA for human TNF- $\alpha$  (Elisakit.com), IL-1 $\beta$  and IL-18 (Instant ELISA, eBioscience, San Diego, CA, USA). The cell pellets were harvested and analysed for unprenylated Rab proteins and Rap1A (as described above) to assess the effect of these drug concentrations on protein prenylation.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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