



OPEN

## Mechanism of TNF $\alpha$ -induced downregulation of salt-inducible kinase 2 in adipocytes

Magdaléna Vaváková<sup>1</sup>, Kaisa Hofwimmer<sup>2</sup>, Jurga Laurencikiene<sup>2</sup> & Olga Göransson<sup>1</sup>✉

Salt-inducible kinase 2 (SIK2) is highly expressed in white adipocytes, but downregulated in individuals with obesity and insulin resistance. These conditions are often associated with a low-grade inflammation in adipose tissue. We and others have previously shown that SIK2 is downregulated by tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), however, involvement of other pro-inflammatory cytokines, or the mechanisms underlying TNF $\alpha$ -induced SIK2 downregulation, remain to be elucidated. In this study we have shown that TNF $\alpha$  downregulates SIK2 protein expression not only in 3T3L1- but also in human in vitro differentiated adipocytes. Furthermore, monocyte chemoattractant protein-1 and interleukin (IL)-1 $\beta$ , but not IL-6, might also contribute to SIK2 downregulation during inflammation. We observed that TNF $\alpha$ -induced SIK2 downregulation occurred also in the presence of pharmacological inhibitors against several kinases involved in inflammation, namely c-Jun N-terminal kinase, mitogen activated protein kinase kinase 1, p38 mitogen activated protein kinase or inhibitor of nuclear factor kappa-B kinase (IKK). However, IKK may be involved in SIK2 regulation as we detected an increase of SIK2 when inhibiting IKK in the absence of TNF $\alpha$ . Increased knowledge about inflammation-induced downregulation of SIK2 could ultimately be used to develop strategies for the reinstatement of SIK2 expression in insulin resistance.

Salt-inducible kinase 2 (SIK2) is a serine/threonine kinase downstream of the master kinase liver kinase B1<sup>1</sup>, with a high expression level in white adipose tissue<sup>2,3</sup>. Previously, we have shown that SIK2 is downregulated in adipose tissue of individuals with obesity and insulin resistance<sup>4</sup>. Pharmacological- or genetic inhibition of SIK isoforms resulted in impairment of insulin signaling and/or glucose uptake in rat and human adipocytes<sup>4,5</sup>. It is thus possible that downregulation of SIK2 in obesity could contribute to the development of obesity-induced insulin resistance.

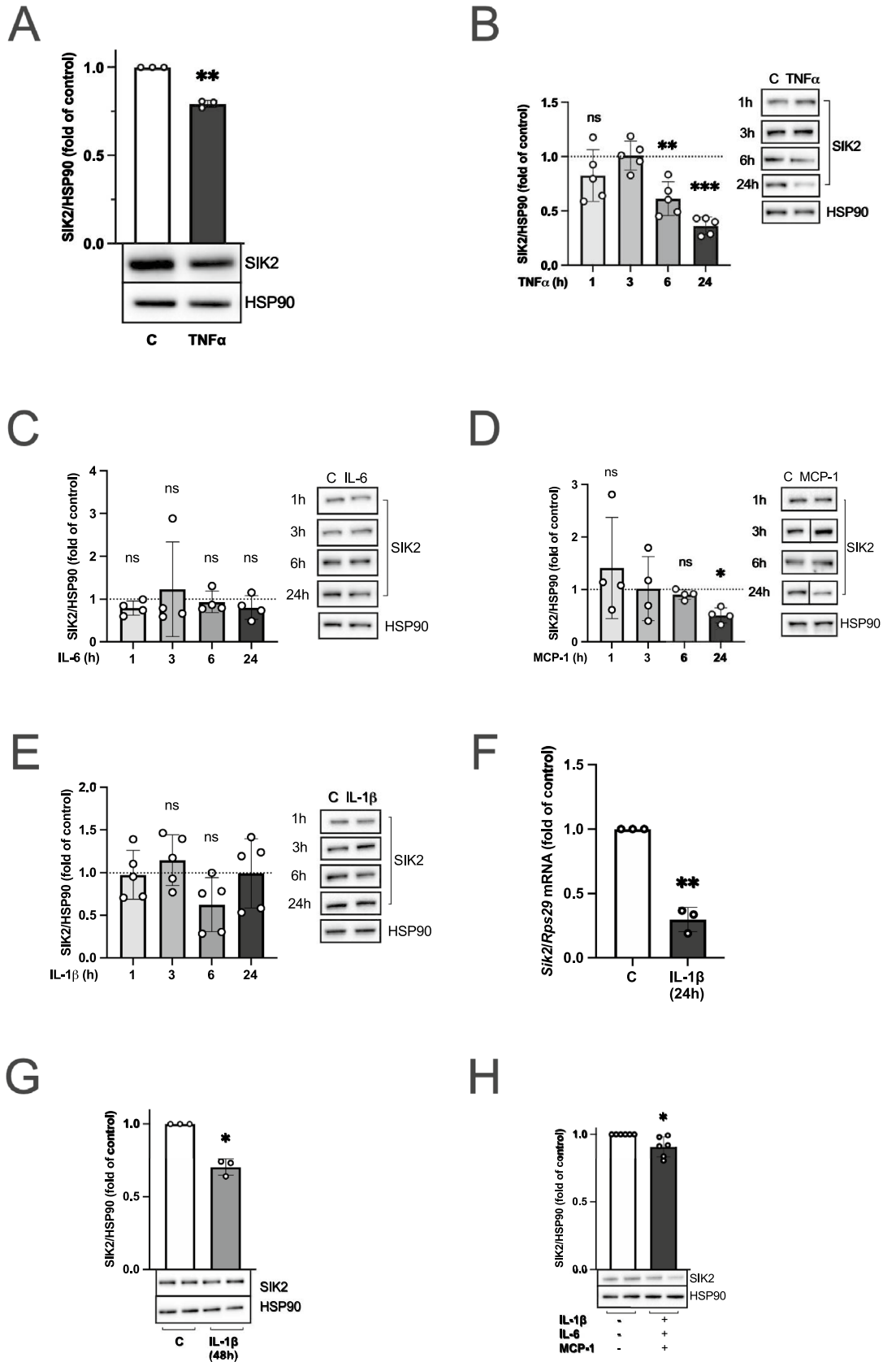
Obesity and insulin resistance is often associated with a low-grade inflammation in adipose tissue and elevated tissue- and plasma levels of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin (IL)-6, IL-1 $\beta$  and monocyte chemoattractant protein-1 (MCP-1)<sup>6,7</sup>. These pro-inflammatory stimuli signal through a number of intracellular pathways involving serine/threonine kinases such as c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), p38 mitogen activated protein kinase (p38) and inhibitor of nuclear factor kappa-B (NF $\kappa$ B) kinase (IKK)<sup>8,9</sup>. IKK in turn phosphorylates inhibitor of NF $\kappa$ B (I $\kappa$ B), which leads to I $\kappa$ B degradation and subsequent translocation of NF $\kappa$ B to the nucleus<sup>10</sup>.

We<sup>4</sup> and others<sup>11</sup> have previously shown that expression of SIK2 mRNA is downregulated by TNF $\alpha$  in human adipocytes and at the mRNA and protein level in 3T3-L1 adipocytes. The mechanism underlying the downregulation of SIK2 by TNF $\alpha$  is not fully understood. Therefore, in this study we investigated involvement of selected key mediators of pro-inflammatory pathways in TNF $\alpha$ -induced SIK2 downregulation in adipocytes. Furthermore, we explored if other cytokines or chemokines have comparable effects on SIK2 to TNF $\alpha$ .

### Results

**SIK2 protein expression is downregulated not only by TNF $\alpha$  but to some extent also by MCP-1 and IL-1 $\beta$ .** In agreement with our previous study investigating the effect of TNF $\alpha$  on SIK2 mRNA levels<sup>4</sup>, SIK2 protein was downregulated in human mesenchymal stem cells (hMSCs) differentiated into adipocytes and treated with TNF $\alpha$  (Fig. 1A). Successful TNF $\alpha$  stimulation was confirmed by means of increased p38 phosphorylation and decreased I $\kappa$ B $\alpha$  levels (Supplementary Fig. S1A,B). Similarly, in the mouse adipocyte like cell line

<sup>1</sup>Protein Phosphorylation Research Group, Section for Diabetes, Metabolism and Endocrinology, Department of Experimental Medical Science, Lund University, Biomedical Centre C11, Klinikgatan 28, 221 84 Lund, Sweden. <sup>2</sup>Lipid Laboratory, Unit of Endocrinology, Department of Medicine Huddinge, Karolinska Institute, Stockholm, Sweden. ✉email: olga.goransson@med.lu.se



**◀Figure 1.** SIK2 protein levels at different timepoints after treatment with TNF $\alpha$ , IL-6, MCP-1, IL-1 $\beta$  and their combination. SIK2 protein levels in hMSCs differentiated into adipocytes (A) and 3T3-L1 adipocytes (B–E, G–H) treated with or without (control, C, dashed line) 50 ng/ml of human TNF $\alpha$  for 24 h (A), 20 ng/ml of mouse TNF $\alpha$  (B), 20 ng/ml IL-6 (C), 20 ng/ml MCP-1 (D) or 20 ng/ml IL-1 $\beta$  (E) for indicated time periods. *Sik2* mRNA levels in 3T3-L1 adipocytes treated with 20 ng/ml IL-1 $\beta$  (F) for 24 h. SIK2 protein levels after 48 h of 20 ng/ml IL-1 $\beta$  (G) or a combination of 100 ng/ml IL-1 $\beta$ , 50 ng/ml IL-6 and 20 ng/ml MCP-1 (H) for 24 h in 3T3-L1 adipocytes. HSP90 was used as a loading control for protein analysis. Representative blots are shown. *Rps29* was used as a housekeeping gene for mRNA analysis. Data are presented as means  $\pm$  SD from multiple independent experiments (A, F–G; n = 3, C, D; n = 4, B, E; n = 5, H; n = 6). Statistical significance was analyzed by one sample t-test (A, F–H) or by one way ANOVA with a Holm–Šidak’s multiple comparisons test (B–E). Uncropped blots are presented in Supplementary Fig. S3.

3T3-L1, TNF $\alpha$  downregulated SIK2 protein- (Fig. 1B) and mRNA expression (Supplementary Fig. S2A). Efficiency of TNF $\alpha$  treatment was demonstrated by a decrease of I $\kappa$ B $\alpha$  levels (Supplementary Fig. S1C).

To assess if SIK2 responds to inflammatory stimuli other than TNF $\alpha$ , but that are also known to be elevated in obesity<sup>6</sup>, we treated 3T3-L1 adipocytes with IL-6, MCP-1 and IL-1 $\beta$  for indicated time periods (Fig. 1C–G, Supplementary Fig. S1D–F, S2B, C). Again, a decrease in I $\kappa$ B $\alpha$  levels (IL-6 and MCP-1) or an increase in the phosphorylation of p38 (IL-1 $\beta$ ), indicated successful cytokine treatment (Supplementary Fig. S1D–F). For IL-6, we did not observe downregulation of SIK2, neither at the protein- (Fig. 1C) nor at the mRNA level (Supplementary Fig. S2B). Notably, we observed significant SIK2 downregulation after 24 h treatment with MCP-1 (Fig. 1D), however without an effect on *Sik2* mRNA at the same time point (Supplementary Fig. S2C). IL-1 $\beta$  did not affect SIK2 protein levels over a 24 h period (Fig. 1E), but significantly reduced *Sik2* mRNA at 24 h (Fig. 1F). To further elucidate this finding, we treated 3T3-L1 adipocytes with IL-1 $\beta$  for 48 h, which, in contrast to the shorter timepoints investigated previously, lead to a 30% downregulation of SIK2 protein levels (Fig. 1G). Finally, to investigate the combined effect of these cytokines on SIK2 protein levels, we co-treated 3T3-L1 adipocytes with IL-1 $\beta$ , IL-6 and MCP-1. This treatment resulted in a 9% decrease in the SIK2 protein level after 24 h (Fig. 1H). Phosphorylation of ERK1/2 and phosphorylation of protein kinase B (PKB) was monitored to determine the effect of the combined cytokine stimulation on inflammatory pathways (Supplementary Fig. S2D,E). Interestingly, the cytokine co-treatment resulted in a decrease of pERK/ERK (Supplementary Fig. S2D). However, we observed a tendency to decreased pPKB/PKB ratio (Supplementary Fig. S2E), which is in line with Yang et al.<sup>12</sup>.

In summary, our results indicate that not only TNF $\alpha$ , but also IL-1 $\beta$  and MCP-1, have negative effects on SIK2 expression. However, IL-1 $\beta$  seems to affect SIK2 protein levels only after extended treatment (48 h) and MCP-1 has no apparent effect on *Sik2* mRNA levels in 3T3-L1 adipocytes. We therefore next focused on investigating involvement of selected key mediators of pro-inflammatory pathways in TNF $\alpha$ -induced SIK2 downregulation.

**TNF $\alpha$ -induced downregulation of SIK2 is not mediated by MEK1, p38, JNK or IKK.** To identify pathways involved in TNF $\alpha$ -induced SIK2 downregulation in 3T3-L1 adipocytes, we pharmacologically inhibited kinases known to be activated downstream of the TNF $\alpha$  receptor, namely mitogen activated protein (MAP) kinase kinase 1 (MEK1), p38, JNK and IKK (Fig. 4).

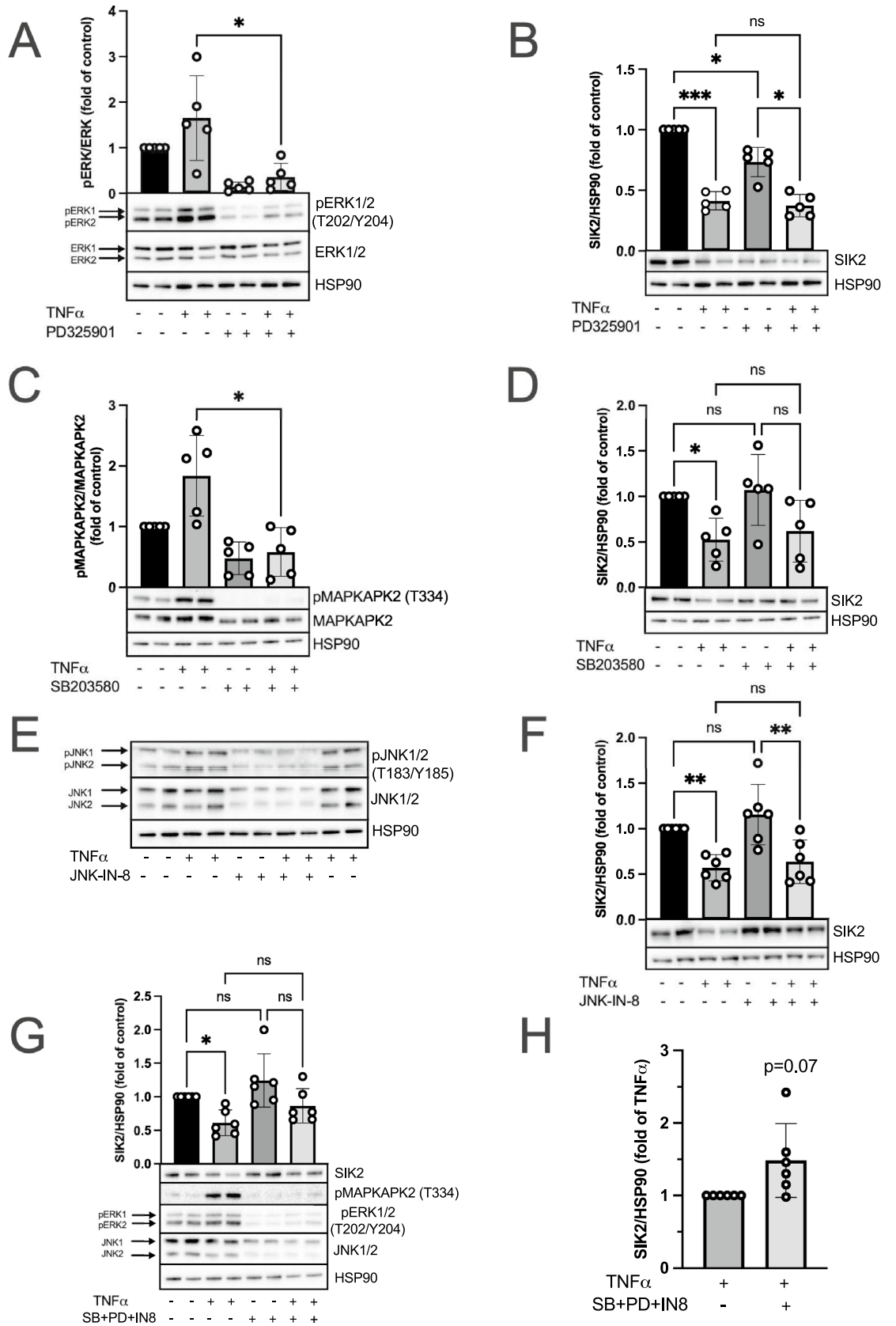
First, MEK1, the upstream kinase for ERK, was inhibited using PD0325901<sup>13</sup> and the efficiency of the inhibition was validated by monitoring the phosphorylation of ERK1/2 (Fig. 2A). Treatment with PD0325901 resulted in a significantly reduced phosphorylation of ERK1/2 in the presence of TNF $\alpha$ —demonstrating successful inhibition of MEK1. As observed previously, TNF $\alpha$  induced a downregulation of SIK2, which was however not prevented by the MEK1 inhibitor (Fig. 2B). On the contrary, we observed a decrease of SIK2 in response to the inhibitor alone (Fig. 2B).

The pharmacological inhibitor SB203580 was used for inhibiting p38 MAPK and the efficiency was assessed by monitoring phosphorylation of the p38 substrate MAP kinase-activated protein kinase 2 (MAPKAPK2)<sup>14</sup> (Fig. 2C). When inhibiting p38, the reduction of SIK2 upon TNF $\alpha$  stimulation was no longer significant (Fig. 2D). However, there was also no significant difference in SIK2 levels when comparing TNF $\alpha$ -treated cells in the absence and presence of the inhibitor, although the inhibitor efficiently reduced MAPKAPK2 phosphorylation (Fig. 2C). There was no effect of the inhibitor alone on SIK2 protein levels (Fig. 2D). All in all, our interpretation of these results is that most likely p38 does not mediate the effect of TNF $\alpha$  on SIK2 and the absence of significant TNF $\alpha$ -mediated downregulation in the presence of the SB203580 is due to low sample size.

Next, JNK was inhibited using JNK-IN-8<sup>15</sup>. Efficiency of the inhibitor was verified by a change in the electrophoretic mobility (gel shift) of JNK1/2, with the inhibitor-bound form of JNK migrating higher up, as shown by Zhang et al.<sup>15</sup> (Fig. 2E). We also observed a reduction in JNK expression over the studied time period (Fig. 2E). However, pre-treatment with JNK-IN-8 did not prevent TNF $\alpha$ -induced downregulation of SIK2 (Fig. 2F). Moreover, JNK-IN-8 alone did not alter SIK2 protein levels (Fig. 2F).

To elucidate a potential compensatory effect of JNK, MEK1 and p38 pathways, when one of them are inhibited, we treated 3T3-L1 adipocytes simultaneously with JNK-IN-8, PD0325901 and SB203580 inhibitors. The effect of this mix on pro-inflammatory signaling pathways was assessed by monitoring pMAPKAPK2 and pERK1/2 as well as by gel shift of JNK1/2 (Fig. 2G). Co-inhibition of these pathways tended to partially prevent TNF $\alpha$ -induced downregulation of SIK2, when only comparing cells treated with TNF $\alpha$  in the absence or presence of the inhibitor, however this did not reach statistical significance (Fig. 2H). SIK2 protein levels remained unchanged when treated with beforementioned inhibitors in the absence of TNF $\alpha$  (Fig. 2G).

Finally, to assess the possible involvement of the IKK/NF $\kappa$ B pathway, we treated 3T3-L1 adipocytes with BI605906<sup>16</sup>, an inhibitor of IKK, and validated successful inhibition by monitoring the pI $\kappa$ B $\alpha$ /I $\kappa$ B $\alpha$  ratio (Fig. 3A). Co-treatment of cells with BI605906 and TNF $\alpha$  did not prevent the downregulation of SIK2 by TNF $\alpha$  (Fig. 3B),



◀**Figure 2.** Effect of pharmacological inhibition of MEK1, p38 and JNK on SIK2 protein levels with or without TNF $\alpha$ . Pharmacological inhibition of MEK1 by 1  $\mu$ M PD325901 (A, B), p38 by 50  $\mu$ M SB203580 (C, D), JNK by 10  $\mu$ M JNK-IN-8 (E, F) and the combination of these three inhibitors (G, H) with or without 20 ng/ml TNF $\alpha$  in 3T3-L1 adipocytes. Cells were incubated with TNF $\alpha$  and their respective inhibitors for 18 h (A–D, G, H) or for 19 h (E, F) which included 1 h of pre-treatment with JNK-IN-8 inhibitor. Efficiency of the inhibitors was monitored by analyzing T202/Y204 pERK1/2/ERK1/2 ratio (A, G), T334 pMAPKAPK2/MAPKAPK2 ratio (C, G) and electrophoretic mobility of JNK1/2 (E, G). SIK2 protein levels were analyzed (B, D, F–H) and HSP90 was used as a loading control. Representative blots are shown. Data are presented as means  $\pm$  SD from multiple independent experiments (A–D; n = 5, F–H; n = 6). Statistical significance was analyzed by a two-tailed paired t-test (A, C), one way ANOVA with a Holm–Šidak’s multiple comparisons test (B, D, F, G) or by one sample t-test (H). Uncropped blots are presented in Supplementary Fig. S5.

although a reduction in pIkBa/IkBa indicated successful inhibition (Fig. 3A). However, inhibition of IKK by BI605906 in the absence of TNF $\alpha$  resulted in significant upregulation of SIK2 on the protein level (Fig. 3B).

## Discussion

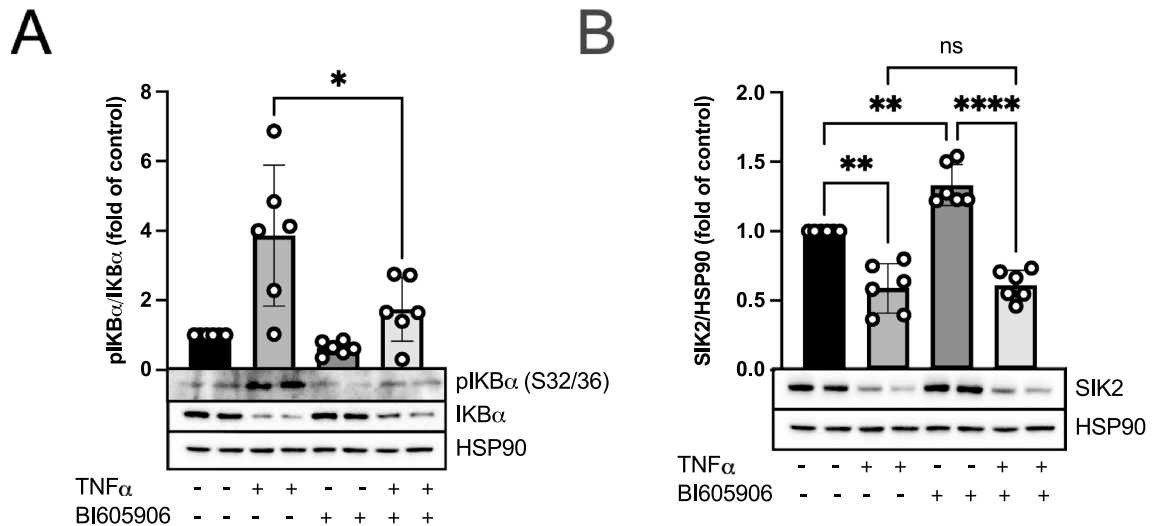
We and others have previously demonstrated that SIK2 is downregulated by TNF $\alpha$  on the protein and mRNA level in the 3T3-L1 mouse adipocyte like cell line<sup>4,11</sup>. We have also shown a downregulation of *Sik2* mRNA by TNF $\alpha$  in in vitro differentiated human adipocytes<sup>4</sup>. In this study, we verified these results, and additionally demonstrated that TNF $\alpha$ -induced downregulation of mRNA translates into lower protein levels of SIK2 in the human cells as well. We have also previously shown that SIK2 expression in adipose tissue is negatively correlated with BMI<sup>4</sup>. Obesity is often associated with low grade inflammation and elevated levels of various pro-inflammatory factors, including TNF $\alpha$ , IL-6, IL-1 $\beta$  and MCP-1<sup>6</sup>. In this study we conclude that, in addition to TNF $\alpha$ , IL-1 $\beta$  and MCP-1 have effects on SIK2 expression (Fig. 4), but these are not as rapid and profound as those of TNF $\alpha$ . We therefore hypothesize that inflammation-associated downregulation of SIK2 is mainly driven by TNF $\alpha$ , but that other cytokines also contribute. In future studies it would be interesting to address if low adipose tissue SIK2 expression in obesity and insulin resistance is indeed a result of low-grade inflammation and the presence of elevated TNF $\alpha$ .

MCP-1 downregulated SIK2 protein levels after 24 h, which was however not associated with changes in mRNA at the same timepoint. This may indicate that *Sik2* mRNA levels were downregulated by MCP-1 in a shorter time period and returned to a basal level after 24 h treatment. We can also speculate that MCP-1 affects protein stability and/or degradation of SIK2. Further studies are needed to answer these questions. mRNA analysis revealed that, not only TNF $\alpha$ , but also IL-1 $\beta$  induced *Sik2* mRNA downregulation, but the latter did not translate into lower SIK2 protein until after 48 h treatment. The reasons for this delayed effect on the protein levels are not clear but the long-time frame suggests that the effects of IL-1 $\beta$  might be indirect and secondary to for example IL-1 $\beta$ -induced production of TNF $\alpha$ <sup>17</sup>. Future studies are needed to elucidate this downregulation of *Sik2* by IL-1 $\beta$  in more detail.

An important focus of our work was to delineate mechanisms whereby TNF $\alpha$  downregulates SIK2. Using pharmacological inhibitors, we showed that neither MEK1, JNK nor p38 activity is required for TNF $\alpha$ -induced SIK2 downregulation (Fig. 4). In fact, inhibition of MEK1 in the absence of TNF $\alpha$  resulted in *downregulation* of SIK2 on protein level, suggesting, if anything, that MEK signaling to some extent promotes SIK2 expression. During the preparation of this manuscript, Yoon et al. showed involvement of IKK in TNF $\alpha$ -induced *Sik2* mRNA downregulation, using the inhibitor IKK16<sup>11</sup>, whereas in our experiments, inhibition of IKK with BI605906 did not reverse the reduction in SIK2 expression following TNF $\alpha$ . The reason for this discrepancy is not clear but could involve different time points [18 h (our study) vs. 6 h (Yoon et al.<sup>11</sup>) of co-treatment with TNF $\alpha$  and the inhibitor], different cell models (3T3-L1 vs. primary mouse adipocytes) or different materials analyzed (protein vs mRNA). We attempted to investigate effects of the IKK16 inhibitor on SIK2 protein levels in 3T3-L1 adipocytes, but observed significant cell death at the concentration used by Yoon et al.<sup>11</sup>. Moreover, we did not observe efficient IKK inhibition at lower concentrations (data not shown). Noteworthy, BI605906 is an IKK2-selective inhibitor<sup>16</sup>, whereas IKK16 inhibits both IKK1, IKK2<sup>18</sup> as well as an unrelated leucine-rich repeat kinase 2 (LRRK2)<sup>19</sup>, suggesting that IKK1 or LRRK2 could be mediating TNF $\alpha$ -induced SIK2 downregulation. Interestingly, we did detect a significant upregulation of SIK2 protein level when inhibiting IKK in the absence of TNF $\alpha$  stimuli. This, together with the data by Yoon et al.<sup>11</sup>, supports involvement of IKK in the regulation of SIK2 expression, at least in the basal state (Fig. 4). Another possibility that could be addressed in future studies is the involvement of non-canonical TNF $\alpha$  signaling, centered around NF $\kappa$ B-inducing kinase (NIK)<sup>20</sup>.

Mechanisms for transcriptional regulation of SIK2, such as the transcription factors involved, have been scarcely studied, but Yoon et al.<sup>11</sup> demonstrated that overexpression of CCAAT/enhancer-binding protein alpha (C/EBP $\alpha$ ) induced SIK2 protein expression as well as the activity of an SIK2 luciferase reporter, proposing that C/EBP $\alpha$  is a direct transcriptional activator of SIK2. Moreover, they showed that overexpression of NF $\kappa$ B, which is activated downstream of IKK<sup>21</sup>, reduced C/EBP $\alpha$  reporter activity. Together, this indicates that the downregulation of SIK2 by TNF $\alpha$  could be mediated via NF $\kappa$ B-induced reduction of C/EBP $\alpha$ . However, considering the rapid effect of TNF $\alpha$  on SIK2 expression, an intriguing possibility is that NF $\kappa$ B directly suppresses SIK2 transcription—a hypothesis that could be tested using SIK2 reporter assays and chromatin immunoprecipitation in future studies.

In summary, we have further established that TNF $\alpha$  downregulates SIK2 expression both at the mRNA- and protein level in mouse as well as in human adipocytes. Furthermore, our results support that other pro-inflammatory cytokines can contribute to regulation of SIK2 expression but that the effects of TNF $\alpha$  are more profound. Neither inhibition of MEK1, p38, JNK nor IKK reversed TNF $\alpha$ -induced SIK2 protein downregulation (Fig. 4).



**Figure 3.** Effect of pharmacological inhibition of IKK on SIK2 protein levels with or without TNF $\alpha$ . Pharmacological inhibition of IKK by 10  $\mu$ M BI605906 (**A**, **B**) for 18 h with or without 20 ng/ml TNF $\alpha$  in 3T3-L1 adipocytes. Efficiency of BI605906 was monitored by analyzing S32/36 pIKB $\alpha$ /IKB $\alpha$  ratio (**A**). SIK2 protein levels were analyzed (**B**) and HSP90 was used as a loading control. Representative blots are shown. Data are presented as means  $\pm$  SD from multiple independent experiments ( $n = 6$ ). Statistical significance was analyzed by a two-tailed paired t-test (**A**) or by a one way ANOVA with Holm–Sidak’s multiple comparisons test (**B**). Uncropped blots are presented in Supplementary Fig. S5.

However, IKK inhibition increased SIK2 in the absence of TNF $\alpha$ , suggesting that IKK may indeed be involved in the regulation of SIK2 expression (Fig. 4).

A limitation of this study is the potential off target effects of pharmacological inhibitors. In the future, siRNA silencing could be used to further study the involvement of various kinases in the downregulation of SIK2 by TNF $\alpha$ .

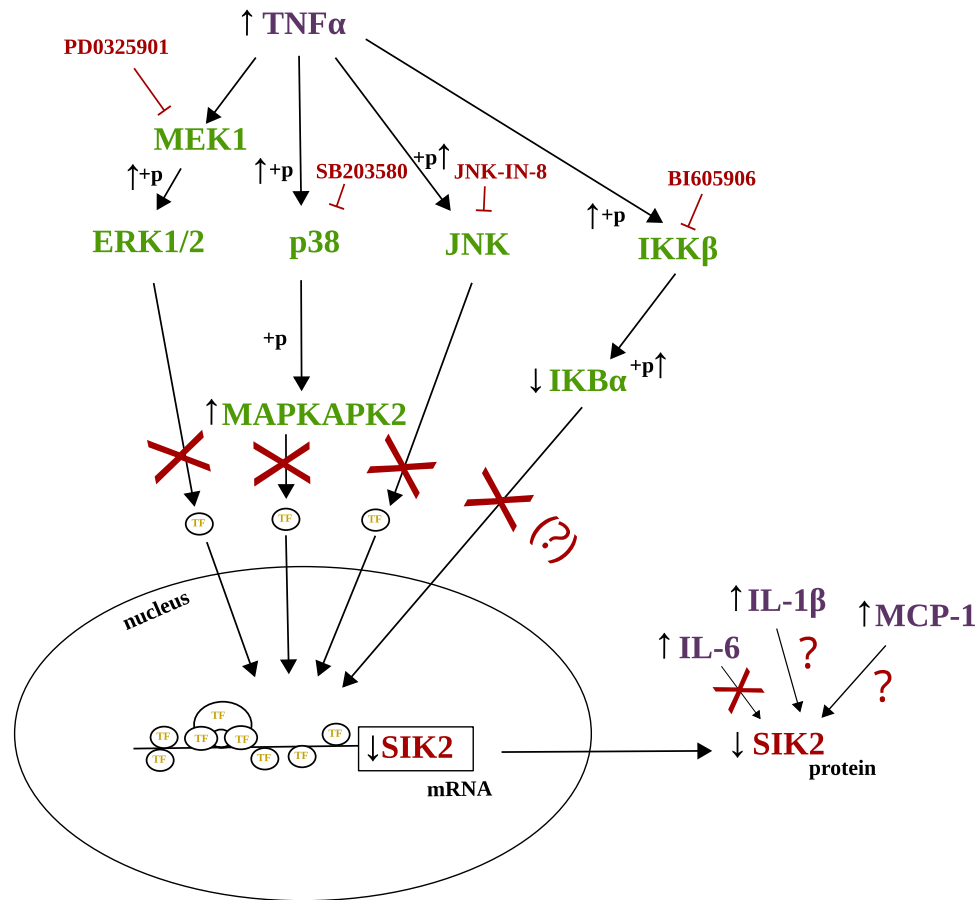
## Methods

**Chemicals and reagents.** Mouse TNF $\alpha$  (#T539), human TNF $\alpha$  (#T6674), mouse IL-1 $\beta$  (#SRP8033), mouse IL-6 (#SRP3330) and mouse MCP-1 (#SRP3215) were purchased from Sigma-Aldrich. PD0325901 (#HY-10254), SB203580 (#HY-10256), JNK-IN-8 (#HY-13319), BI605906 (#HY-13019) and IKK16 (#HY-13687) were obtained from MedChemExpress and used in concentrations and time points indicated in figure legends.

**Cell culture.** 3T3-L1 murine fibroblasts obtained from American Type Culture Collection (ATCC, CL-173) were cultured in high glucose DMEM media (Sigma, #D6429) supplemented with 10% fetal bovine serum (FBS, Sigma, #F7524) and 100 U/ml penicillin/streptomycin (Sigma, #P0781) in 5% CO $_2$  humidified atmosphere at 37  $^{\circ}$ C, at sub-confluence. Two days post-confluent cells were exposed to 1  $\mu$ M dexamethasone (Sigma-Aldrich #D4902), 0.52 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, #I7018) and 1.74  $\mu$ M insulin (Sigma-Aldrich, #I2643) for 72 h to induce differentiation. From day three, dexamethasone, IBMX and insulin were removed from the media. Cells were treated as indicated in figures and collected 9–14 days post-differentiation. hMSCs were isolated from a human multipotent stem cell population originating from adipose tissue, cultured and differentiated into adipocytes in vitro as described previously<sup>22</sup>.

**Cell stimulation, lysis and protein extraction.** Cells were treated with cytokines and/or inhibitors as indicated in figure legends. Treatment conditions were controlled with an equal volume of H $_2$ O (TNF $\alpha$ , IL-1 $\beta$ ), phosphate buffered saline (PBS) (IL-6, MCP-1) or dimethyl sulfoxide (DMSO) (inhibitors). Cellular proteins were collected in lysis buffer containing 50 mM Tris–HCl pH 7.5, 1% NP40, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), 5 mM Na-pyrophosphate, 0.27 M sucrose, 50 mM NaF, 1 mM Na-orthovanadate, 1 mM dithiothreitol (DTT) and cOmplete protease inhibitors (Roche, #11697498,001) (3T3-L1) or RIPA buffer (Thermo Scientific, #89901) supplemented with protease inhibitors (Millipore, #539134) and phosphatase inhibitors (Roche, #04906837,001) (hMSCs). Protein concentration was determined by the Bradford method for protein quantification<sup>23</sup>.

**Western blot.** Cell lysates (10–30  $\mu$ g) mixed with 4xLDS sample buffer (Thermo Scientific) were heated for 5 min in 95  $^{\circ}$ C, loaded on pre-cast Novex Bis–Tris 4–12% polyacrylamide gels (Thermo Scientific), transferred to nitrocellulose membrane (Amersham™ Protran™ Western blotting membrane, Sigma-Aldrich), blocked for 60 min in 10% (w/v) skimmed milk in tris-buffered saline containing Tween 20 (TBS-T, 50 mM Tris–HCl pH 7.6, 137 mM NaCl and 0.1% (w/v) Tween 20, Sigma-Aldrich) and incubated overnight at 4  $^{\circ}$ C in primary antibodies diluted in 5% (w/v) bovine serum albumin (BSA, Sigma-Aldrich) in TBS-T. Horseradish peroxidase-coupled anti-mouse (Sigma-Aldrich, #NA931) and anti-rabbit (Thermo Scientific, #A16096) secondary antibodies,



**Figure 4.** Schematic summary of investigated pathways and main conclusions. We hypothesized that SIK2 is downregulated through one or several highlighted pathways downstream of TNF $\alpha$ , and used a number of pharmacological agents (in red) to inhibit these pathways. We also investigated the potential effects of cytokines other than TNF $\alpha$ , on the expression of SIK2. We conclude that TNF $\alpha$ -induced downregulation of SIK2 is likely not mediated by MEK1, p38, JNK or IKK. However, we do not completely rule out the involvement of IKK. Moreover, SIK2 expression is to some extent downregulated by IL-1 $\beta$  and MCP-1, albeit not as rapidly and robustly as by TNF $\alpha$ . ERK1/2: Extracellular signal-regulated kinase 1/2; I $\kappa$ B $\alpha$ : nuclear factor kappa-B inhibitor alpha; IKK $\beta$ : Inhibitor of nuclear factor kappa-B kinase subunit beta; JNK: c-Jun N-terminal kinase 1; MAPKAPK2: mitogen activated protein (MAP) kinase-activated protein kinase 2; MEK1: MAP kinase kinase 1; SIK2: Salt-inducible kinase 2; TF: transcription factor; TNF $\alpha$ : tumor necrosis factor  $\alpha$ ; IL-6: interleukin 6; IL-1 $\beta$ : interleukin 1  $\beta$ , MCP-1: monocyte chemoattractant protein-1; + p: phosphorylation;  $\downarrow$ : downregulation;  $\uparrow$ : upregulation;  $\perp$ : inhibition.

together with enhanced chemiluminescence reagent (SuperSignal West Pico or Femto Chemiluminescent Substrates, Thermo Scientific) were used for developing the signals. Bio-Rad Chemidoc CCD camera and Image Lab software (Bio-rad Laboratories) were used for detection and quantification. Signals were normalized against the loading control (HSP90) and a control condition (cells treated with H<sub>2</sub>O, PBS or DMSO) loaded on the same gel.

Primary antibodies used in this study included anti-HSP90 (BD Biosciences, #BD610418), anti-SIK2 (produced in house by Innovagen, Lund, as described in Henriksson et al.<sup>24</sup>), anti-ERK1/2 [Cell Signaling Technology (CST), #9102], anti-T202/Y204 p-ERK1/2 (CST, #9101), anti-JNK1/2 (Upstate, #06-748), anti-T183/Y185 p-JNK1/2 (CST, #4668), anti-I $\kappa$ B $\alpha$  (CST, #4814), anti-S32/36 p-I $\kappa$ B $\alpha$  (CST, #9246), anti-p38 (CST, #9212), anti-T180/Y182 p-p38 (CST, #9211), anti-MAPKAPK-2 (CST, #12155), anti-T334 p-MAPKAPK-2 (CST, #3007), anti-PKB (CST, #9272) and anti-S473 p-PKB (Thermo Fisher, #44621G).

**RNA extraction and qPCR.** Cells were lysed in Qiazol lysis reagent (Qiagen) and RNA was extracted according to the manufacturer's instructions using the RNeasy Mini kit (Qiagen). One step real time PCR was performed using Quantifast SYBR Green RT-PCR kit (Qiagen) in a QuantStudio 5 Real Time PCR system (Applied Biosystems). The results were calculated using the  $2^{-\Delta\Delta CT}$  method, normalized to the 40S ribosomal protein S29 (*Rps29*) and expressed as fold of a control condition. Primers for *Sik2* (#QT00129269) and *Rps29* (#QT02328858) were obtained from Qiagen.

**Statistics.** Data for SIK2 protein levels in samples treated with 20 ng/ml TNF $\alpha$  for 18 h and their representative controls were pooled and tested for normality by Shapiro–Wilk test ( $n = 28$ ,  $p > 0.1$ ). Normal distribution of the data was thereafter assumed across the study. Data are presented as means  $\pm$  standard deviation (SD). The statistical significance was analyzed by one-way ANOVA with Holm–Šidák’s multiple comparisons test, two tailed paired t-test or by one sample t-test, as stated in the legends. Statistical analysis as well as visualization was performed using GraphPad Prism 9. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

### Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Received: 17 February 2023; Accepted: 20 June 2023

Published online: 29 June 2023

### References

- Lizcano, J. M. *et al.* LKB1 is a master kinase that activates 13 kinases of the AMPK subfamily, including MARK/PAR-1. *Embo J.* **23**, 833–843. <https://doi.org/10.1038/sj.emboj.7600110> (2004).
- Horike, N. *et al.* Adipose-specific expression, phosphorylation of Ser794 in insulin receptor substrate-1, and activation in diabetic animals of salt-inducible kinase-2. *J. Biol. Chem.* **278**, 18440–18447. <https://doi.org/10.1074/jbc.M211770200> (2003).
- Du, J., Chen, Q., Takemori, H. & Xu, H. SIK2 can be activated by deprivation of nutrition and it inhibits expression of lipogenic genes in adipocytes. *Obesity (Silver Spring)* **16**, 531–538. <https://doi.org/10.1038/oby.2007.98> (2008).
- Säll, J. *et al.* Salt-inducible kinase 2 and -3 are downregulated in adipose tissue from obese or insulin-resistant individuals: Implications for insulin signalling and glucose uptake in human adipocytes. *Diabetologia* **60**, 314–323. <https://doi.org/10.1007/s00125-016-4141-y> (2017).
- Henriksson, E. *et al.* SIK2 regulates CRTCs, HDAC4 and glucose uptake in adipocytes. *J. Cell Sci.* **128**, 472–486. <https://doi.org/10.1242/jcs.153932> (2015).
- Makki, K., Froguel, P. & Wolowczuk, I. Adipose tissue in obesity-related inflammation and insulin resistance: Cells, cytokines, and chemokines. *ISRN Inflamm.* **2013**, 139239. <https://doi.org/10.1155/2013/139239> (2013).
- Wellen, K. E. & Hotamisligil, G. S. Inflammation, stress, and diabetes. *J. Clin. Invest.* **115**, 1111–1119. <https://doi.org/10.1172/jci25102> (2005).
- Tanti, J. F. & Jager, J. Cellular mechanisms of insulin resistance: Role of stress-regulated serine kinases and insulin receptor substrates (IRS) serine phosphorylation. *Curr. Opin. Pharmacol.* **9**, 753–762. <https://doi.org/10.1016/j.coph.2009.07.004> (2009).
- Meng, D. *et al.* p38 $\alpha$  deficiency in T cells ameliorates diet-induced obesity, insulin resistance, and adipose tissue senescence. *Diabetes* **71**, 1205–1217. <https://doi.org/10.2337/db21-0653> (2022).
- Karin, M. How NF- $\kappa$ B is activated: The role of the I $\kappa$ B kinase (IKK) complex. *Oncogene* **18**, 6867–6874. <https://doi.org/10.1038/sj.onc.1203219> (1999).
- Yoon, Y. S. *et al.* Activation of the adipocyte CREB/CRTC pathway in obesity. *Commun. Biol.* **4**, 1214. <https://doi.org/10.1038/s42003-021-02735-5> (2021).
- Yang, P. *et al.* Paradoxical effect of rapamycin on inflammatory stress-induced insulin resistance in vitro and in vivo. *Sci. Rep.* **5**, 14959. <https://doi.org/10.1038/srep14959> (2015).
- Sebolt-Leopold, J. S. & Herrera, R. Targeting the mitogen-activated protein kinase cascade to treat cancer. *Nat. Rev. Cancer* **4**, 937–947. <https://doi.org/10.1038/nrc1503> (2004).
- Cuenda, A. *et al.* SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364**, 229–233. [https://doi.org/10.1016/0014-5793\(95\)00357-f](https://doi.org/10.1016/0014-5793(95)00357-f) (1995).
- Zhang, T. *et al.* Discovery of potent and selective covalent inhibitors of JNK. *Chem. Biol.* **19**, 140–154. <https://doi.org/10.1016/j.chembiol.2011.11.010> (2012).
- Clark, K. *et al.* Novel cross-talk within the IKK family controls innate immunity. *Biochem. J.* **434**, 93–104. <https://doi.org/10.1042/bj20101701> (2011).
- Clark, K. *et al.* Phosphorylation of CRTC3 by the salt-inducible kinases controls the interconversion of classically activated and regulatory macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 16986–16991. <https://doi.org/10.1073/pnas.1215450109> (2012).
- Waelchli, R. *et al.* Design and preparation of 2-benzamido-pyrimidines as inhibitors of IKK. *Bioorg. Med. Chem. Lett.* **16**, 108–112. <https://doi.org/10.1016/j.bmcl.2005.09.035> (2006).
- Hermanson, S. B. *et al.* Screening for novel LRRK2 inhibitors using a high-throughput TR-FRET cellular assay for LRRK2 Ser935 phosphorylation. *PLoS ONE* **7**, e43580. <https://doi.org/10.1371/journal.pone.0043580> (2012).
- Sun, S. C. The non-canonical NF- $\kappa$ B pathway in immunity and inflammation. *Nat. Rev. Immunol.* **17**, 545–558. <https://doi.org/10.1038/nri.2017.52> (2017).
- Hoffmann, A., Natoli, G. & Ghosh, G. Transcriptional regulation via the NF- $\kappa$ B signaling module. *Oncogene* **25**, 6706–6716. <https://doi.org/10.1038/sj.onc.1209933> (2006).
- Pettersson, A. M. *et al.* LXR is a negative regulator of glucose uptake in human adipocytes. *Diabetologia* **56**, 2044–2054. <https://doi.org/10.1007/s00125-013-2954-5> (2013).
- Kruger, N. J. The Bradford method for protein quantitation. *Methods Mol. Biol.* **32**, 9–15. <https://doi.org/10.1385/0-89603-268-x:9> (1994).
- Henriksson, E. *et al.* The AMPK-related kinase SIK2 is regulated by cAMP via phosphorylation at Ser358 in adipocytes. *Biochem. J.* **444**, 503–514. <https://doi.org/10.1042/bj20111932> (2012).

### Acknowledgements

We thank Professor Philip Cohen, University of Dundee, UK, for recommendation of selective inhibitors used in this study.

### Author contributions

M.V. and O.G. were responsible for, and K.H. and J.L. contributed to, the conception and design of the study. M.V. performed most of the experiments. K.H. performed experiments on hMSCs. M.V. and O.G. analyzed and interpreted the data. M.V. prepared the figures and drafted the manuscript. O.G., K.H. and J.L. edited and revised the manuscript. O.G. is the guarantor of this work. All authors approved the final version of the manuscript.



## Funding

Open access funding provided by Lund University. This work was financially supported by The Swedish Research Council (project Grant Dnr 2017-01295, the Strategic Research Area Exodiab Dnr 2009-1039), The Swedish Foundation for Strategic Research (Dnr IRC15-0067), The Swedish Diabetes Foundation and the Royal Physiographic Society of Lund.

## Competing interests

The authors declare no competing interests.

## Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-023-37340-5>.

**Correspondence** and requests for materials should be addressed to O.G.

**Reprints and permissions information** is available at [www.nature.com/reprints](http://www.nature.com/reprints).

**Publisher's note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



**Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2023