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ARTICLE

Nuclear factor κB (NF-κB) suppresses food intake and energy expenditure in mice by directly activating the *Pomc* promoter

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Abstract
Aims/hypothesis While chronic low-grade inflammation is associated with obesity, acute inflammation reduces food intake and leads to negative energy balance. Although both types of inflammation activate nuclear factor κB (NF-κB) signalling, it remains unclear how NF-κB activation results in opposite physiological responses in the two types of inflammation. The goal of this study was to address this question, and to understand the link between inflammation and leptin signalling.

Methods We studied the ability of NF-κB to modulate *Pomc* transcription, and how it impinges on signal transducer and activator of transcription 3 (STAT3)-mediated leptin signalling by using a combination of animal models, biochemical assays and molecular biology.

Results We report that suppression of food intake and physical movement with acute inflammation is not dependent on STAT3 activation in pro-opiomelanocortin (POMC) neurons. Under these conditions, activated NF-κB independently leads to increased *Pomc* transcription. Electrophoretic mobility shift assay and chromatin immunoprecipitation (ChIP) experiments reveal that NF-κB v-rel reticuloendotheliosis viral oncogene homologue A (avian) (RELA [also known as p65]) binds to the *Pomc* promoter region between −138 and −88 bp, which also harbours the trans-acting transcription factor 1 (SP1) binding site. We found significant changes in the methylation pattern at this region and reduced *Pomc* activation under chronic inflammation induced by a high-fat diet. Furthermore, RELA is unable to bind and activate transcription when the *Pomc* promoter is methylated. Finally, RELA binds to STAT3 and inhibits STAT3-mediated promoter activity, suggesting that RELA, possibly together with forkhead box-containing protein 1 (FOXO1), may prevent STAT3-mediated leptin activation of the *Pomc* promoter.

Conclusions/interpretation Our study provides a mechanism for the involvement of RELA in the divergent regulation of energy homeostasis in acute and chronic inflammation.

Keywords Appetite · Energy homeostasis · FOXO1 · Inflammation · NF-κB · Obesity · STAT3

Abbreviations

A*STAR	Agency for Science, Technology and Research
ChIP	Chromatin immunoprecipitation
DKO	Double-knockout
EMSA	Electrophoretic mobility shift assay
FOXO1	Forkhead box-containing protein 1

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HFD	High-fat diet
IKK	IκB kinase
LFD	Low-fat diet
LPS	Lipopolysaccharide
NF-κB	Nuclear factor κB
NPY	Neuropeptide Y
P-KO	POMC-neuron-specific deletion of *Stat3* (*Pomc/Stat3*$^{F/F}$)
POMC	Pro-opiomelanocortin
PTP1B	Protein-tyrosine kinase 1B
RAP1	Ras-related protein 1
RELA	V-rel reticuloendotheliosis viral oncogene homologue A (avian)
RER	Respiratory exchange ratio
SOCS3	Suppressor of cytokine signalling 3
SP1	Trans-acting transcription factor 1
STAT3	Signal transducer and activator of transcription 3

Introduction

The maintenance of energy homeostasis is regulated by neurohormonal signals. Leptin, a key hormone involved in this process, is released from adipocytes [1]. Through a saturated transport mechanism, circulating leptin enters the brain and elicits specific downstream signalling pathways to inhibit food intake and energy expenditure by acting on at least the following neurons in the hypothalamus: the orexigenic neuropeptide Y (NPY) and the anorexigenic pro-opiomelanocortin (POMC) neurons [1, 2]. In the case of POMC neurons, leptin binds to the long-form leptin receptor [3], leading to phosphorylation and nuclear translocation of signal transducer and activator of transcription 3 (STAT3). Activated STAT3 then binds to the trans-acting transcription factor 1 (SP1)–*Pomc* promoter complex to activate *Pomc* transcription to suppress appetite [2, 4].

However, the leptin signalling can be impaired in pathological conditions, such as in obesity models, resulting in a phenomenon known as leptin resistance [5]. A number of models have been proposed to account for leptin resistance, for example, suppression of STAT3 phosphorylation and thus its downstream actions by suppressor of cytokine signalling 3 (SOCS3) [6, 7] and protein-tyrosine phosphatase 1B (PTP1B) [8, 9]. Previously, we have identified that forkhead box-containing protein 1 (FOXO1) may act at a step downstream of STAT3 phosphorylation to prevent activated STAT3 from interacting with the SP1–*Pomc* promoter complex, thereby repressing transcriptional activation [10]. FOXO1 may also inhibit phosphorylated STAT3 (pSTAT3) action directly at the promoter level [11, 12].

Other non-physiological conditions, such as inflammation, can also have direct and profound effects on food intake and energy expenditure [13]. Acute inflammation such as that caused by bacterial lipopolysaccharide (LPS) activates TNF-α, IL-1β, and IL-6 in the hypothalamic–pituitary cytokine network and subsequently induces *Pomc* expression, leading to reduced food intake and increased energy expenditure [14]. In contrast, chronic low-grade inflammation is often associated with unsuppressed appetite and increased obesity, along with other metabolic disorders, such as insulin resistance and beta cell dysfunction, although inflammatory cytokines are also elevated under this condition.

Inflammatory cytokines bind to cell surface toll-like receptors (TLRs) to activate the IκB kinase (IKK) kinase complex, which consists of the catalytic subunits IKKα and IKKβ, and the regulatory subunits IKKγ (also known as NF-κB essential modulator [NEMO]), Ras-related protein 1 (RAP1) [15] and ELKS (also known as ELKS/RAB6-interacting/CAST family member 1 [ERC1]) [16]. IKKβ plays a key role in metabolic regulation. For example, mice with brain- and hypothalamus-specific deletion of *Ikkβ* (also known as *Ikbkb*) show decreased food intake, and are protected against diet-induced obesity, insulin resistance and glucose intolerance [17–19]. The IKK kinase complex is crucial for activation of nuclear factor κB (NF-κB) to drive the expression of a large number of target genes, which may intersect with other signalling cascades such as those controlled by leptin. The fact that both acute and chronic inflammation activate NF-κB, but result in opposite physiological responses, indicates that NF-κB does not simply antagonise leptin signalling.

To understand the role of NF-κB in energy regulation, and to tease out the link between inflammation and leptin signalling pathways, we studied *Pomc* transcriptional regulation by v-rel reticuloendotheliosis viral oncogene homologue A (avian) (RELA [also known as p65]), the most abundant subunit of NF-κB [20], and STAT3, a key regulator in leptin signalling. Here, we show that RELA activation in acute inflammatory conditions directly promotes *Pomc* transcription. The direct activation of *Pomc* promoter, however, is impaired under chronic inflammatory conditions, as RELA is unable to bind to methylated *Pomc* promoter, which occurs under chronic inflammation. At the same time, like FOXO1, RELA also attenuates leptin action by binding to STAT3. The combined effect under chronic inflammatory conditions is loss of *Pomc* regulation by either RELA or STAT3. These in vitro findings support our model of divergent regulation of *Pomc* transcription by NF-κB, which sheds new light on how inflammation may interfere with leptin signalling. Future in vivo studies are needed to validate this model.

Methods

Animal welfare All experiments involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of Agency for Science, Technology and Research (A*STAR). The mice were supplied by the A*STAR animal facility in Singapore.

Oxymax/Comprehensive Lab Animal Monitoring System Oxymax/Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA) was used to measure and determine oxygen consumption (volume of O_2 [VO_2]), carbon dioxide production (volume of CO_2 [VCO_2]), activity, food intake, basal metabolic rate, respiratory exchange ratio (RER) and movements of individual mice [21, 22]. Paired mice were individually housed in chambers maintained at 24±1°C, and given free access to chow and water. All the measurements were taken every 15 min for 5 days after the mice were acclimatised for 1 day. All data collected were averaged from 5 days' monitoring.

Mouse tissue preparation, DNA extraction and bisulfite modification We used male C57Bl/6 mice fed with a high-fat diet (HFD) containing 60% of energy from fat, or a low-fat diet (LFD) (Research Diets, New Brunswick, NJ, USA) for 20 weeks (n=20 per group). Mouse brains were quickly removed and frozen using Mouse Brain Slicer Matrix (Zivic Instruments, Pittsburgh, PA, USA) on dry ice. Coronal sections, 500 μm containing the arcuate (Arc) region of the hypothalamus were collected by microdissection knife for genomic DNA isolation by DNeasy kit (Qiagen, Valencia, CA, USA), followed by bisulfate modification and purification using the EpiTect Bisulfite Kit (Qiagen).

Amplification of the Pomc promoter region, ligation, cloning and sequencing The region between −269 and −99 bp in the *Pomc* promoter was amplified with the following primers (5′-TTG TTT AGT TTT AAG TGG AGA TTT AAT ATT-3′ and 5′-AAA ACT ATC CAA AAC TAA AAC ACC CT-3′) using bisulfite-modified DNA as templates. After purification with QIAquick Gel Extraction Kit (Qiagen), the PCR products were ligated into the TA vectors (pCT2.1-TOPO, Qiagen). Sequencing for the *Pomc* promoter region was performed on 15–20 positive clones per mouse.

Quantitative real-time PCR The cDNAs were diluted ten times for PCR analysis in triplicates. A 10 μl volume of reaction mixture contained 5 μl SYBR Green, PCR Master Mix, 300 nmol/l specific target gene primers and 2 μl of cDNA. The reactions were performed on an ABI Prism 7500 sequence detector system. SYBR Green primers were designed by Primer Express software from Applied Biosystems (Carlsbad, CA, USA); the sequences are provided in electronic supplementary material (ESM) Table 1.

DNA constructs The *Pomc* promoter-luciferase construct (*Pomc*-Luc) was a generous gift from D. Accili (Columbia University, New York, NY, USA). The following plasmids were generated in our laboratories and have been described previously: pcDNA3.1-*Rela-Myc*, pXJ40-*Flag-STAT3*, pN3-*Flag-Sp1* [10].

Cell culture and luciferase assay Flp-In 293-OBRb (293-OBRb) cells [23] were cultured in Dulbecco's Minimal Essential Medium (Invitrogen, Carlsbad, CA, USA) containing 10% (vol./vol.) fetal bovine serum in a 37°C incubator with 5% CO_2. Transfection and cell treatment were done as previously described [10]. Luciferase activity was measured from cell extracts on an Lmax II (Molecular Devices, Sunnyvale, CA, USA). The firefly luciferase activity was normalised to Renilla luciferase activity.

Cytosolic and nucleus extracts preparation The cells were washed twice and collected in cold PBS. The cell suspension was centrifuged at 200g for 5 min. The resulting pellet was then resuspended with hypotonic buffer containing 20 mmol/l HEPES, pH 7.9, 10 mmol/l KCl, 1 mmol/l EDTA, 1 mmol/l Na_3VO_4, 10% glycerol, 0.2% Nonidet P-40, 20 mmol/l NaF, 1 mmol/l dithiothreitol and ×1 complete protease inhibitor (Roche Applied Science, Indianapolis, IN, USA) and rocked at 4°C for 10 min. The mixture was then centrifuged at 20,000g for 30 s, the supernatant fraction was collected as the cytosolic fraction, and the pellet was resuspended with high-salt buffer (20% glycerol, 420 mmol/l NaCl, 1 mmol/l Na_3VO_4, 1 mmol/l dithiothreitol and ×1 complete protease inhibitor in hypotonic buffer without Nonidet P-40). After 1 h rocking, the mixture was centrifuged at 20,000g for 10 min at 4°C. The supernatant fraction was then collected as the nuclear extract.

Co-immunoprecipitation assays 293-OBRb cells were transfected with expression vectors pcDNA3.1-*Rela-Myc* (full length or deletion mutants) and pXJ40-*Flag-Stat3* (full length or deletion mutants) by using Lipofectamine 2000 (Invitrogen) and harvested 48 h after transfection with whole-cell lysis buffer (20 mmol/l Hepes pH 7.9, 280 mmol/l KCl, 1 mmol/l EDTA, 0.1 mmol/l Na_3VO_4, 10% Glycerol, 0.5% NP40, 1 mmol/l DTT, 1 mmol/l PMSF, protease inhibitor cocktail). Cell lysate, 1,000 μg, was incubated with antibody against FLAG (Sigma-Aldrich, St Louis, MO, USA), MYC (Roche Applied Science) or control mouse IgG overnight, followed by immunoprecipitation with 1:1 protein A/G-sepharose beads (Invitrogen) for 1 h. The beads were washed four times with lysis buffer, mixed

with ×2 SDS loading buffer, and the resulting samples were loaded and resolved using 10% SDS-PAGE.

Chromatin immunoprecipitation assays Preparation of chromatin DNA from 293-OBRb cells expressing *Flag-Rela* or *Flag-Sp1* and subsequent chromatin immunoprecipitation (ChIP) assays were done with a (ChIP) assay kit (Upstate Biotechnology, Waltham, MA, USA) according to the manufacturer's instructions. *Rela*-DNA or *Sp1*-DNA complexes were precipitated by anti-FLAG antibody (Sigma-Aldrich). Precipitated DNA was amplified by real-time PCR using primers flanking the potential RELA binding site (−138 to −88 bp) in the *Pomc* promoter (ESM Table 2). Rabbit IgG was used as the negative control.

Electrophoretic mobility shift assays Nuclear, cytosolic or whole-cell protein extracts were collected using an NE-PER Nuclear and Cytoplasmic Extraction Reagent Kit (Thermo Scientific, Waltham, MA, USA). Normal, mutant and methylated probes (ESM Table 3) were annealed and labelled by biotin (LightShift Chemiluminescent EMSA Kit, Thermo Scientific). For 20 μl binding reaction, 2–3 μl of NE-PER nuclear extract was used. To each 20 μl binding reaction, 5 μl of ×5 loading buffer was added, and then 20 μl samples were loaded onto 6% polyacrylamide gel in ×0.5 Tris/borate/EDTA (TBE). After membrane transfer and crosslinking, supersensitive x-ray films (Kodak, Rochester, NY, USA) were exposed for 2–5 min to detect the biotin-labelled DNA by chemiluminescence.

SDS-PAGE and western blot Electrophoresis of protein samples was done under denaturing reducing conditions using 10% polyacrylamide gels with SDS running buffer (Invitrogen). The blotted membranes were probed with antibodies against phosphorylated-STAT3 (pSTAT3 [Tyr705]), total STAT3, phosphorylated RELA (pRELA [Ser536]), RELA (Cell Signaling, Boston, MA, USA), FLAG (Sigma-Aldrich) and MYC (Roche Applied Science).

Statistics The data are presented as mean ± SEM. Comparisons of data were made using two-tailed Student's *t* test for independent data. The significance limits are displayed as $^{*}p<0.05$, and $^{**}p<0.01$.

Results

LPS-induced acute inflammation suppresses appetite and physical activity independent of STAT3-mediated Pomc activation Under physiological conditions, leptin is the major regulator of food intake by activating the Janus kinase 2 (JAK2)–STAT3 pathway [3]. However, activation of hypothalamic NF-κB signalling can also promote *Pomc* transcription and induce weight loss and food intake reduction [24]. Moreover, constitutively active RELA triggers severe TNF-α-dependent inflammation, which leads to small sized and lean mice on a *Tnfr1* (also known as *Tnfrsf1a*)$^{-/-}$ background [25]. To investigate whether acute inflammation-induced appetite suppression through NF-κB signalling in the hypothalamus depends on a leptin-induced STAT3–SP1–POMC pathway, we examined food intake in mice with POMC-neuron-specific deletion of *Stat3* (*Pomc/Stat3*$^{F/F}$) (P-KO) [26] in response to i.p. LPS injection, and compared them with *Stat3*$^{F/F}$ control mice. LPS injection caused a significant decrease in food intake, RER and locomotor activity when compared with the group receiving BSA injections. Furthermore, LPS-induced responses, such as food intake (Fig. 1a, b), cumulative food intake (Fig. 1c), RER (Fig. 1d) and horizontal movement (Fig. 1e) were indistinguishable between P-KO and control groups. We also examined *Pomc* transcript levels in these mice, and found the level was increased to a similar extent in P-KO mice as in the control group after LPS treatment (Fig. 1f), suggesting that LPS-induced signalling may directly regulate *Pomc* transcription, and that such regulation is independent of the STAT3 pathway.

RELA directly activates Pomc transcription Given that inflammation also induces NF-κB signalling, we next evaluated the role of this signalling cascade in the observed appetite suppression. To determine whether NF-κB signalling subunit RELA affects *Pomc* promoter activity, and whether this pathway is related to leptin signalling, we performed reporter gene assays in 293-OBRb cells [23]. The dual luciferase reporter system contained the *Pomc* promoter from −646 to +65 bp that was inserted upstream of the luciferase gene [10]. Expression of *Rela* alone resulted in enhanced *Pomc* promoter activity, even in the absence of leptin treatment, indicating that RELA can directly regulate *Pomc* transcription (Fig. 2a). With increasing amounts of RELA, we did not observe a dose-dependent increase in *Pomc* transcription without leptin treatment, suggesting that the lowest dose used in our experiments was at the saturating level in activating *Pomc* transcription (white bars in Fig. 2a). We then tested the crosstalk between RELA and leptin signalling in the regulation of *Pomc* activation, and found that there was no further increase in *Pomc* transcription with leptin treatment in the presence of RELA (Fig. 2a, b). At least two non-exclusive models can account for the above findings: first, RELA induces maximal *Pomc* activation, and leptin signalling does not have any additive effect; and second, RELA completely inhibits leptin signalling. The fact that even a very low dose of RELA could result in a saturated level of *Pomc* activation supports the first model. Nonetheless, we tested the second hypothesis, which may work in parallel with the first one, by examining the pSTAT3 levels in the cytosolic and nuclear fractions of

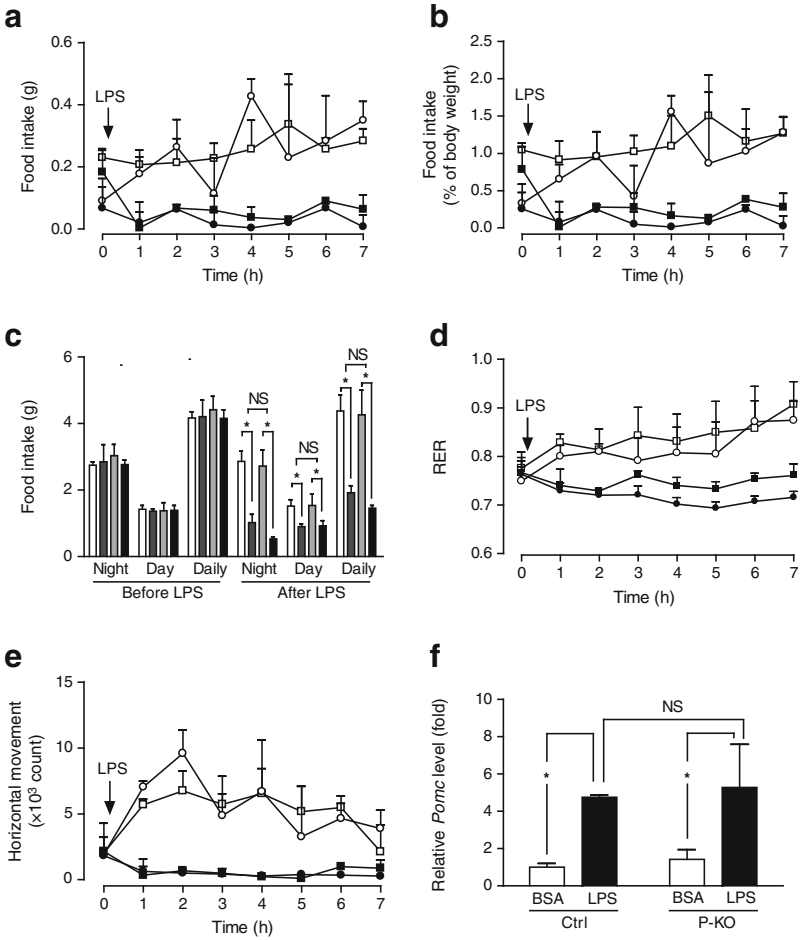

Fig. 1 LPS-induced acute inflammation suppresses appetite and physical activity independent of STAT3-mediated *Pomc* activation. (**a**–**c**) Acute inflammation induced by i.p. injection of LPS reduced food intake and energy expenditure in both P-KO and control (*Stat* $^{F/F}$) mice. The LPS effect was similar between P-KO and control mice. (**a**) Food intake during the first 8 h after i.p. injection of LPS. (**b**) Normalised food intake (% of body weight) during the first 8 h after LPS injection. (**c**) Food intake before and after LPS injection. White bars, control with BSA treatment; dark-shaded bars, control with LPS treatment; light-shaded bars, P-KO with BSA treatment; black bars, P-KO with LPS treatment. (**d**) LPS treatment reduced RER during the first 8 h after i.p. injection of LPS. (**e**) Horizontal movement was reduced by LPS treatment during the first 8 h after LPS injection. (**f**) *Pomc* mRNA levels in response to LPS treatment in P-KO and control groups. Data are presented as mean ± SEM. n=4 per group. $^{*}p<0.05$. For a, b, d and e: white squares, control with BSA treatment; black squares, control with LPS treatment; white circles, P-KO with BSA treatment; black circles, P-KO with LPS treatment. Ctrl, control

leptin-stimulated 293-OBRb cells. Although STAT3 may directly interact with RELA [27–30] (also see Fig. 5a), increasing amounts of RELA did not affect pSTAT3 nuclear translocation (Fig. 2c). Together, these data suggest that NF-κB RELA may directly and maximally activate *Pomc* transcription under acute inflammation, regardless of leptin action and nuclear levels of pSTAT3.

RELA regulates Pomc transcription through direct interaction To investigate whether NF-κB RELA regulates *Pomc* activity by direct interaction with the promoter, we first generated a series of deletion mutations (mutants 1–5) in the promoter region of *Pomc* to determine the essential regions for RELA regulation. RELA failed to activate *Pomc* transcription in mutant constructs 1 and 5, which lacked the region between −138 and −88 bp (black box), while the activity remained intact in the other mutants that contained this region (Fig. 3a). We further tested the effect of RELA using a point-mutation construct (mutant 6; sequence shown in Fig. 3b), and found that mutations within the region between −138 and −88 bp were sufficient to abolish the RELA activity (Fig. 3a). These results suggest that the identified *Pomc* promoter region may be the target binding site for RELA in regulating *Pomc* transcription. We tested this notion by electromobility shift assay (EMSA) using nuclear extracts from HEK293 cells transfected with *Rela*. The nuclear RELA protein bound to wild-type probes (lanes 2 and 3, Fig. 3c), but not to the mutant probes derived from the mutant 6 construct (lanes 4 and 5, Fig. 3c). The RELA binding activity was specifically inhibited by unlabelled wild-type probes (lanes 6 and 7, Fig. 3c), and the complex was super-shifted with the addition of the RELA antibody

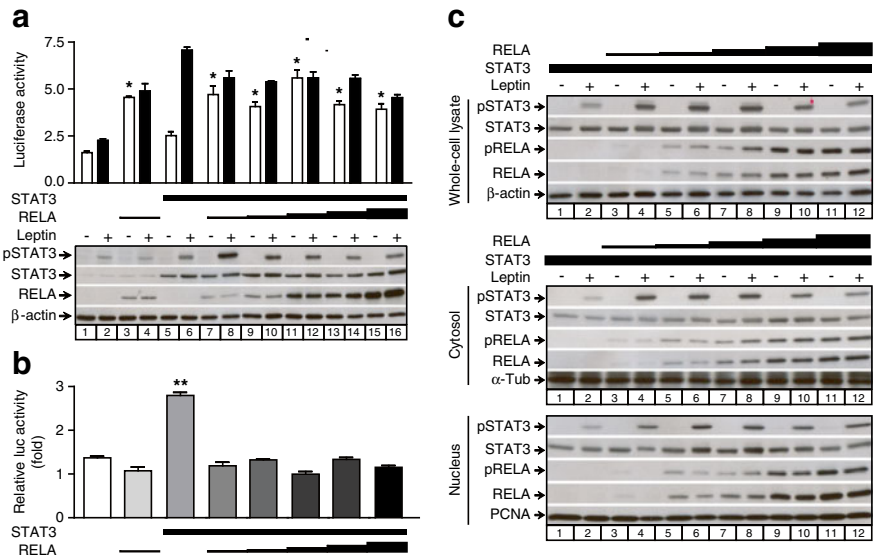

Fig. 2 RELA directly activates *Pomc* transcription. (**a**, **b**) 293-OBRb cells were transfected with the same amount of *Flag-Stat3* and *Pomc*-Luc, with an increasing amount of RELA from 0.01 to 1 μg, as indicated by the black bars. Empty vector pCDNA3.1-*Myc* was transfected in place of *Pomc*-Luc as baseline control. Cell lysate was used for luciferase activity measurement, and firefly luciferase activity was normalised to Renilla luciferase activity. The remaining lysate was used for immunoblotting using antibodies against pSTAT3, total STAT3, and RELA. β-actin was used as the loading control. Data are presented as mean ± SEM; n=3 per group. (**a**) White bars, vehicle treatment; black bars, leptin treatment. $^{*}p<0.05$ vs vehicle control (white bars) without *Stat3* or *Rela* overexpression. (**b**) Relative luciferase activity was calculated as the ratio of leptin treatment to vehicle treatment with the same transfection condition. $^{**}p<0.01$ vs control (white bars) without *Stat3* or *Rela* overexpression. (**c**) 293-OBRb cells were transfected with the same amount of *Flag-Stat3* plus increasing amounts of RELA from 0.1 to 9 μg, as indicated by the black bars. Whole-cell, cytosolic and nuclear fractions were immunoprobed using antibodies against pSTAT3, STAT3, pRELA and RELA. β-actin was used as whole-cell lysis loading control, α-tubulin as cytosol loading control, and proliferating cell nuclear antigen as the nuclear loading control. PCNA, proliferating cell nuclear antigen; α-Tub, α-tubulin

(lanes 8 and 9, Fig. 3c). We further tested RELA-*Pomc* promoter binding by ChIP assay. The quantitative ChIP results showed apparent RELA binding activity to the *Pomc* promoter (Fig. 3d). The same *Pomc* promoter region between −138 and −88 bp was previously identified to be important for STAT3-SP1-mediated leptin regulation of *Pomc* transcription [10]. Consistently, ChIP experiments confirmed SP1 binding to this region (Fig. 3d). We performed sequence analysis of this region and found apparent sequence conservation for the RELA and STAT3–SP1 binding sites and the same spatial arrangement of the two binding sites in several species across mammals (Fig. 3b), suggesting that this may be an important architectural feature. These data demonstrate that NF-κB regulation of *Pomc* transcription is through direct RELA binding with the *Pomc* promoter.

Reduced Pomc expression levels in mice lacking Rela To examine whether RELA regulates *Pomc* transcription in vivo, we studied mice lacking RELA on the background of *Tnfr1* knockout. Mice with *Rela* deletion die in utero at embryonic day 14–15 because of massive liver destruction, with large areas of apoptosis [31–33], but can survive to about 40 days when tumour necrosis factor-receptor 1 (TNFR1)-mediated signalling is abolished by deleting *Tnfr1*. The $Tnfr1^{-/-}RelA^{-/-}$ double-knockout (DKO) mice lack the development of lymph nodes, organised spleen structure, and T cell responses [34], and eventually die from acute infiltration of immature neutrophils in the liver [35, 36]. We performed real-time PCR to measure the mRNA levels in the hypothalamus of DKO mice and compared them with the $Tnfr1^{-/-}$ mice as control. *Pomc* expression levels decreased by ~80% in the DKO mice, while expression of neuropeptides agouti-related peptide (AgRP) and NPY, and inflammatory cytokines IL-6 and TNF-α were not changed (ESM Fig. 1). These in vivo findings strongly suggest a specific role of RELA in the regulation of *Pomc* transcription in the hypothalamus.

Increased Pomc promoter methylation in mice fed an HFD Obesity, characterised by increased appetite and reduced energy expenditure, is associated with chronic low-grade inflammation [4, 5]. Mice fed on an HFD exhibit activated NF-κB signalling in the hypothalamus [37]. However, their POMC levels are lower. Epigenetic changes such as DNA methylation have a major effect on transcription factor binding to the affected DNA sequence [38]. Moreover, the methylation levels of the *Pomc* promoter are highly dynamic under physiological and pathological conditions. For example, *Pomc* promoter methylation is significantly higher in tissues that do not express *Pomc* [39], or is altered in human patients with anorexia nervosa [40], in SL rats with early overfeeding [41], and in ectopic expression in the

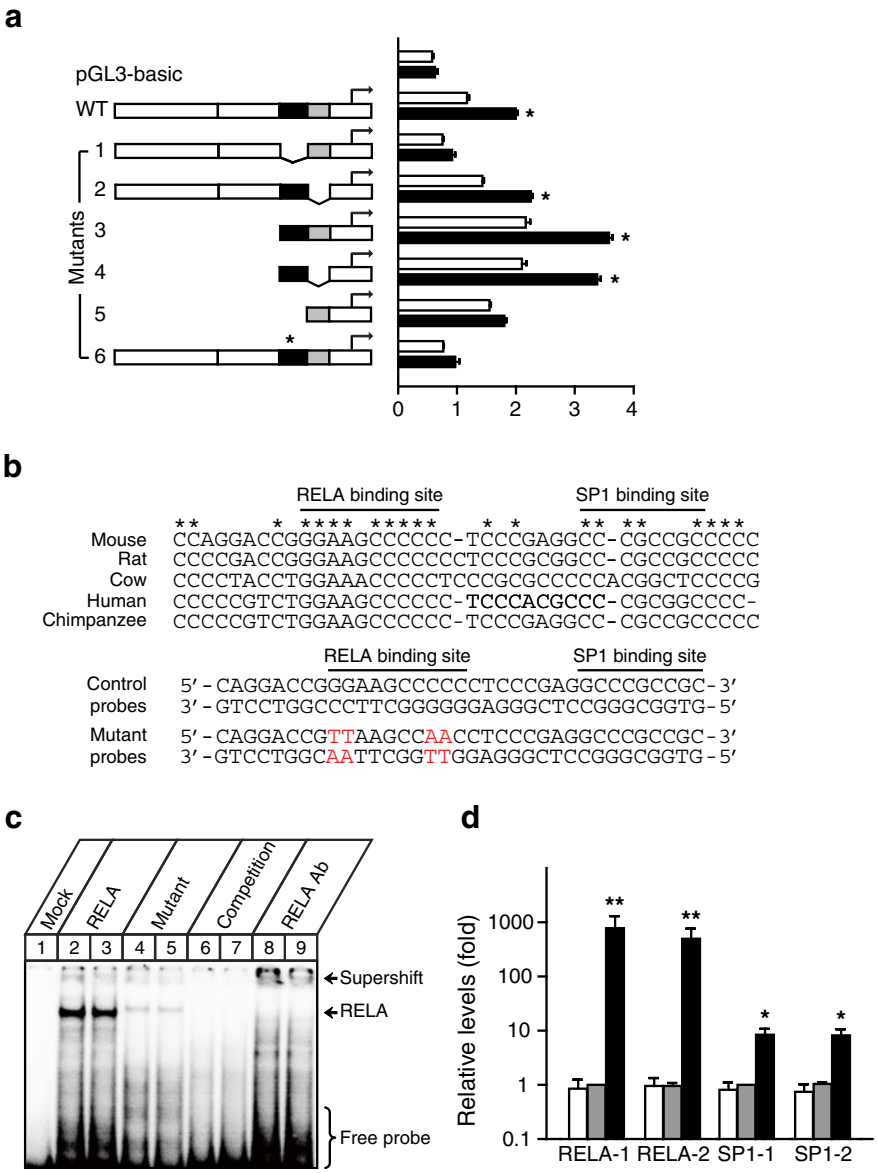

Fig. 3 RELA regulates *Pomc* transcription through direct interaction with the promoter region. (**a**) Essential DNA element in *Pomc* promoter (−646 to +65) mediating RELA regulation of *Pomc* promoter activity. A series of *Pomc* promoter deletion and point mutants were cloned to pGL3 basic plasmid. Wild-type, full length (−656 to +65) *Pomc*-Luc; mutant 1, deletion of fragment between −220 and −132; mutant 2, deletion of fragment between −132 and −88; mutant 3, deletion of fragment between −646 and −132; mutant 4, deletion of fragments between −646 and −132 and between −88 and −57; mutant 5, deletion of fragment between −646 and −88; and mutant 6, full length with point mutations at the putative RELA binding site. Firefly luciferase activity was measured and normalised to Renilla luciferase activity. White bars, control; black bars, RELA. Data are presented as mean ± SEM; n=3. $^{*}p<0.05$. (**b**) Sequence analysis of *Pomc* promoter region between −138 and −88 bp in mammals and sequences of EMSA probes. The RELA and SP1 binding sites are within a stretch of 50 bp. RELA binding site mutations are highlighted in red. (**c**) EMSAs with control and mutant probes were carried out using nuclear extracts of 293T cells. See the Results section for details. (**d**) ChIP samples were prepared from 293T cells by using antibodies against RELA or SP1 separately, followed by real-time PCR using two pairs of primers that target the potential RELA or SP1 binding sites in the *Pomc* promoter region. White bars, control; grey bars, IgG; black bars, antibody. Data are presented as mean ± SEM. n=3. $^{*}p<0.05$, $^{**}p<0.01$ vs control. WT, wild type

thymic carcinoids [42]. Therefore, we decided to examine the methylation status of the *Pomc* promoter from HFD-fed mice. We dissected the Arc region of the hypothalamus of 4-month-old C57Bl6 mice fed with either an HFD or LFD, extracted DNA and performed bisulfite sequencing to map the methylation status of the cytosines of CpG dinucleotides in the *Pomc* promoter region. The 300 bp *Pomc* promoter region that we examined contains more than 15 CpG dinucleotides, and covers both the RELA and SP1 binding sites. We found that overall DNA methylation showed an upward trend, with a significant increase at or near the RELA and SP1 binding sites in the HFD group when compared with

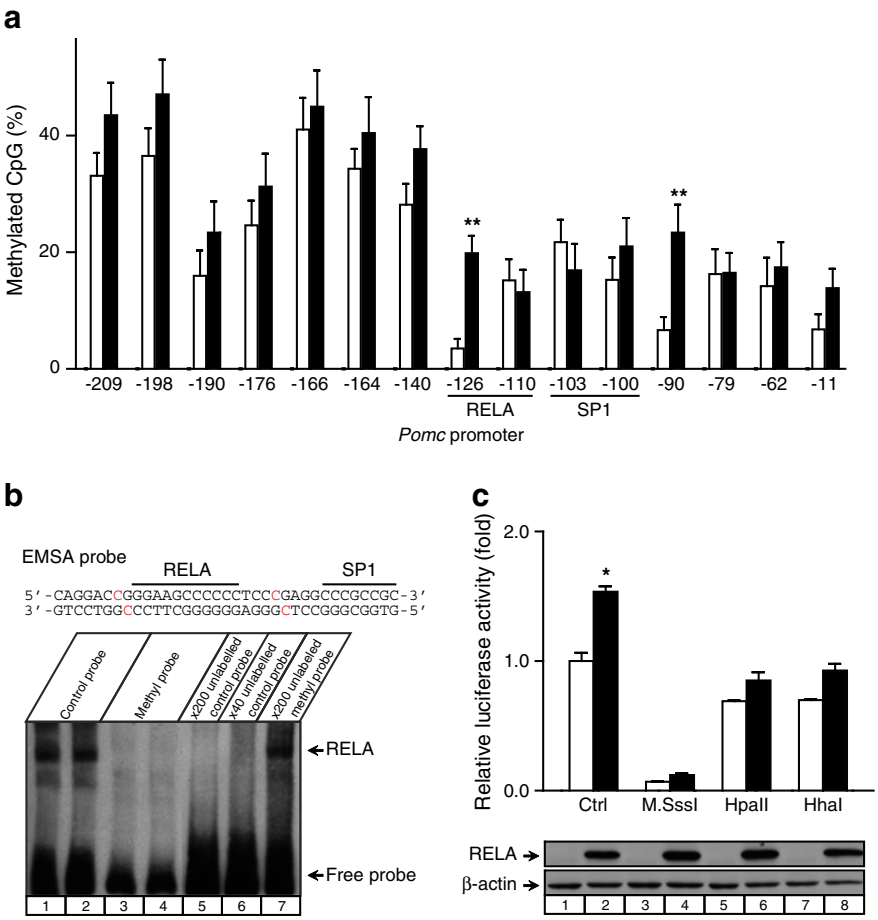

Fig. 4 Increased hypothalamic *Pomc* promoter methylation under HFD-induced chronic inflammation. (**a**) Methylation analysis of the *Pomc* promoter in the hypothalamic Arc region of C57Bl6 fed on an HFD (black bars) or LFD (white bars) for more than 20 weeks. The percentage of CpG residues was measured by bisulfite sequencing from at least 15 positive clones per mouse. Data are presented as mean ± SEM. n=20 mice per group. $^{**}p<0.01$ vs LFD. (**b**) Analysis of RELA binding with *Pomc* promoter by EMSA using nuclear extracts from 239-OBRb cells. Methylated cytosines were chemically synthesised at two CpG sites (highlighted in red) within the RELA binding site as the methylated probes. The RELA binding was abolished by unlabelled control probes but not by methylated probes. (**c**) *Pomc*-Luc reporter plasmids were treated with three DNA methyltransferases—M.SssI, HpaII and HhaI—and then transfected into 293-OBRb cells. Specific recognition sequences for the methyltransferases are: M.SssI (CG); HpaII (CCGG); and HhaI (GCGC). Firefly luciferase activity was normalised to that of Renilla luciferase. White bars, control; black bars, RELA. Data are presented as mean ± SEM. n=3. $^{*}p<0.05$ vs control (white bars). Immunoblots were done with the remaining lysates after the luciferase assay. β-actin was used as loading control

the LFD group (Fig. 4a). To test whether increased methylation affects RELA interaction with the *Pomc* promoter, we performed EMSA with nuclear extracts from HEK293 cells transfected with the *Rela* plasmid. Nuclear RELA protein bound to unmodified wild-type probes (lanes 1 and 2, Fig. 4b), but not to the probes containing chemically synthesised methylated cytosine at indicated CpG sites (lanes 3 and 4, Fig. 4b), suggesting that RELA binding to the *Pomc* promoter is sensitive to methylation states in this region. We then treated the *Pomc*-luciferase plasmid with DNA methyltransferases that target CapG of specific sequences, and co-transfected each modified luciferase construct with RELA into 293-OBRb cells. Hypermethylation at the *Pomc* promoter region abolished transcriptional activation by RELA (Fig. 4c). Together, these results suggest that increased methylation levels during HFD-induced chronic inflammation inhibit RELA binding to the *Pomc* promoter, and the consequent activation of *Pomc* transcription.

RELA inhibits leptin-stimulated POMC activity through STAT3 interaction Although RELA did not affect STAT3 phosphorylation or nuclear translocation, it may inhibit nuclear pSTAT3 from interacting with the SP1–*Pomc* promoter complex by sequestering STAT3 through direct interaction, similar to FOXO1 [10]. Consistent with previous reports [27–30], STAT3 and RELA were co-immunoprecipitated from 293-OBRb cells (Fig. 5a). We further investigated whether the interaction was dependent on leptin signalling by separately examining STAT3–RELA interaction in the cytosolic and nuclear

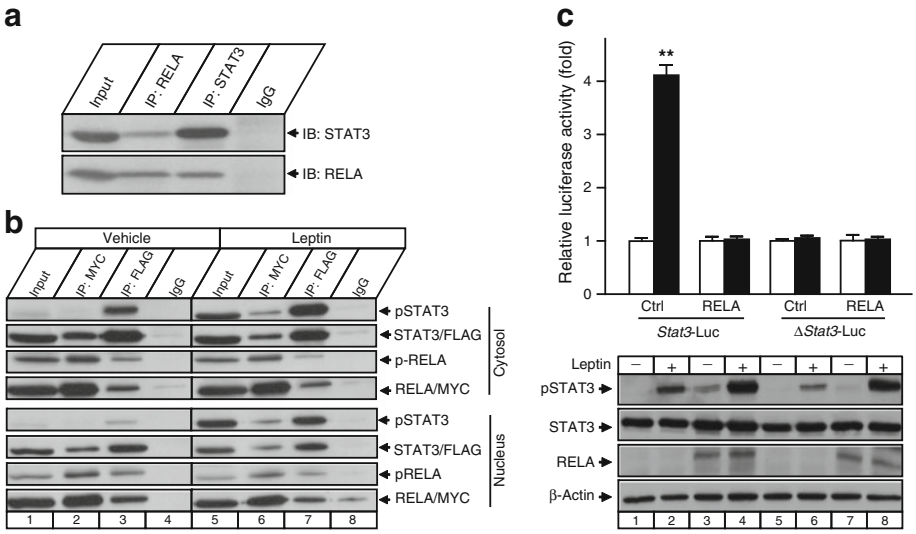

Fig. 5 RELA inhibits leptin-stimulated *Pomc* activity through STAT3 interaction. (**a**) Co-immunoprecipitation using lysates of 293-OBRb cells transfected with *Rela* and *Stat3* expression plasmids. Antibodies against RELA and STAT3 were used to immunoprecipitate the respective proteins, while normal IgG was used as negative control. (**b**) Co-immunoprecipitation using lysates of 293-OBRb cells transfected with *Rela-Myc* and *Flag-Stat3* expression plasmids. Cytosol and nuclear extracts from leptin-treated cells were mixed with antibodies against MYC or FLAG to immunoprecipitate RELA or STAT3, respectively. Immunoprecipitated proteins were detected by using antibodies against pSTAT3, FLAG (total STAT3), pRELA and MYC (total RELA). (**c**) 293-OBRb cells were transfected with the same amount of *Flag-Stat3* and *Stat3*-Luc, with either *Rela* or pCDNA3.1. Δ*Stat3*-Luc was used in place of *Stat3*-Luc as negative control. After 20 h of leptin treatments, cells were harvested for immunoblotting using antibodies against pSTAT3, total STAT3 and RELA. β-actin was used as loading control. White bars, control; black bars, leptin. Data are presented as mean ± SEM. n=3 per group. $^{**}p<0.01$ vs vehicle treatment. Ctrl, control; IB, immunoblot; IP, immunoprecipitant

fractions. No apparent difference was observed between the cytosolic and nuclear fractions, suggesting that the STAT3–RELA interaction was not dependent on leptin stimulation (Fig. 5b). To investigate the effects of the STAT3–RELA interaction on STAT3-mediated leptin regulation of gene transcription, we used STAT3-luciferase as the reporter in 293-OBRb cells. *Rela* expression abolished leptin-stimulated STAT3 regulation of transcription, without affecting the pSTAT3 levels (Fig. 5c). These results suggest that RELA can inhibit STAT3 from regulating *Pomc* transcription through direct interaction.

Discussion

The first aim of this study was to understand how the two types of inflammatory response lead to opposite physiological effects in food intake and energy expenditure, even though both acute and chronic inflammation activates NF-κB

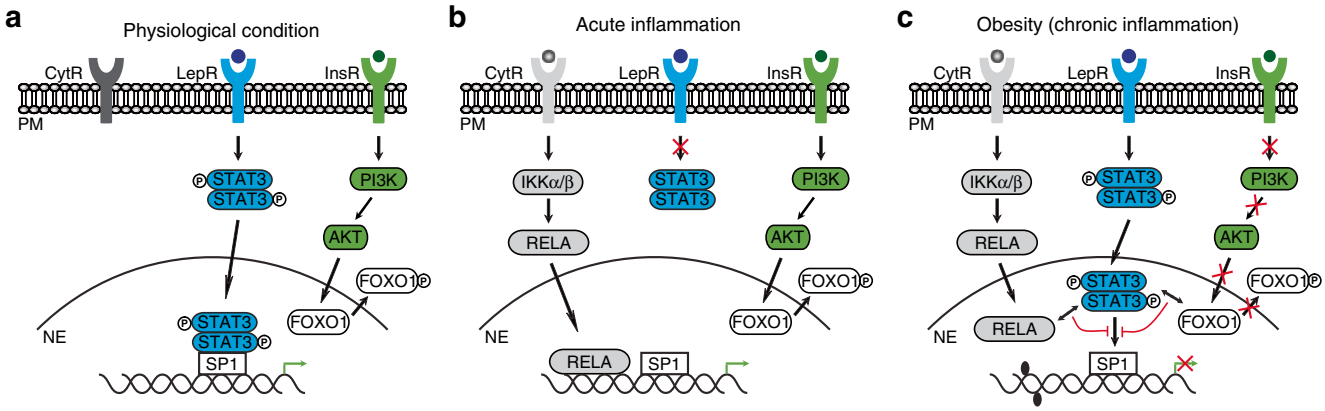

Fig. 6 Divergent *Pomc* transcription regulation under acute and chronic inflammatory conditions. Proposed models for the regulation of *Pomc* transcription under physiological conditions (**a**), acute inflammation (**b**) and chronic inflammation (**c**). CytR, cytokine receptor; LepR, leptin receptor; InsR, insulin receptor; PM, plasma membrane; NE, nuclear envelope. See text for details

signalling. Considering the critical importance of POMC neurons and the neuropeptides released from these neurons [2, 4, 43, 44], we focused on the regulation of *Pomc* transcription by various signalling pathways, in particular NF-κB signalling and leptin-induced STAT3 signalling.

Under normal physiological conditions, leptin controls *Pomc* transcription through the JAK2–STAT3 pathway [3]. On STAT3 phosphorylation and nuclear translocation, it activates *Pomc* transcription by binding to the SP1–*Pomc* complex [10]. Although FOXO1 and NF-κB RELA may inhibit STAT3 action under pathological conditions, normal insulin signalling reduces nuclear accumulation of FOXO1, and RELA remains in the cytosol in the absence of inflammatory stimulation (Fig. 6a)—conditions that allow leptin to efficiently regulate *Pomc* transcription and the downstream physiological responses.

At the onset of acute inflammation, such as viral infection, NF-κB RELA is released and translocates to the nucleus, where it binds directly to the *Pomc* promoter to induce *Pomc* transcription (Fig. 6b). Under this condition, the leptin level is very low. In the absence of leptin signalling, STAT3 is not phosphorylated, and remains in the cytosol, and thus does not contribute to the appetite suppression during acute inflammation.

The situation is more complex during chronic inflammation, which is often associated with dysregulated food intake and energy expenditure, i.e. leptin resistance. So why does activated NF-κB signalling in the hypothalamus induced by chronic inflammation fail to promote *Pomc* transcription? Numerous studies have established a clear link between epigenetic changes, including DNA methylation changes, and gene transcription activities [38, 45–47]. For example, the methylation status of the *Pomc* promoter was reported to change under unfavourable metabolic conditions [39–42]. Our current study identified increased methylation at and near the RELA binding site within the *Pomc* promoter using tissues from the Arc region of HFD-fed mice (Fig. 4a). Moreover, RELA is unable to activate *Pomc* transcription when the promoter is hypermethylated, possibly because of an impaired interaction between RELA and the *Pomc* promoter (Fig. 4b, c). Therefore, even though RELA can be activated and translocated into the nucleus during chronic inflammation, increased DNA methylation prevents RELA from interacting with the *Pomc* promoter, resulting in the failure of NF-κB-signalling-induced *Pomc* transcription (Fig. 6c).

The second aim of the study was to investigate how chronic inflammation contributes to leptin resistance. To address this, we studied the crosstalk between NF-κB signalling and leptin signalling in the regulation of *Pomc* transcription. Two non-exclusive models may underlie leptin resistance depending on the steps relative to STAT3 phosphorylation. Leptin resistance may occur at a step upstream of STAT3 phosphorylation, leading to impaired or failed STAT3 activation. Molecules involved in this model include an SH2-domain-containing protein tyrosine phosphatase (SHP2) [3], SOSC3 [6, 7], and PTP1B [8, 9]. For example, the feedback inhibition of STAT3 by leptin–STAT3-pathway-induced upregulation of SOCS3 is a model for late-stage leptin resistance [48, 49]. Besides the classic activation of *Socs3* transcription and expression by STAT3, NF-κB is another direct positive regulator of *Socs3* transcription [19]. Under chronic inflammation, the activated NF-κB signalling may thus lead to elevated SOCS3, which in turn suppresses STAT3 and the leptin signalling. Leptin resistance can also occur downstream of STAT3 phosphorylation. For example, FOXO1 inhibits pSTAT3 binding to the SP1–*Pomc* complex by sequestering STAT3 through direct interaction [10]. The fact that RELA interacts with STAT3 [27–30] and inhibits STAT3-mediated promoter activity further highlights the connection between inflammation and leptin signalling, and offers a novel direct link between the inflammatory pathway and leptin resistance. Similar to FOXO1 action in leptin resistance, RELA may also block leptin-induced POMC activity through direct interaction (Fig. 6c). Thus, our current work and the study by Zhang et al [19] provide a linkage between NF-κB and leptin signalling, and offer a model of how two parallel pathways acting both upstream and downstream of STAT3 activation may lead to leptin resistance in obesity. In our study, we used mice fed an HFD long term as a model for chronic inflammation. A recent study reported a hypothalamic inflammatory response in rodents within 1 to 3 days of HFD onset [50]. Whether similar changes occur because of short-term HFD exposure remains to be determined.

In summary, in conditions of acute inflammation, RELA can directly activate *Pomc* transcription, whereas during chronic inflammation, increased promoter methylation leads to excess free RELA, which blocks leptin-induced signalling by interaction with STAT3. Our current in vitro findings support a novel mechanism for the divergent regulation of appetite control by NF-κB signalling under different inflammatory conditions, and for leptin resistance involving the crosstalk between NF-κB RELA and leptin signalling.

Author contributions WH and GKR designed the research. XS, XW, QL, MS, EC, ETW performed the experiments. XS, XW, ZL, VT and WH analysed the data. XS, VT and WH wrote the manuscript. All authors were involved in the discussion and approved the manuscript.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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