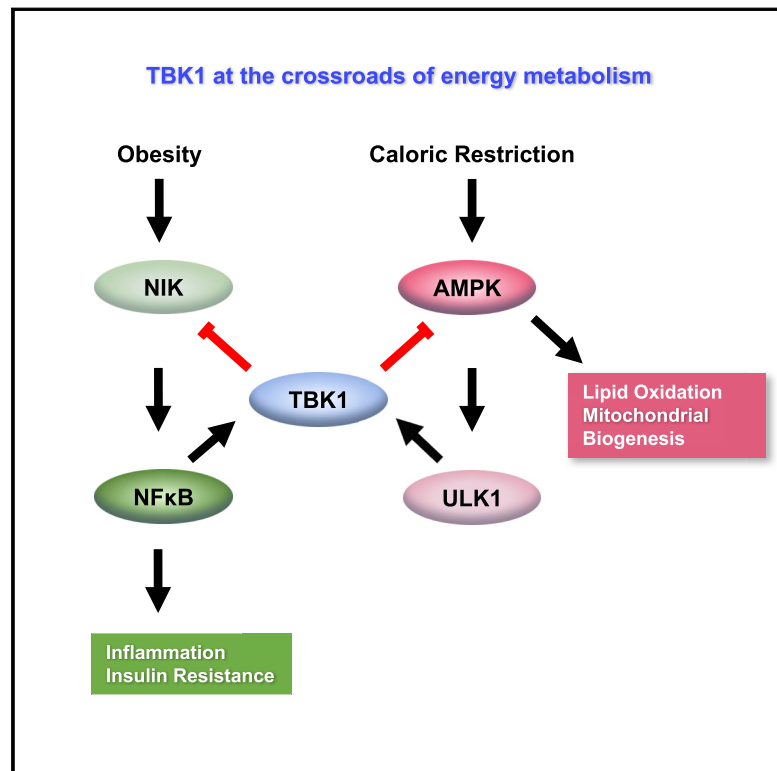


TBK1 at the Crossroads of Inflammation and Energy Homeostasis in Adipose Tissue

Graphical Abstract



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In Brief

Adipose tissue inflammation plays an important role in reducing energy expenditure in obesity.

Highlights

- TBK1 operates at the intersection of energy expenditure and inflammation
- TBK1 deficiency attenuates HFD-induced obesity but exaggerates inflammation
- TBK1 represses energy expenditure by phosphorylating and inhibiting AMPK
- TBK1 attenuates NF- κ B activation and mediates the anti-inflammatory effect of AMPK



TBK1 at the Crossroads of Inflammation and Energy Homeostasis in Adipose Tissue

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SUMMARY

The noncanonical IKK family member TANK-binding kinase 1 (TBK1) is activated by pro-inflammatory cytokines, but its role in controlling metabolism remains unclear. Here, we report that the kinase uniquely controls energy metabolism. *Tbk1* expression is increased in adipocytes of HFD-fed mice. Adipocyte-specific TBK1 knockout (ATKO) attenuates HFD-induced obesity by increasing energy expenditure; further studies show that TBK1 directly inhibits AMPK to repress respiration and increase energy storage. Conversely, activation of AMPK under catabolic conditions can increase TBK1 activity through phosphorylation, mediated by AMPK's downstream target ULK1. Surprisingly, ATKO also exaggerates adipose tissue inflammation and insulin resistance. TBK1 suppresses inflammation by phosphorylating and inducing the degradation of the IKK kinase NIK, thus attenuating NF- κ B activity. Moreover, TBK1 mediates the negative impact of AMPK activity on NF- κ B activation. These data implicate a unique role for TBK1 in mediating bidirectional crosstalk between energy sensing and inflammatory signaling pathways in both over- and undernutrition.

INTRODUCTION

Obesity results from a positive energy balance, when food intake exceeds energy expenditure. Although energy intake and expenditure are tightly coupled to ensure homeostasis (Hall et al., 2012), even a slight mismatch over time can result in massive weight gain and glucose intolerance. While short-term nutritional overload is associated with increased energy expenditure in both rodents (Pazos et al., 2015) and humans (Rasmussen et al., 2007), sustained obesity produces a decrease in energy expenditure (Bachman et al., 2002; Choi et al., 2015; Coleman, 1978; Leibel et al., 1995; Ravussin et al., 1988; Roberts and Leibel, 1998). Caloric restriction also reduces energy expenditure, resulting in lower basal metabolic rate, in an attempt to defend body weight (Leibel et al., 1995).

Thus, both over- and undernutrition are associated with reduced energy expenditure.

A great deal of basal energy metabolism occurs in adipose tissue, due to both nonshivering thermogenesis and lipid oxidation (Guilherme et al., 2008). Among the mechanisms that control energy expenditure are activation of AMP-activated protein kinase (AMPK), a key regulator that senses energy status and responds by increasing lipid oxidation and mitochondrial biogenesis, while reducing lipogenesis and glycogenesis (Daval et al., 2006; Hardie, 2011). AMPK is a heterotrimeric protein activated by liver kinase B1 (LKB1) via phosphorylation at Thr172 in the AMPK α subunit (Shaw et al., 2004). The enzyme is also allosterically activated in response to energy stress by sensing an increased AMP/ATP ratio (Hardie et al., 2012; Mihaylova and Shaw, 2011). Activation of AMPK increases lipid oxidation, represses lipogenesis, and significantly increases mitochondrial biogenesis through peroxisome proliferator-activated receptor gamma co-activator 1 α (PGC1 α) (Cantó and Auwerx, 2009; Puigserver et al., 1998; Wan et al., 2014). AMPK can also increase autophagy via phosphorylation and activation of Unc-51-like autophagy-activating kinase 1 (ULK1) (Kim et al., 2011). The AMPK/ULK1 axis is required for maintaining mitochondrial homeostasis and also increases the browning of inguinal white adipose tissues (Mottillo et al., 2016). Moreover, studies have demonstrated a correlation between decreased AMPK activity and increased inflammation (Gauthier et al., 2011), while activation of AMPK decreases expression of proinflammatory mediators and attenuates inflammation (Huang et al., 2015; O'Neill and Hardie, 2013; Salminen et al., 2011). Interestingly, AMPK activity is reduced in both obese rodents (Martin et al., 2006) and patients (Viollet et al., 2010; Xu et al., 2012), in part explaining reduced energy expenditure in obesity.

Numerous studies in both rodent models and humans indicate that obesity generates a state of chronic inflammation in adipose tissue, which in turn leads to insulin resistance and type 2 diabetes (Reilly and Saltiel, 2017; Weisberg et al., 2003; Xu et al., 2003). This inflammation is generated by recruitment and activation of immune cells, especially proinflammatory macrophages (Lumeng and Saltiel, 2011; Weisberg et al., 2003). Monocyte chemoattractant protein 1 (MCP1/*Ccl2*) is produced by adipocytes during obesity and is required for macrophage infiltration (Kamei et al., 2006; Kanda et al., 2006). In turn, proinflammatory cytokines secreted by immune cells in adipose tissue disrupt insulin action in adipocytes (Hotamisligil et al., 1995; Hotamisligil et al., 1994, 1996).



The nuclear factor κ B (NF- κ B) signaling pathway plays an important role in the development of inflammation and insulin resistance in adipose tissue (Arkan et al., 2005; Baker et al., 2011; Chiang et al., 2009). NF- κ B is activated by the canonical I κ B kinases (IKKs), IKK α and IKK β , which phosphorylate I κ B, leading to its degradation and release of NF- κ B (Lawrence, 2009). Unlike IKK α and β , the noncanonical IKKs, IKK ϵ , and TBK1 are induced downstream of NF- κ B activation (Reilly et al., 2013). TANK-binding kinase 1 (TBK1) also can be activated by phosphorylation in response to proinflammatory cytokines or other substances that bind to Toll-like receptors 3 and 4 (Perry et al., 2004), although the upstream kinase that phosphorylates TBK1 remains unknown. Previous studies from our group showed that the activities of IKK ϵ and TBK1 are significantly increased in adipose tissue of HFD-fed mice (Reilly et al., 2013) and further that IKK ϵ deficiency partially protects mice from HFD-induced systemic inflammation, insulin resistance, and hepatic steatosis (Chiang et al., 2009). However, how TBK1 might influence glucose metabolism and energy homeostasis remains uncertain.

Here, we report that *Tbk1* expression and activity are significantly increased in mature adipocytes of HFD-fed mice. To investigate the roles of adipocyte TBK1, we generated adipocyte-specific TBK1 knockout (ATKO) mice, because whole-body KO of TBK1 is lethal (Delhase et al., 2012). Adipose-specific TBK1 deficiency attenuates HFD-induced obesity, due to dramatically increased oxygen consumption and energy expenditure in white adipose tissue of obese, but not normal weight, mice. This was explained by increased activity of AMPK. *In vitro* studies revealed that AMPK is a direct substrate of TBK1 and further that TBK1 mediates the tumor necrosis factor alpha (TNF- α)-induced repression of AMPK activity. At the same time, AMPK activation reciprocally increased the activity of TBK1; this effect is indirect and mediated by the AMPK downstream kinase ULK1, which directly phosphorylates TBK1 at Ser172. This infers a negative feedback loop in which AMPK activity is limited when activated, perhaps explaining in part the repression of energy expenditure during caloric restriction. Surprisingly, TBK1 KO in adipocytes led to increased activation of NF- κ B, with upregulation of MCP1 production from adipocytes and macrophage infiltration into adipose tissue, accompanied by exaggerated insulin resistance in HFD-fed mice. The anti-inflammatory effect of TBK1 is mediated by phosphorylation of NIK, which results in its degradation and reduced activity of the atypical NF- κ B pathway. The loss of TBK1 thus substantially attenuates the AMPK-induced negative regulation of NF- κ B, showing that TBK1 is essential for the inhibitory crosstalk between AMPK and proinflammatory signaling. Taken together, these data reveal an unexpected role for TBK1 in both feedback and bidirectional crosstalk between energy sensing and inflammatory pathways in adipocytes to ensure energy homeostasis.

RESULTS

Adipocyte-Specific TBK1 Deficiency Increases Energy Expenditure and Attenuates HFD-Induced Obesity

We previously demonstrated that TBK1 activity is induced by TNF- α and increased in adipose tissue of high-fat diet (HFD)-fed mice (Reilly et al., 2013). Here, we show that *Tbk1* expression

is significantly upregulated in mature adipocytes derived from both inguinal (iWAT) and epididymal WAT (eWAT) in HFD-fed mice compared to normal diet (ND)-fed mice (Figure 1A). We generate adipocyte-specific TBK1 KO mice by crossing floxed TBK1 (Flox) mice with adiponectin-Cre mice. ATKO mice have greater than a 50% decrease of *Tbk1* mRNA in both eWAT and iWAT, without any change of expression in the liver (Figure 1B), reflecting complete reduction of TBK1 in adipocytes, with residual protein present in other adipose tissue cells (Jeffery et al., 2014).

Adipocyte-specific TBK1 KO has no effect on body weight or composition in ND-fed mice (Figures S1A–S1E). However, TBK1-deficient mice tend to be leaner and have a lower body weight when fed a HFD, although this difference was not statistically significant (Figures 1C–1E). DEXA scanning revealed that ATKO mice have significantly less fat mass (Figures 1D, 1F, and 1G). In addition, weights of both eWAT and iWAT were significantly reduced in ATKO mice, with no difference in brown adipose tissue (BAT) or liver (Figures 1C and 1H).

While TBK1 deficiency did not affect oxygen consumption rate (OCR) or energy expenditure (EE) in ND-fed mice (Figures 1I and 1J), ATKO mice exhibited dramatically increased OCR and EE after HFD (Figures 1K and 1L). Although a HFD inhibited OCR and EE in both Flox and ATKO mice, TBK1 deficiency significantly attenuated the reduction of OCR observed after a HFD. TBK1 KO did not affect respiratory exchange rate (RER) or activity in either ND- or HFD-fed mice (Figures S1F–S1I). Moreover, adipocyte-specific TBK1 KO had no effect on food intake (Figure S1J) or body temperature (Figure S1K) of ND- or HFD-fed mice.

TBK1 Deficiency Upregulates Mitochondrial Biogenesis by Attenuating the Inflammation-Induced Inhibition of AMPK Activity in Adipose Tissues in Mice on a HFD

Adipocyte size was significantly decreased in HFD-fed ATKO mice (Figure 2A), suggesting that TBK1 deficiency attenuates HFD-induced adipocyte hypertrophy. Loss of TBK1 significantly increased *ex vivo* OCR in both WATs of HFD-fed mice (Figure 2B). This increase was accompanied by a significant upregulation of mitochondrial biogenesis genes such as *Ppargc1a*, as well as the mitochondrial genes *Ndufs7*, *Cox4i1*, *Cox5b*, *Cox8a*, and *Atp5d*, all of which belong to different complexes in the respiratory chain (Figures 2C and 2D). Moreover, expression of the thermogenic gene *Ucp1* did not change (Figure 2D), a finding consistent with no major change in body temperature in ATKO mice. There was no effect on mitochondrial biogenic and thermogenic genes expression in BAT (Figure S2A).

Recent studies have identified a creatine-driven pathway that increases EE and thermogenesis (Kazak et al., 2015), and mice lacking this cycle have reduced EE and greater HFD-induced obesity (Kazak et al., 2017). There was significant upregulation of *Ckmt1* expression in eWAT and *Gamt* expression in iWAT, suggesting increased activity of the creatine cycle in ATKO mice (Figures S2B and S2C). Staining of WATs with MitoTracker also confirmed a substantial increase of mitochondria in adipocytes of HFD-fed ATKO mice (Figure 2E). Likewise, NAD/NADH ratio was significantly increased in eWAT, and only slightly increased in iWAT from ATKO mice (Figure 2F). The significant upregulation of hormone sensitive lipase (HSL/*Lipe*) and adipose triglyceride lipase (ATGL/*Pnpla2*) in iWAT, but not eWAT (Figures

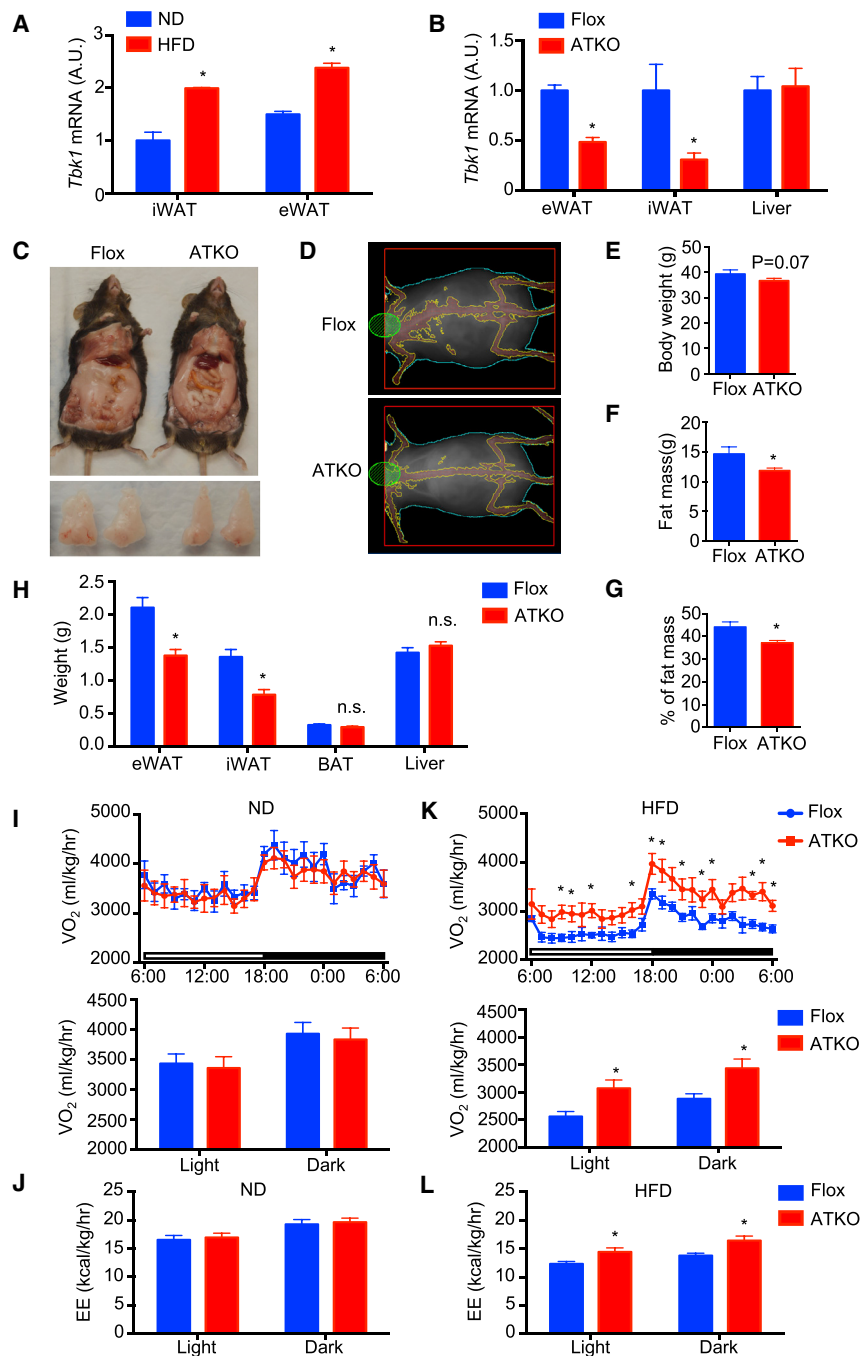


Figure 1. Adipocyte-Specific TBK1 Deficiency Increases Energy Expenditure and Attenuates HFD-Induced Obesity

(A) *Tbk1* expression in mature adipocytes from iWAT and eWAT of 16 weeks ND or HFD-fed mice. N = 3 from 9 mice.

(B) Expression of *Tbk1* in eWAT, iWAT, and liver of Flox and ATKO mice. N = 6–7.

(C) Photo of dissected mice and eWAT from 16 weeks HFD-fed Flox and ATKO mice.

(D) DEXA imaging of HFD-fed Flox and ATKO mice.

(E) Body weight of 16 weeks HFD-fed Flox and ATKO mice. N = 11–13.

(F and G) Fat mass (F) or percentage of fat mass (G) in 16 weeks HFD-fed Flox and ATKO mice. N = 10–11.

(H) Tissue weight of HFD-fed Flox and ATKO mice. N = 11–13.

(I and K) OCR of 16 weeks ND (I) or HFD (K)-fed Flox and ATKO mice. N = 5–6.

(J and L) EE of ND-fed (J) and HFD-fed (L) Flox and ATKO mice. N = 5–6. Data are represented as mean ± SEM. *p < 0.05. See also Figure S1.

stimulation (Figures S2G and S2H), suggesting that TBK1 plays no role in controlling catecholamine sensitivity, unlike that proposed for IKK ϵ (Mowers et al., 2013). While a slight increase of basal lipolysis in iWAT was observed because of upregulated expression of *Lipe* and *Pnpla2* (Figure S2H and S2E), TBK1 deficiency did not affect serum triglycerides and non-esterified fatty acid (NEFA) concentrations (Figures S2I and S2J). However, TBK1 deficiency substantially upregulated AMPK Thr172 phosphorylation in both eWAT and iWAT, but not BAT, of HFD-fed (Figures 2G, 2H, and S2K), but not ND-fed (Figures S2M and S2N), mice. The lack of effect on BAT is likely because TBK1 expression is not significantly induced in BAT that has lower inflammation (Figure S2L). There was no difference in AMP/ATP or ADP/ATP ratios between HFD-fed ATKO and Flox mice (Figures S2O and S2P).

HFD feeding significantly decreased AMPK Thr172 phosphorylation in adipose tissue (Figure 2I). TNF- α decreased AMPK Thr172 phosphorylation within 30min in adipocytes (Figure 2J). TBK1 deficiency in both differentiated primary preadipocytes and murine embryonic fibroblasts (MEFs) abrogated inhibition of AMPK activation by TNF- α (Figures 2K and S2Q). Thus, since TBK1 levels and activity are increased during obesity, these *in vivo* and *in vitro* observations suggest that the decrease in AMPK activity observed in obesity is mediated by TBK1, which is elevated by HFD-induced adipose inflammation,

S2D and S2E), could lead to elevation of lipolysis and lipid oxidation in iWAT, potentially attenuating the elevation of NAD/NADH ratio produced by increased mitochondrial biogenesis. Taken together, our data indicate a mitochondria-driven increase of catabolism in WATs of ATKO mice.

Sympathetic activation of adipose tissue increases cAMP levels, and induces mitochondrial biogenesis (Wu et al., 1999). TBK1 KO did not affect cAMP levels in HFD-fed mice (Figure S2F), nor was there a change in response to β -adrenergic

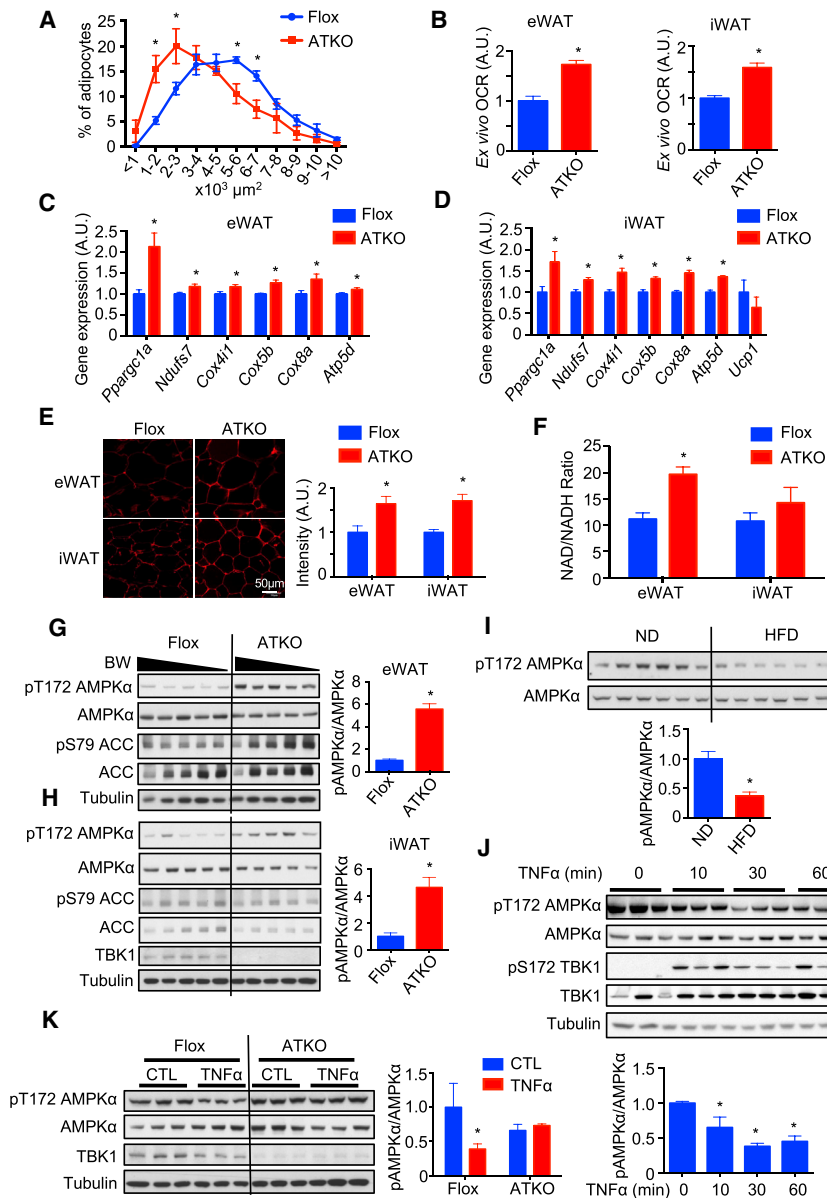


Figure 2. TBK1 Deficiency Upregulates Mitochondrial Biogenesis via Attenuating Inflammation-Induced Inhibition of AMPK Activity in Adipose Tissues of 16 Weeks HFD-Fed Mice

(A) Analysis of adipocyte size in eWAT of HFD-fed Flox and ATKO mice. N = 4–5. (B) Ex vivo OCR of eWAT and iWAT from HFD-fed Flox and ATKO mice. N = 5–6. (C and D) Mitochondrial biogenic genes expression in eWAT (C) and iWAT (D) of HFD-fed Flox and ATKO mice. N = 9. (E) Mitotracker staining of eWAT and iWAT of HFD-fed Flox and ATKO mice. Scale bar, 50 μm. N = 4–6. (F) NAD/NADH ratio in eWAT and iWAT of HFD-fed Flox and ATKO mice. N = 8–10. (G and H) Immunoblot (IB) analysis of eWAT (G) and iWAT (H) in HFD-fed Flox and ATKO mice. N = 5. (I) IB analysis of eWAT in ND or HFD-fed mice. N = 6. Data are represented as mean ± SEM. *p < 0.05. (J) IB analysis of 3T3-L1 adipocytes serum-starved in low-glucose DMEM and treated with TNF-α (10 nM) for the indicated time. (K) IB analysis of differentiated primary preadipocytes (Flox and ATKO) serum-starved in low-glucose DMEM and treated with TNF-α (10 nM) for 45 min. Data are represented as mean ± SD. *p < 0.05. See also Figure S2.

The AMPKα1 protein from cells overexpressing AMPKα1/TBK1 WT and AMPKα1/TBK1 KD was subject to phospho-amino acid analysis by LC-MS/MS, revealing two phosphorylation sites (Ser459 and Ser476) on AMPKα1 from cells overexpressing AMPKα1 with TBK1 WT, but not the KD mutant (Figures 3B and S3A–S3C; Table S1). Both Ser459 and Ser476 are conserved between AMPKα1 (PRKAA1) and AMPKα2 (PRKAA2) isoforms (Figure 3C) and among species from *Drosophila* to human (Figure 3D). We generated Ser/Ala nonphospho-mimetic (SS/AA) and Ser/Asp phospho-mimetic (SS/DD) mutants for both

and thus may represent a major mechanism of the obesity-dependent reduction in AMPK activity in fat tissue.

TBK1 Directly Phosphorylates AMPK α Subunit to Inhibit AMPK Activity

We examined whether TBK1 interacts with AMPKα to inhibit its activity. Overexpression of TBK1 wild-type (WT), but not TBK1 K38A kinase-dead mutant (KD), substantially decreased AMPKα1 Thr172 phosphorylation. AMPKα1 directly interacts with both TBK1 WT and TBK1 KD (Figure 3A). Moreover, overexpression of TBK1 WT, but not TBK1 KD, resulted in a shift of AMPKα1 band (Figure 3A). Lambda protein phosphatase treatment of immunoprecipitated AMPKα1 abolished the shift produced by TBK1 WT (Figure 3A), indicating that TBK1 directly interacts with and phosphorylates the AMPKα1 subunit to inhibit its activation.

Overexpression of HA-AMPKα1 WT or mutants with Flag-AMPKβ1 and Myc-AMPKγ1 demonstrated that the SS/AA mutation increased, while the SS/DD mutation decreased AMPKα1 Thr172 phosphorylation (Figures 3E and 3F), suggesting that TBK1-induced phosphorylation of AMPKα1 Ser459 and Ser476 inhibits AMPK activation, in turn repressing energy expenditure in obesity (Figure 3G). The TBK1 phosphorylation sites in the AMPKα subunit are close to, but distinct from, inhibitory sites phosphorylated by Akt and GSK-3, both of which also inhibit AMPK activity (Suzuki et al., 2013). Both phosphorylation sites identified by our study are located close to β subunit-interacting domain and ST loop of the AMPKα subunit. Previous studies have shown that the interaction between AMPKα and β subunits affect AMPK Thr172 phosphorylation and activation (Garcia-Haro et al., 2010), and AMPKβ KO causes a loss of

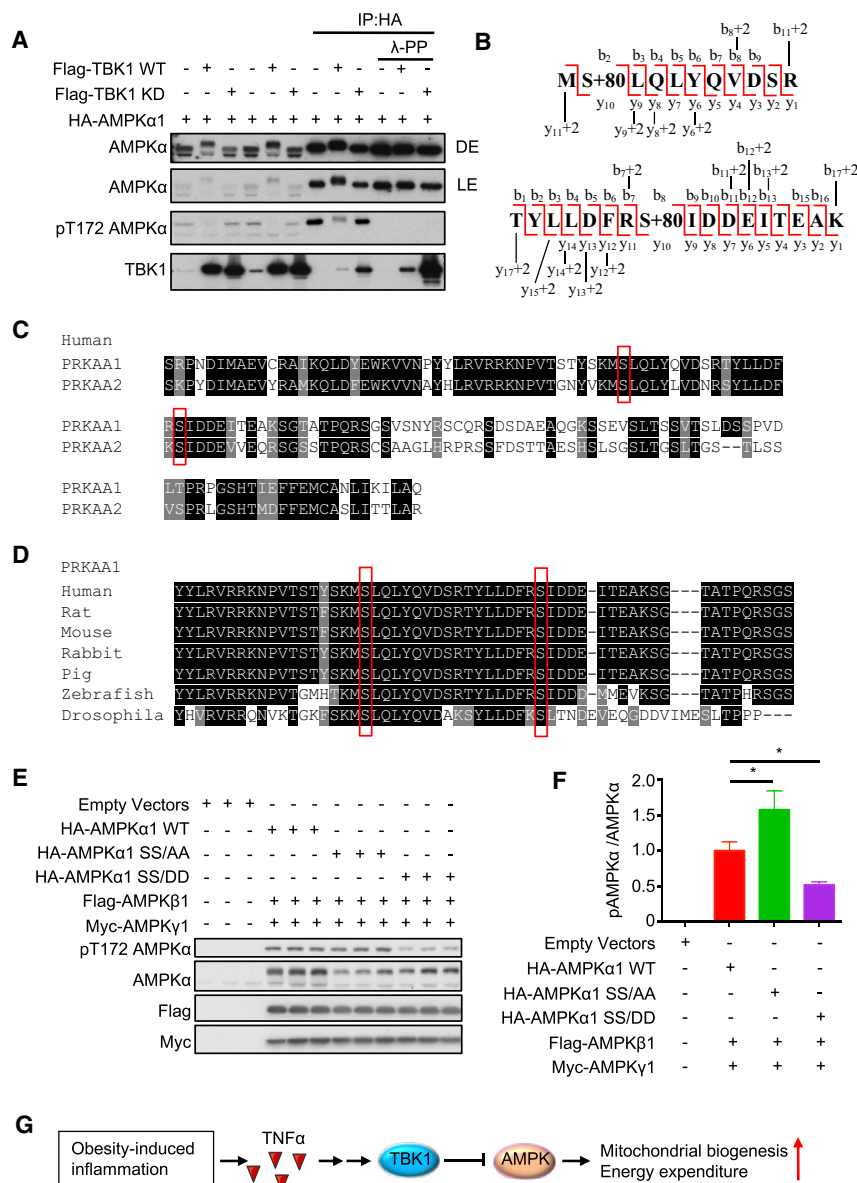


Figure 3. TBK1 Directly Phosphorylates AMPK α Subunit to Inhibit AMPK Activity

(A) IB analysis of whole-cell lysate from HEK293T cells overexpressing HA-AMPK α 1 with Flag-TBK1 WT or Flag-TBK1 kinase-dead mutant (KD) (left part). IB analysis of product from immunoprecipitation with HA-antibody treated with lambda protein phosphatase or not (right part).

(B) Phosphopeptide derived from mass spectrometry (MS) chromatogram showing AMPK α 1 Ser459 and Ser476 phosphorylation (S+80).

(C) Alignment of AMPK α 1 (PRKAA1) and AMPK α 2 (PRKAA2) sequence.

(D) Alignment of AMPK α 1 (PRKAA1) sequences in different species. Identified Ser phosphorylation sites are highlighted with red box.

(E) IB analysis of HEK293T cells overexpressing HA-AMPK α 1 WT or SS/AA or SS/DD mutant with Flag-AMPK β 1 and Myc-AMPK γ 1.

(F) Quantification of pAMPK α /AMPK α ratio in (E). Data are represented as mean \pm SD. * p < 0.05.

(G) Proposed model of TBK1-mediated repression of AMPK and EE.

See also Figure S3 and Table S1.

adipocytes with AICAR also increased TBK1 phosphorylation, and this increase was attenuated by pretreatment with the AMPK inhibitor compound C (Figure 4B). Co-overexpression of AMPK α 1 or α 2 with AMPK β 1 and γ 1 subunits increased TBK1 phosphorylation in HEK293T cells (Figures S4A and S4B). These data indicate that activation of AMPK stimulates TBK1 activating phosphorylation *in vitro*.

To examine AMPK-stimulated TBK1 phosphorylation *in vivo*, we generated adipocyte-specific AMPK α 1/ α 2 double-KO mice (α 1/ α 2 AKO). AICAR injection induced TBK1 Ser172 phosphorylation in adipose tissue of AMPK FL/FL mice, but not α 1/ α 2 AKO mice (Figure 4C). Moreover, we found that *Tbk1* expression is significantly induced by fasting (Figure S4C).

Taken together, these data demonstrate that activation of AMPK under catabolic conditions produces TBK1 phosphorylation and activation both *in vitro* and *in vivo*, suggesting that TBK1 activity is increased by both inflammatory and catabolic signals.

We were unable to demonstrate a direct phosphorylation of TBK1 by recombinant AMPK, suggesting existence of an intermediate kinase. Since both TAK1 and ULK1 have been reported as downstream effectors of AMPK, we pretreated 3T3-L1 adipocytes with the TAK1 inhibitor oxozeanol (Oxo) or the ULK1 inhibitor SBI-0206965 (SBI), followed by treatment with AICAR. Inhibition of ULK1 with SBI-0206965 attenuated AICAR-induced TBK1 phosphorylation, while Oxo had no effect (Figure 4D), suggesting that ULK1 mediates the phosphorylation of TBK1 stimulated by AMPK. Moreover, AICAR-stimulated TBK1 phosphorylation was completely abrogated in ULK1-deficient MEFs

AMPK α in fat tissue (Mottillo et al., 2016). Phosphorylation within the ST loop may induce a conformational change (Hawley et al., 2014), or recruit a phosphatase to decrease Thr172 phosphorylation (Suzuki et al., 2013).

TBK1 Is Stimulated by Activation of AMPK

Since caloric restriction reduces energy expenditure to defend body weight (Martin et al., 2007), we sought to determine whether TBK1 might contribute to the reduction of energy expenditure during caloric restriction via repressing AMPK. We treated WT and AMPK α 1/ α 2 KO MEFs with the TLR4 agonist lipopolysaccharides (LPS) or the AMPK activator AICAR. AICAR treatment resulted in activating phosphorylation of TBK1 at Ser172 in WT, but not AMPK α 1/ α 2 KO, MEFs, while LPS remained effective (Figure 4A). Additionally, treatment of 3T3-L1

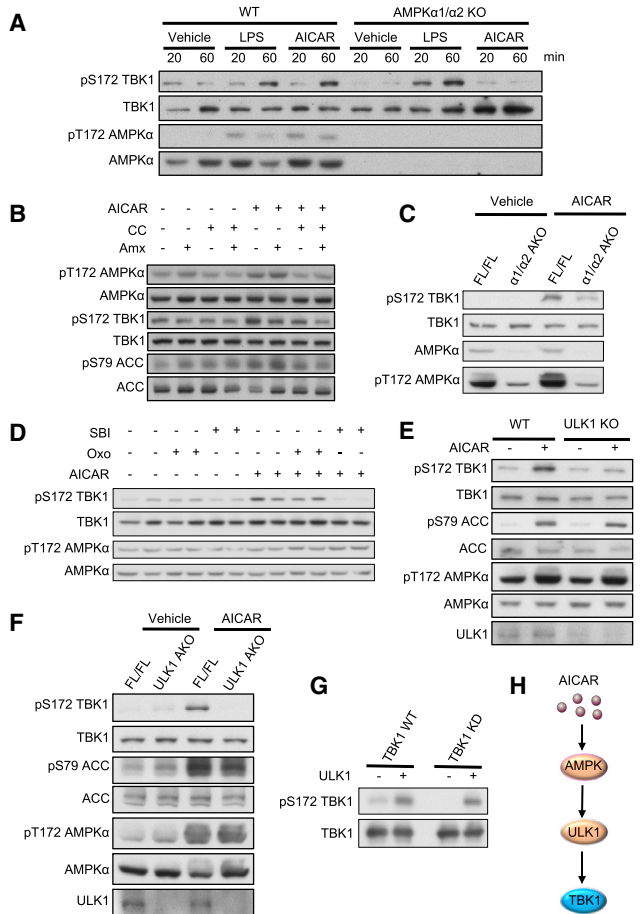


Figure 4. Activation of AMPK Induces TBK1 Phosphorylation via ULK1 *In Vitro* and *In Vivo*

(A) IB analysis of WT and AMPK α 1/ α 2 KO MEFs treated with vehicle, LPS (100 ng/ml), or AICAR (500 μ M) for the indicated time. (B) IB analysis of 3T3-L1 adipocytes pretreated with vehicle, compound C (CC, 5 μ M), or amlexanox (Amx, 50 μ M) for 1 hr and then treated with AICAR (500 μ M) for 45 min. (C) IB analysis of eWAT from FL/FL and α 1/ α 2 AKO mice (12–14 weeks old) intraperitoneally injected with vehicle or AICAR (500 mg/kg) for 45 min. (D) IB analysis of 3T3-L1 adipocytes pretreated with vehicle, oxozeaenol (Oxo, 300 nM), or SBI-0206965 (SBI, 10 μ M) for 1 hr and then treated with AICAR (500 μ M) for 45 min. (E) IB analysis of WT and ULK1 KO MEFs treated with vehicle or AICAR (500 μ M) for 45 min. (F) IB analysis of eWAT from FL/FL and ULK1 AKO mice (12–14 weeks old) intraperitoneally injected with vehicle or AICAR (500 mg/kg) for 45 min. (G) IB of *in vitro* kinase assay with ULK1 active kinase and immunoprecipitated Flag-TBK1 WT or Flag-TBK1 KD. Immunoprecipitated Flag-TBK1 WT were pretreated with lambda-protein phosphatase before performing kinase assay. Each panel is a representative experiment that was repeated three times. (H) Proposed model of AMPK-induced activation of TBK1. See also Figure S4.

(Figure 4E). We generated adipocyte-specific ULK1 KO (ULK1 AKO) mice, and injected ULK1 FL/FL and ULK1 AKO mice with AICAR. While AICAR treatment stimulated TBK1 phosphorylation in adipose tissue of ULK1 FL/FL mice, loss of ULK1 abrogated the effect of AICAR (Figure 4F). Additionally, an *in vitro*

kinase assay was performed to test if ULK1 directly phosphorylates TBK1 Ser172. Immunoprecipitated TBK1 WT was pretreated with lambda protein phosphatase to reduce basal phosphorylation, and then incubated with ULK1. Active ULK1 directly phosphorylated both TBK1 WT and its KD mutant (Figure 4G). These data conclusively demonstrate that ULK1 directly phosphorylates TBK1 Ser172 and mediates the activation of TBK1 phosphorylation produced by AMPK activation both *in vitro* and *in vivo* (Figure 4H).

We sought to examine if the ULK1/TBK1 axis mediates negative feedback regulation of AMPK. 2-hr glucose starvation induced AMPK Thr172 phosphorylation in both WT and ULK1 KO MEFs, but was significantly higher in ULK1 KO cells (Figure S4D). Additionally, glucose starvation increased TBK1 Ser172 phosphorylation in WT, but not in ULK1 KO, MEFs (Figure S4D). These results indicate that upon its phosphorylation and activation by AMPK, ULK1 initiates a negative-feedback loop to regulate AMPK activity by phosphorylation and activation of TBK1. These data suggest that the ULK1/TBK1 axis normally limits AMPK activity to preserve energy storage.

Adipocyte-Specific TBK1 KO Mice Have Exaggerated HFD-Induced Glucose Intolerance and Insulin Resistance, along with Increased Adipose Inflammation

TBK1 deficiency did not significantly affect fasting glucose or insulin levels in ND mice. However, both fasting blood glucose and insulin tended to be slightly higher in HFD-fed ATKO mice (Figures S5A and S5B). Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) showed no difference between the genotypes in ND-fed mice (Figures S5C and S5D). Surprisingly, TBK1 deficiency exaggerated glucose intolerance and insulin resistance in HFD-fed mice (Figures 5A and 5B), without significant effects on insulin levels during GTT (Figure S5E). Because there was no difference in hepatic steatosis and liver gene expression in ATKO mice on HFD (Figures S5F and S5G), we examined whether glucose intolerance was due to decreased insulin-stimulated glucose uptake in adipose tissues by *in vivo* glucose uptake assay. Adipose tissue glucose uptake was significantly decreased in both eWAT and iWAT of HFD-fed ATKO mice (Figure 5C). This was accompanied by reduced insulin-stimulated Akt phosphorylation in adipose tissue of TBK1-deficient mice (Figure 5D).

Increased adipose inflammation and macrophage infiltration inhibit insulin-stimulated glucose uptake and result in insulin resistance (Hotamisligil et al., 1993; Weisberg et al., 2003). Adipose expression of macrophage markers F4/80 (*Adgre1*), CD11c (*Itgax*), and CD206 (*Mrc1*) were significantly induced by HFD feeding in Flox mice. TBK1 deficiency further increased expression of macrophage marker genes (Figure 5E). H&E and F4/80 staining of adipose tissue showed a substantial increase of adipose macrophage infiltration in ATKO mice (Figure 5F). Staining and fluorescence-activated cell sorting (FACS) analysis of stromal vascular fractions (SVFs) from eWAT with F4/80 antibody revealed a significant increase of macrophages in HFD-fed ATKO mice (Figures 5G and 5H). MCP1 (*Ccl2*) plays an essential role in the induction of adipose macrophage infiltration (Kanda et al., 2006). *Ccl2* expression was significantly upregulated in adipose tissue (Figure 5I) and

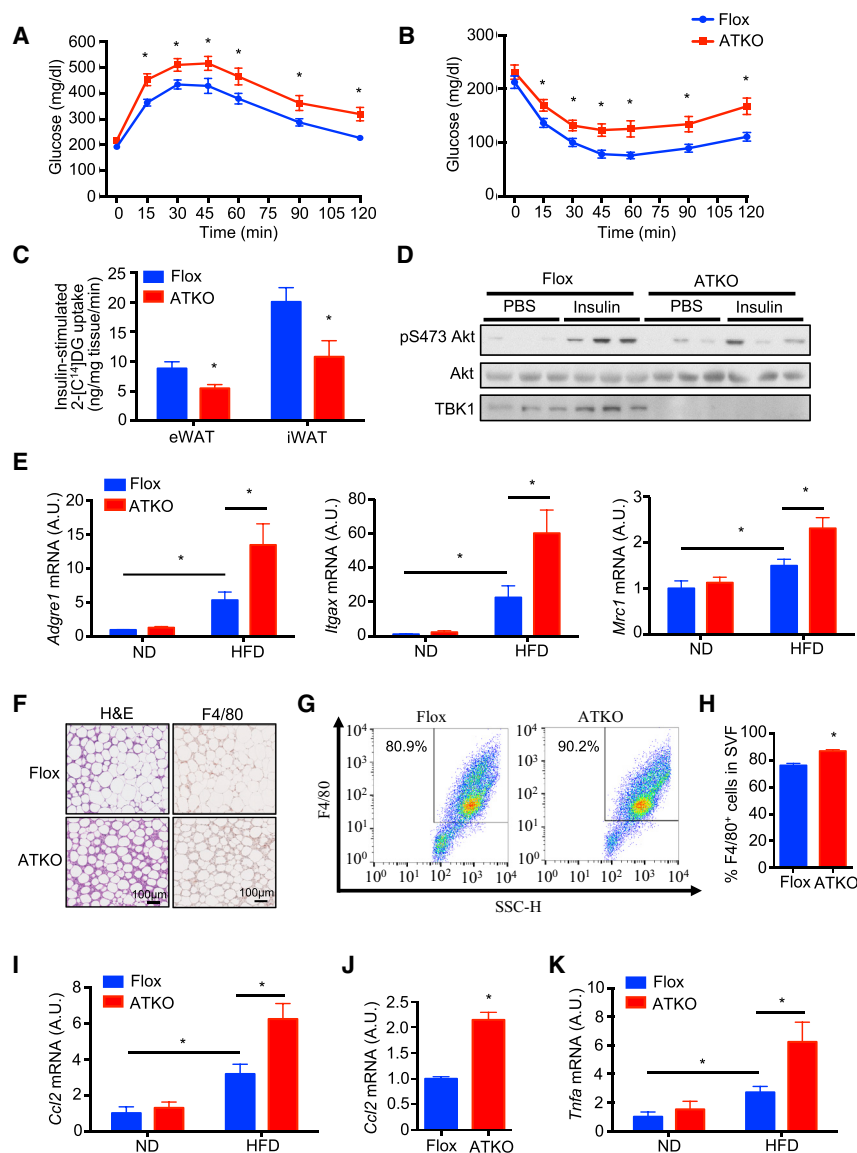


Figure 5. Adipocyte-Specific TBK1 KO Mice Have Exaggerated HFD-Induced Glucose Intolerance and Insulin Resistance, along with Increased Adipose Inflammation

(A) Glucose (1.2 g/kg BW) tolerance test on 12 weeks HFD-fed Flox and ATKO mice. N = 10–12. (B) Insulin (1.2 U/kg BW) tolerance test on 12 weeks HFD-fed Flox and ATKO mice. N = 10–12.

(C) *In vivo* glucose uptake in eWAT and iWAT of 14 weeks HFD-fed Flox and ATKO mice. Each mouse was injected with 1.2 g/kg glucose, 1 U/kg insulin, and 180 μ Ci/kg 2-[C^{14}]-deoxyglucose for 1 hr. N = 4–5. (D) IB analysis of eWAT in HFD-fed Flox and ATKO mice injected with PBS or 1.2 U/kg insulin.

(E) Expression of macrophage marker genes in eWAT of 16 weeks ND or HFD-fed Flox and ATKO mice. N = 6–8. (F) H&E (left) and F4/80 (right) staining of eWAT in HFD-fed Flox and ATKO mice. Scale bar, 100 μ m.

(G and H) FACS analysis of F4/80⁺ cells in stromal vascular fraction (SVF) from eWAT of HFD-fed Flox and ATKO mice. N = 4 from 10 mice. (H) is statistical analysis of (G). (I) *Ccl2* (MCP1) expression in eWAT of 16 weeks HFD-fed Flox and ATKO mice. N = 6–8.

(J) *Ccl2* expression in mature adipocyte fraction from eWAT of HFD-fed Flox and ATKO mice. N = 6 from 12 mice. (K) *Tnfa* expression in eWAT of HFD-fed Flox and ATKO mice. N = 6–8. Data are represented as mean \pm SEM. * p < 0.05.

See also Figure S5.

in mature adipocytes of HFD-fed ATKO mice (Figure 5J). Further analysis revealed that TBK1 deficiency exaggerated HFD-induced expression of *Tnfa* in eWAT (Figure 5K). Taken together, these data indicate that TBK1 deficiency in adipocytes increases the recruitment of proinflammatory macrophages into adipose tissue, releasing inflammatory cytokines such as TNF- α to block insulin action.

Loss of TBK1 Increases TNF- α -Induced NF- κ B Activation in HFD-Fed Mice

To understand the underlying mechanism of increased inflammation in ATKO mice, we examined NF- κ B signaling in adipose tissues. Compared to Flox controls, there was an increase in p52, the active form of NF- κ B, as well as phosphorylation of p105 in eWAT and iWAT, but not BAT, of HFD-fed ATKO mice or WATs of ND-fed ATKO mice (Figures 6A, 6B, and S6A–S6C). To deter-

mine whether the hyperactivation of NF- κ B caused by TBK1 deficiency was cell autonomous, we differentiated primary preadipocytes from Flox and ATKO mice and treated with TNF- α for 2 or 8 hr. TBK1 KO significantly increased TNF- α -induced p52 generation and p105 phosphorylation (Figures 6C and S6D). Furthermore, *Ccl2* expression induced by 16 hr TNF- α treatment was significantly increased in TBK1-deficient differentiated preadipocytes and MEFs (Figures 6D and S6E), although TBK1 deficiency did not affect acute NF- κ B activation up to 1 hr of TNF- α treatment (Figure S6F). Taken together, these data indicate that TBK1 KO affects TNF- α -induced NF- κ B activity only after 2 hr of treatment, strongly suggesting that the effects of TBK1 KO are dependent on gene expression or protein degradation.

NF- κ B-inducing kinase (NIK) phosphorylates and activates IKK α homodimers and to a lesser extent IKK α / β heterodimers. The activated IKK α /IKK α homodimer phosphorylates p100 to induce its cleavage and generate p52, while the activated IKK α /IKK β heterodimer phosphorylates p105 (Sun, 2011; Zarnegar et al., 2008). A previous study suggested that TBK1 phosphorylates NIK, and induces its degradation to attenuate NF- κ B activation (Jin et al., 2012). Overexpression of TBK1 WT, but not KD, decreased NIK protein (Figure S6G).

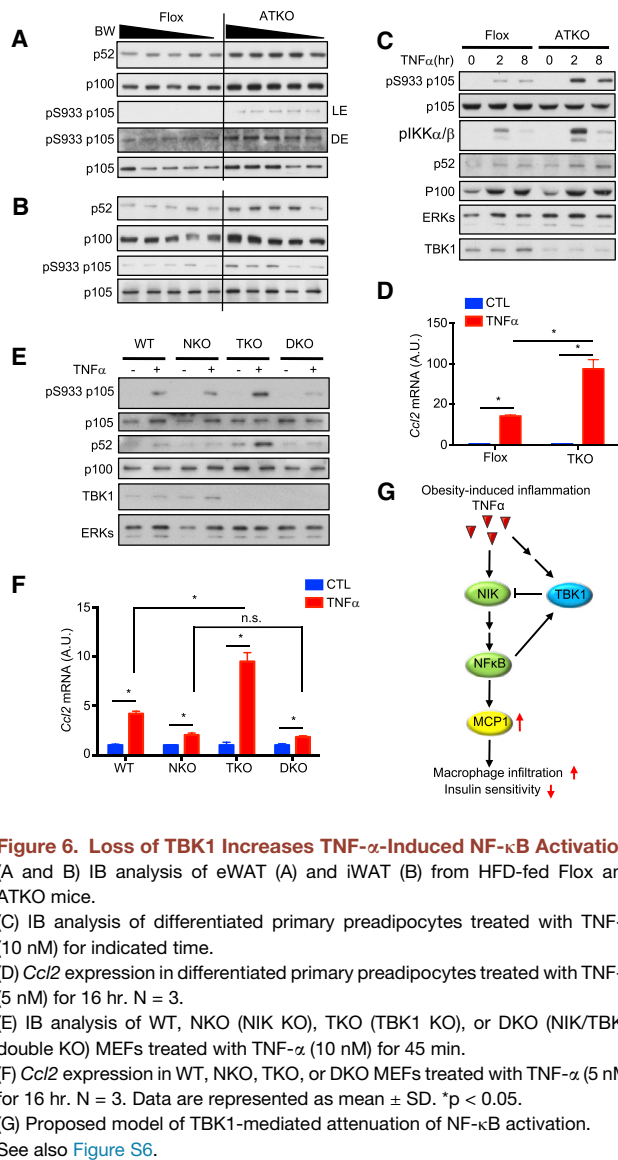


Figure 6. Loss of TBK1 Increases TNF- α -Induced NF- κ B Activation (A and B) IB analysis of eWAT (A) and iWAT (B) from HFD-fed Flox and ATKO mice. (C) IB analysis of differentiated primary preadipocytes treated with TNF- α (10 nM) for indicated time. (D) *Ccl2* expression in differentiated primary preadipocytes treated with TNF- α (5 nM) for 16 hr. N = 3. (E) IB analysis of WT, NKO (NIK KO), TKO (TBK1 KO), or DKO (NIK/TBK1 double KO) MEFs treated with TNF- α (10 nM) for 45 min. (F) *Ccl2* expression in WT, NKO, TKO, or DKO MEFs treated with TNF- α (5 nM) for 16 hr. N = 3. Data are represented as mean \pm SD. *p < 0.05. (G) Proposed model of TBK1-mediated attenuation of NF- κ B activation. See also Figure S6.

Co-immunoprecipitation experiments confirmed that NIK directly interacts with both TBK1 WT and its KD mutant (Figure S6H). Unfortunately, the currently available NIK antibody was not able to detect endogenous NIK without MG132 treatment. We used CRISPR/Cas9 to knock out NIK in immortalized WT and TBK1 KO MEFs (Figure S6I). NIK KO attenuated TNF- α -induced activation of NF- κ B, and abrogated NF- κ B hyperactivation caused by TBK1 deficiency (Figure 6E). NIK KO significantly attenuated TNF- α -induced *Ccl2* expression, and abrogated increased *Ccl2* expression in TBK1 KO cells (Figure 6F). These results demonstrate that TBK1 attenuates TNF- α -induced NF- κ B activation and *Ccl2* expression via regulating NIK degradation, and strongly suggest that increased inflammation in TBK1 KO adipocytes is due to increased levels of NIK and hyperactivation of NF- κ B. Our study thus reveals a counter-inflammatory role of TBK1, which controls TNF- α -induced NF- κ B activation in a negative feedback loop, and

maintains inflammation at low levels, typical of obesity-induced inflammation (Figure 6G).

TBK1 Deficiency Attenuates Inhibition of NF- κ B Signaling by AMPK

Activation of AMPK reduces production of inflammatory cytokines and attenuates inflammation (Huang et al., 2015; Salminen et al., 2011), although the underlying mechanism is unknown. Because TBK1 can be activated downstream of AMPK, and since loss of TBK1 abrogates the feedback inhibition of inflammation, we explored the hypothesis that TBK1 mediates the anti-inflammatory effect of AMPK activation. We pretreated adipocytes with AICAR, and then treated cells with TNF- α to examine activation of NF- κ B signaling. AICAR attenuated TNF- α -induced phosphorylation of p105 and IKK α/β in adipocytes (Figure 7A), and decreased TNF- α -induced *Ccl2* expression (Figure 7B). TBK1 deficiency abrogated the inhibitory effect of AICAR on TNF- α -induced p105 and IKK α/β phosphorylation (Figures 7C and S7A). AICAR-induced inhibition of *Ccl2* expression was significantly attenuated in TBK1 KO cells (Figures 7D and S7B). To confirm that attenuation of the anti-inflammatory effects of AICAR is due to loss of TBK1, immortalized TBK1 KO MEFs were transfected to express GFP or TBK1. In TBK1 KO MEFs, expression of TBK1 restored inhibition of p105 and IKK α/β phosphorylation by AMPK activation (Figure 7E). We conclude that TBK1 mediates the anti-inflammatory effects of AMPK activation via attenuation of NF- κ B activity (Figure 7F). Taken together, our findings demonstrate that TBK1 operates at the intersection of two crucial pathways to mediate both the repression of energy expenditure by inflammation and the attenuation of inflammation by AMPK activation.

DISCUSSION

We demonstrate here a previously unappreciated role for the protein kinase TBK1; upon its induction and activation during obesity and caloric restriction, TBK1 represses energy expenditure while also attenuating inflammation in adipocytes and surrounding cells in adipose tissue, thus operating at the intersection of inflammation and energy homeostasis. We conclude that TBK1 mediates both feedback and bi-directional inhibitory crosstalk between AMPK and inflammation (Figure S7C). Numerous studies have demonstrated that obesity in both rodents and patients is associated with reduced energy expenditure (Hill et al., 2012). However, the mechanisms by which energy expenditure is reduced in obesity is unknown. Data shown here suggest that adipose tissue inflammation may play an important role, at least partially through the induction of TBK1.

Several studies have implicated a central role for the NF- κ B pathway in triggering and sustaining the deleterious effects of overnutrition on energy homeostasis (Baker et al., 2011). Although TBK1 displays significant sequence homology to the other IKKs (Clément et al., 2008), and although TBK1 is activated by pro-inflammatory stimuli, the kinase is completely dispensable for NF- κ B activation. Interestingly, we found that *Tbk1* expression is significantly upregulated in mature adipocytes of

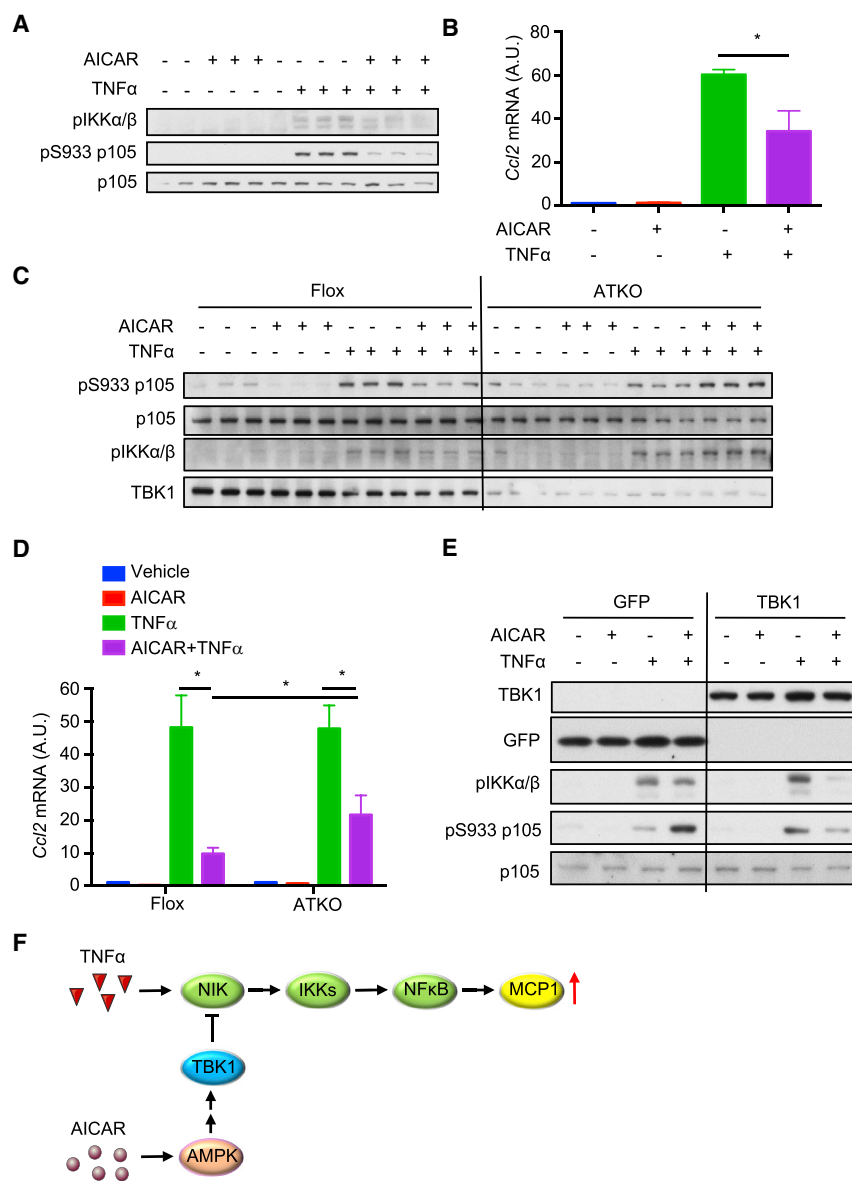


Figure 7. TBK1 Deficiency Attenuates Inhibition of NF- κ B Signaling by AMPK

(A) IB analysis of 3T3-L1 adipocytes pretreated with vehicle or AICAR (250 μ M) for 7 hr and then treated with TNF- α (10 nM) for 45 min.

(B) *Ccl2* expression in 3T3-L1 adipocytes pretreated with vehicle or AICAR (250 μ M) for 7 hr and then treated with TNF- α (10 nM) for 2 hr. * p < 0.05.

(C) IB analysis of differentiated primary pre-adipocytes (Flox and ATKO) pretreated with vehicle or AICAR (250 μ M) for 7 hr and then treated with TNF- α (10 nM) for 45 min.

(D) *Ccl2* expression in differentiated primary pre-adipocytes (Flox and ATKO) pretreated with vehicle or AICAR (250 μ M) for 7 hr and then treated with TNF- α (10 nM) for 2 hr. Data are represented as mean \pm SD. * p < 0.05.

(E) IB analysis of TKO MEFs overexpressing GFP or TBK1. MEFs were pretreated with vehicle or AICAR (500 μ M) for 7 hr and then treated with TNF- α (10 nM) for 45 min.

(F) Proposed model of TBK1-mediated anti-inflammatory effect of AMPK.

See also Figure S7.

What is the mechanism by which attenuation of AMPK activity by TBK1 represses energy expenditure? Numerous studies have indicated that AMPK increases lipid oxidation, while inhibiting lipogenesis through reduced activity of mTORC1 (Gwinn et al., 2008), and increases mitochondrial biogenesis (Reznick and Shulman, 2006). Increased AMPK activity in HFD-fed ATKO mice increases expression of *Pparc1a* and other genes involved in respiration, including a substantial increase of mitochondrial biogenesis in adipocytes. Thus, these findings illuminate how obesity-dependent chronic inflammation reduces energy metabolism via TNF- α -induced inhibition of AMPK activity in adipose tissues.

TBK1 Ser172 phosphorylation is increased by pro-inflammatory cytokines

HFD-fed mice, suggesting that it might be an effector of inflammation. Moreover, the specific IKK ϵ /TBK1 inhibitor amlexanox alleviates many of the effects of HFD feeding and genetic obesity, improving weight, insulin sensitivity, inflammation, and hepatic steatosis (Reilly et al., 2013), along with a substantial, clinically relevant improvement in blood sugar and glucose homeostasis in a subset of obese type 2 diabetic patients with underlying inflammation (Oral et al., 2017). As we have already studied IKK ϵ KO mice (Chiang et al., 2009), these findings prompted us to create adipocyte-specific TBK1 KO mice to evaluate the role of TBK1 in metabolic homeostasis. Adipocyte-specific TBK1 KO ameliorates HFD-induced obesity via increasing oxygen consumption rates and energy expenditure, and like the IKK ϵ /TBK1 inhibitor amlexanox (Reilly et al., 2013), has no effect on normal weight mice.

and ligands that bind to TLR3 and TLR4 (Perry et al., 2004), and structural studies show that TBK1 is activated by trans-autophosphorylation (Ma et al., 2012). We show here that TBK1 is surprisingly activated by AMPK via its downstream protein kinase ULK1. ULK1 is thus the first TBK1 kinase discovered. Although ULK1 is a key regulator of autophagy (Russell et al., 2013), the discovery of the AMPK/ULK1/TBK1 axis indicates that ULK1 may have other important functions. More importantly, under conditions in which TBK1 is expressed, AMPK activation may be self-limiting through this feedback loop. Moreover, TBK1 may limit energy expenditure during caloric restriction (Leibel et al., 1995), in which absolute restriction of food intake in patients resulted in reduced energy expenditure, in an attempt to defend body weight. This finding also has important implications for the discovery

and development of AMPK activators for the treatment of metabolic disease, and suggests that a combination of AMPK activators with TBK1 inhibitors such as amlexanox might be synergistic.

Despite increasing energy expenditure, TBK1 KO exaggerated HFD-induced glucose intolerance and insulin resistance, likely due to enhanced adipose inflammation and macrophage infiltration in HFD-fed ATKO mice (Hotamisligil et al., 1993; Weisberg et al., 2003). TBK1 deficiency increased TNF- α -induced p105 phosphorylation and generation of p52 in a cell-autonomous manner, and upregulated TNF- α -induced *Ccl2* expression. Increased MCP1 production is the primary signal for macrophage infiltration in adipose tissue in obesity (Kamei et al., 2006). KO of NIK by CRISPR/Cas9 confirmed that the TBK1 deficiency-induced hyper-response to TNF- α is dependent on NIK. However, while NIK KO did not completely abrogate TNF- α -induced NF- κ B activation and *Ccl2* expression, it completely abolished the difference between WT and TBK1 deficient cells, suggesting that TBK1 mainly controls the atypical pathway for NF- κ B activation. Moreover, these data also indicate that TBK1 does not affect the initiation of inflammation in obese mice, but limits its development, perhaps accounting for the low-grade nature of adipose tissue inflammation in obesity (Xu et al., 2003). In addition, studies have shown that extracellular ATP exerts a pro-inflammatory effect (Cauwels et al., 2014), synergistic with the effect of TNF- α (Schnurr et al., 2000). Thus, in HFD-fed ATKO mice, increased AMPK-induced ATP synthesis and TNF- α -stimulated ATP release (Lohman et al., 2015) may exacerbate adipose inflammation via increasing extracellular ATP. The increased ATP release might also explain why intracellular ATP in adipocytes of HFD-fed ATKO mice remains stable, despite a dramatic increase in mitochondrial number and activity.

It is important to note that the IKK ϵ /TBK1 inhibitor amlexanox also increases energy expenditure in rodents (Reilly et al., 2013), and was effective in lowering blood sugar in a subset of obese patients with type 2 diabetes (Oral et al., 2017). Interestingly, analysis of gene expression changes in adipose tissue from responding patients revealed many of the same changes in mitochondrial respiration genes observed here in ATKO mice, suggesting that at least a part of amlexanox's beneficial effects might be due to increased AMPK activity via TBK1 inhibition. However, unlike the ATKO mice, amlexanox treatment improves catecholamine sensitivity in adipose tissue (Mowers et al., 2013), while reducing insulin resistance and adipose tissue inflammation (Reilly et al., 2013). We reported that IKK ϵ KO increases thermogenesis and energy expenditure, while reducing adipose inflammation (Chiang et al., 2009). It is likely that IKK ϵ is responsible for adipocyte catecholamine resistance via phosphorylation of PDE3B (Mowers et al., 2013), and must be inhibited to improve glucose homeostasis and inflammation. However, IKK ϵ has no effect on AMPK phosphorylation. During HFD-feeding, expression of both *Ikkbe* and *Tbk1* are upregulated by inflammation. While IKK ϵ phosphorylates and activates PDE3B to induce catecholamine resistance, TBK1 inhibits AMPK activity to reduce catabolism via this pathway. The combination of IKK ϵ and TBK1 activation thus decreases energy expenditure

to exaggerate energy storage and obesity. Thus, we propose that the beneficial metabolic effects of amlexanox require inhibition of both IKK ϵ and TBK1.

Previous studies showed that activation of AMPK by energy stresses, such as caloric restriction, reduces adipose inflammation and improves glucose metabolism (Cantó and Auwerx, 2011; Larson-Meyer et al., 2006). We show here that loss of TBK1 disrupts the anti-inflammatory effects of AMPK activation. As TBK1 induces NIK degradation to attenuate NF- κ B activation, we conclude that TBK1 mediates the anti-inflammatory functions of AMPK via negatively regulating NF- κ B activation.

Taken together, these data have introduced a new player in the control of energy homeostasis under conditions of both over- and undernutrition. Upon its activation, TBK1 can repress respiration by inhibiting AMPK activity, perhaps explaining in part why energy expenditure is reduced in states of obesity. At the same time, TBK1 can attenuate inflammation after it is induced by obesity, maintaining the inflammatory state at a low-grade level. Finally, TBK1 plays a crucial role in a feedback loop, both limiting the activity of AMPK under conditions, such as caloric restriction, in which it is activated but also mediating the anti-inflammatory effects of this important energy sensor. While many questions remain, these insights open a new line of investigation concerning how TBK1 modulates energy homeostasis.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and two tables and can be found with this article online at <https://doi.org/10.1016/j.cell.2018.01.007>.

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AUTHOR CONTRIBUTIONS

P.Z. and A.R.S. conceived the project. P.Z. designed the study and performed all the experiments. K.i.W. assisted with most of the experiments. X.S. contributed to Figures 1C, 1H, 2E, 2F, 5A–5C, S1E, S1K, S2I, S2J, S5C, S5D, and S7C. S.M.R. generated the ATKO mice. M.U. performed the preliminary experiments. Z.L. contributed to Figures 2E, 3E, 3F, 6E, and 6F. Y.S. contributed to Figure 5C. P.Z. and A.R.S. wrote the manuscript.

DECLARATION OF INTERESTS

A.R.S. is a named inventor on several patent applications that include amlex-anox. All other authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals and Recombinant Peptides		
Recombination Mouse TNF α	Thermo Scientific	PMC3014
Amlexanox	Abcam	ab142825
AICAR	Cell Signaling Technology	9944
Compound C	Calbiochem	171260
SBI-0206965	Sigma-Aldrich	SML1540
5Z-7-Oxozeaenol	Sigma-Aldrich	O9890
MG132	Sigma-Aldrich	M8699
Lipopolysaccharide	Sigma-Aldrich	L4391
Protease inhibitor tablet	Sigma-Aldrich	11873580001
2-[C ¹⁴]-deoxyglucose	PerkinElmer	NEC720A250UC
ULK1 active kinase	Signal Chem	U01-11G-10
Antibodies		
TBK1 antibody	Cell Signaling Technology	3013
Phospho-TBK1 Ser172 antibody	Cell Signaling Technology	5483
AMPK α antibody	Cell Signaling Technology	2532
Phospho-AMPK α Thr172 antibody	Cell Signaling Technology	2535
p100/p52 antibody	Cell Signaling Technology	4882
Phosphor-IKK α / β antibody	Cell Signaling Technology	2078
p105 antibody	Cell Signaling Technology	4717
Phosphor-p105 Ser933 antibody	Cell Signaling Technology	4806
ULK1 antibody	Cell Signaling Technology	8054
NIK antibody	Cell Signaling Technology	4994
ACC antibody	Cell Signaling Technology	3662
Phospho-ACC Ser79 antibody	Cell Signaling Technology	3661
MAPK (Erk1/2) antibody	Cell Signaling Technology	9102
β -tubulin antibody	Cell Signaling Technology	2146
Myc antibody	Santa Cruz Biotechnology	sc-40
Flag antibody	Sigma-Aldrich	F1804
Oligonucleotides		
Primers for Q-PCR, see Table S2	This paper	N/A
Critical Commercial Assays		
Ultra-sensitive mouse insulin ELISA kit	Crystal Chem	90080
Triglycerides liquid reagents	Pointe Scientific	T7532
NEFA-HR(2) assay	Wako	434-91795 434-91995
Cyclic AMP XP assay kit	Cell Signaling Technology	4339
Free glycerol determination kit	Sigma-Aldrich	F6428
Extracellular oxygen consumption assay	Abcam	ab197243
NAD/NADH assay kit	Abcam	ab65348
AMP-Glo assay	Promega	V6930

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
ADP-Glo assay	Promega	V5011
ENLITEN(R) ATP assay	Promega	PAFF2000
Recombinant DNA		
pcDNA-Flag-TBK1	Gift from Kate Fitzgerald	N/A
pcDNA-HA-AMPK α 1	Gift from Ken Inoki	N/A
pcDNA-HA-AMPK α 2	Gift from Ken Inoki	N/A
pcDNA-Flag-AMPK β 1	Gift from Ken Inoki	N/A
pcDNA-Myc-AMPK γ 1	Gift from Ken Inoki	N/A
pLentiCRISPR-v2-NIK-gRNA	Gift from Michael Karin	N/A
Experimental Models: Cell Lines		
TBK1 WT/KO MEFs	Gift from Kate Fitzgerald	N/A
AMPK α 1/ α 2 WT/KO MEFs	Gift from Ken Inoki	N/A
Immortalized ULK1 WT MEFs	Sigma-Aldrich	14050804-1VL
Immortalized ULK1 KO MEFs	Sigma-Aldrich	14050807-1VL
Experimental Models: Organisms/Strains		
Mouse: <i>Tbk1</i> ^{fl/fl}	Gift from Kate Fitzgerald	N/A
Mouse: <i>Prkaa1</i> ^{fl/fl} , <i>Prkaa2</i> ^{fl/fl}	Gift from Ken Inoki	N/A
Mouse: <i>Ulk1</i> ^{fl/fl}	Jackson Laboratory	017976
Mouse: Adiponectin-cre	Jackson Laboratory	028020
Mouse: C57BL/6J	Jackson Laboratory	000664
Software		
ImageJ	NIH	N/A
GraphPad Prism 6	GraphPad Software	N/A
Other		
High-Fat Diet (45% kcal% fat)	Research Diet, Inc.	D12451

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Alan R. Saltiel (asaltiel@ucsd.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Tbk1^{fl/fl} mice were bred with adiponectin-cre mice to generate adipocyte-specific TBK1 KO mice. Adipocyte-specific AMPK α 1/ α 2 double KO mice were generated by breeding *Prkaa1*^{fl/fl} mice and *Prkaa2*^{fl/fl} mice to adiponectin-cre mice. Adipocyte-specific ULK1 KO mice were created by breeding *Ulk1*^{fl/fl} mice to adiponectin-cre mice. All the mice have C57BL/6J background. Only male mice were used for experiments. We fed mice with normal chow diet or high fat diet consisting of 45% of calories from fat (D12451 Research Diets Inc.) for 12-16 weeks, starting at 6-8 weeks of age. During metabolic studies, ear tag numbers were used to identify animals. Flox and KO mice are littermates and cage mates. Researchers performing test and collecting data were blinded during experiments. Animals in each cohort were produced from 20 breeding pairs to minimize the birthdate range. Mice were housed in a specific pathogen-free facility with a 12-h light, 12-h dark cycle, and given free access to food and water, except for fasting period. All animal use was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California-San Diego. For metabolic study, mice are subject to CLAMs indirect calorimetry and dual energy X-ray absorptionmetry (DEXA) scanning at ACP phenotyping core of UCSD. Rectal temperature was measured by Model 4600 Thermometer (Alpha Technics). Serum insulin was measured with Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem).

Cells

3T3-L1 adipocytes

Murine 3T3-L1 preadipocytes were grown to 2 days after confluence in Dulbecco's Modified Eagle's Media (DMEM) with 10% newborn calf serum (NCS). 0.5mM 3-isobutyl-methylxanthine, 1 μ M dexamethasone, and 1 μ g/ml insulin (MDI) cocktail were used to induce preadipocytes differentiation in DMEM containing 10% fetal bovine serum (FBS) for the first 3 days. Cells were cultured in media containing insulin for another 3 days. After 48hrs, the media was replaced by DMEM with 10% FBS. Finally, differentiation was completed in the media containing 10% FBS (Zhao and Stephens, 2013). For serum deprivation, media was change to DMEM containing 0.2% BSA or low-glucose DMEM containing 0.2% BSA. Only cultures in which > 90% of cells displayed adipocyte morphology were used.

Differentiation of primary preadipocytes

Inguinal adipose tissues of 4-6 weeks old male mice were minced and digested with 1mg/ml type II collagenase for 25 min at 37°C with gentle agitation. Cells were washed, filtered through 100 μ m filter. After centrifugation, SVF was plated on 10cm collagen-coated cell culture plate. After 2 rounds of culture, Preadipocytes were grown to confluence and induced to differentiate with 0.5mM 3-isobutyl-methylxanthine, 1 μ M dexamethasone, 1 μ g/ml insulin (MDI) cocktail and 1 μ M rosiglitazone (Cayman).

Immortalization of TBK1 KO MEFs

To immortalize cells, they were transduced with retroviruses encoding a temperature-sensitive (tsa58) mutant A438V of large T antigen. After antibiotic selection with 100 μ g/ml G418 at the permissive temperature of 33°C for two weeks, cells were trypsinized and transferred to new culture dishes. Cells were moved to 37°C incubation 48hrs before experiments to eliminate expression of SV40 large T antigen.

Generation of NIK KO MEFs

Immortalized WT or TBK1 KO MEFs were transduced with viral supernatant to express gRNA/Cas9 (gRNA: 5'-GGCAAATGGC CCGTGTGTGC-3'). Supernatants were centrifuged to remove cell debris, and supplemented with Polybrene (8 μ g/ml) before adding to cells. After antibiotic selection with 2.5 μ g/ml puromycin for two weeks, cells were trypsinized and transferred to new culture dishes.

Reconstitution of TBK1 in TBK1 KO MEFs

Immortalized TBK1 KO MEFs were transduced with viral supernatants to express GFP or TBK1. Supernatants were centrifuged to remove cell debris, and supplemented with Polybrene (8 μ g/ml) before adding to cells. 48hrs after infection, expression of GFP and TBK1 was examined by Microscopy and western blot.

METHOD DETAILS

Gene expression analysis

Tissues were homogenized in TRIzol Reagent (Life Technologies). RNA was isolated with PureLink RNA mini kit (Life Technologies). 1 μ g of purified RNA was used for reverse transcription-PCR to generate cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem). $\Delta\Delta$ Ct real time PCR with Power SYBR Green PCR Master Mix (Life Technologies) and QuantStudio 5 Real-Time PCR System were used to analyze cDNA. *Gapdh* was used as endogenous control.

Transfection

1 μ g DNA for each plasmid was used to transfect HEK293T cells with Lipofectamine 3000 (Life Technologies), according the manufacturer's instruction. 24hrs later, cells were harvested and subject to western blot analysis.

Immunoprecipitation and lambda protein phosphatase treatment

Cells were harvested in IP buffer containing 100mM Tris (pH 7.5), 5mM MgCl₂, 130mM NaCl, 1mM EDTA, 1% Triton X-100 with freshly added protease inhibitors tablet (Roche), and phosphatase inhibitor 2 mM sodium vanadate and 5mM sodium fluoride. Immunoprecipitation was performed with anti-HA or Flag affinity gels (Biotool). Affinity gel was washed with TBS for 3 times and incubated with lambda protein phosphatase (NEB) at 30°C for 30min. After 3 times wash with PBS, protein was eluted with SDS loading buffer at 95°C.

Western blot

Tissues or cells were homogenized in a non-denaturing lysis buffer that contained 10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, with freshly added protease inhibitors tablet (Roche), and phosphatase inhibitor 2 mM sodium vanadate and 5mM sodium fluoride. The extract was centrifuged at 9500 g for 10 min at 4°C. Supernatants were collected and analyzed with BCA (Thermo Scientific) to quantify protein content. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). Individual proteins were detected with specific antibodies and visualized on film using horseradish peroxidase-conjugated secondary antibodies (Fisher Scientific) and SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Bands were quantified with ImageJ (NIH).

Glucose tolerance test

Fasting blood glucose were measured after 14hrs fast, using Easy Step Blood Glucose Monitoring System. Mice were then intraperitoneally injected with D-[+]-glucose (Sigma) at a dose of 2g/kg BW for ND-fed mice or 1.2g/kg BW for HFD-fed mice. Blood glucose levels were measured at 15, 30, 45, 60, 90 and 120 min after injection.

Insulin tolerance test

Fasting blood glucose were measured after 4hrs fast, using Easy Step Blood Glucose Monitoring System. Mice were then intraperitoneally injected with insulin (Humulin R) at a dose of 1U/kg BW for ND-fed mice or 1.2U/kg BW for HFD-fed mice. Blood glucose levels were measured at 15, 30, 45, 60, 90 and 120 min after injection.

In vivo 2-[C¹⁴]-deoxyglucose uptake

Mouse was fasted for 16hrs, and then injected with 1.2g/kg glucose, 1U/kg insulin with 180 μ Ci/kg 2-[C¹⁴]-deoxyglucose. Blood was collected at 0, 15, 30, 45 and 60min. Mice were dissected at 60min. Adipose tissues were collected, weighted, and homogenized in perchloric acid. After centrifugation, supernatant was collected and neutralized. Radioactivity in lysate and serum was determined by the Beckman Coulter.

Histology and determination of adipocyte size

Tissues were dissected, and then fixed in 10% formalin. Paraffin-embedding tissues were sectioned and subject to H&E and F4/80 (AbD Serotec) IHC staining at UCSD histology core. Stained tissue was visualized with NanoZoomer Slide Scanner. Size of adipocytes was determined by ImageJ analysis of H&E stained tissues.

Mature adipocytes and SVF separation and FACS

Minced white adipose tissue was digested in RPMI with 0.5% BSA containing 1mg/ml type II collagenase for 25 min at 37 °C with gentle agitation. The cell suspension was filtered through a 100 μ m cell strainer and centrifuged at 500 g for 5 min to separate floating mature adipocytes and SVF. Red blood cells in SVF were lysed with ACK red blood cell lysis buffer for 5min. SVF was washed with RPMI with 0.5% BSA. Fc receptor was blocked with anti-CD16/CD32 antibody (eBioscience). Cells were stained with anti-F4/80-FITC (eBioscience), and analyzed by flow cytometry.

Ex vivo oxygen consumption rate measurement

Adipose tissues were dissected, weighted, quickly rinsed in PBS. Minced tissues were placed in 96-well tissue culture plate. *Ex vivo* oxygen consumption rate was measured with Oxygen Consumption Assay Kit (Abcam) according to the manufacturer's instruction. 150 μ L pre-warmed medium and 10 μ L oxygen consumption reagent were added to each well. Wells were sealed with pre-warmed high sensitivity mineral oil. Fluorescence was measured at 37°C for 30min by Tecan Infinite M200 Pro.

Mitochondrial staining and confocal microscopy

Adipose tissues were dissected and fixed in 10% formalin with 7.5% sucrose. OCT-embedded tissue was sectioned and stained with 200nM Mitotracker Red (Life Technologies) for 45min at room temperature, followed by 3 washes with PBS. Fluorescence was visualized with Olympus FV1000 microscope.

In vitro kinase assay

Immunoprecipitated Flag-TBK1 WT or Flag-TBK1 KD was subject to *in vitro* kinase assay with 40ng/ μ l ULK1 active kinase (SignalChem), kinase assay buffer III (SignalChem) and 100 μ M ATP (SignalChem). After 15min incubation in 30°C water bath, reaction was stopped by adding SDS loading buffer and heating at 95°C. Phospho-TBK1 Ser172 antibody (Cell Signaling) was used to detect phosphorylated TBK1.

NAD/NADH ratio measurement

Tissue NAD/NADH ratio was measured with NAD/NADH Assay kit (abcam). Tissues were dissected, rinsed in PBS, and homogenized in NAD/NADH extraction buffer from the kit. After centrifugation, supernatant was collected. NAD/NADH ratio was measured according to the manufacturer's instruction.

cAMP measurement

Tissue cAMP levels were determined using the cAMP XP Assay kit (Cell Signaling). Tissues were dissected, rinsed in PBS, and homogenized in cell lysis buffer on ice. After centrifugation, supernatant was collected. 50 μ L sample was used to determine cAMP level according to the manufacturer's instruction. cAMP level was normalized to protein content.

ATP, ADP, AMP measurement

Tissues were homogenized in 2% trichloroacetic acid (TCA) buffer. After centrifugation, supernatant was collected and neutralized with pH8.0 Tris-HCl. ATP was measured with ENLITEN ATP Assay System (Promega). ADP was measured with ADP-Glo Kinase Assay (Promega). AMP was measured with AMP-Glo Assay (Promega), according to the manufacturer's instructions.

Ex vivo lipolysis measurement

Adipose tissues were dissected, weighted, and quickly rinsed with PBS. Minced tissues were cultured in 12-well plate in the absence or presence of CL-316,243 (10 μ M) for 2hrs. Glycerol in supernatant was measured with Free Glycerol Determination kit (Sigma).

Mass spectrometry

In gel digestion followed by LC-MS/MS was performed by the Biomolecular and Proteomics Mass Spectrometry Facility at University of California-San Diego. Peptides were resolved on a nano-C18 column and sprayed into Orbitrap mass spectrometer (Thermo fusion lumos). Peak Studio 8 was used to search against human protein database (Uniprot).

Site-direct mutagenesis

HA-AMPK α 1 SS/AA and HA-AMPK α 1 SS/DD plasmids were generated using Kapa Hifi DNA polymerase with 0.5ng HA-AMPK α 1 WT as template DNA in 50ul reaction. Template DNA was digested by DpnI (NEB). Stellar competent cell (Takara) was used for transformation and plasmids amplification. Plasmid was purified with Wizard Plus SV Minipreps DNA purification system (Promega). Mutation was confirmed by DNA sequencing (Eton Bioscience).

QUANTIFICATION AND STATISTICAL ANALYSIS

All data of animal study are shown as mean \pm SEM, while data from *in vitro* studies are shown as mean \pm SD. Replicates are indicated in figure legends. N represents the number of experimental replicates. *F*-test was performed to determine the equality of variance. When comparing two groups, statistical analysis was performed using a two-tailed Student's *t* test, except when the *f*-test suggested that variances are statistically different. For analysis of more than two groups, we used analysis of variance (ANOVA) to determine equality of variance. Comparisons between groups were performed with Tukey-Kramer post hoc analysis (Reilly et al., 2013). For all tests, $p < 0.05$ was considered statistically significant.

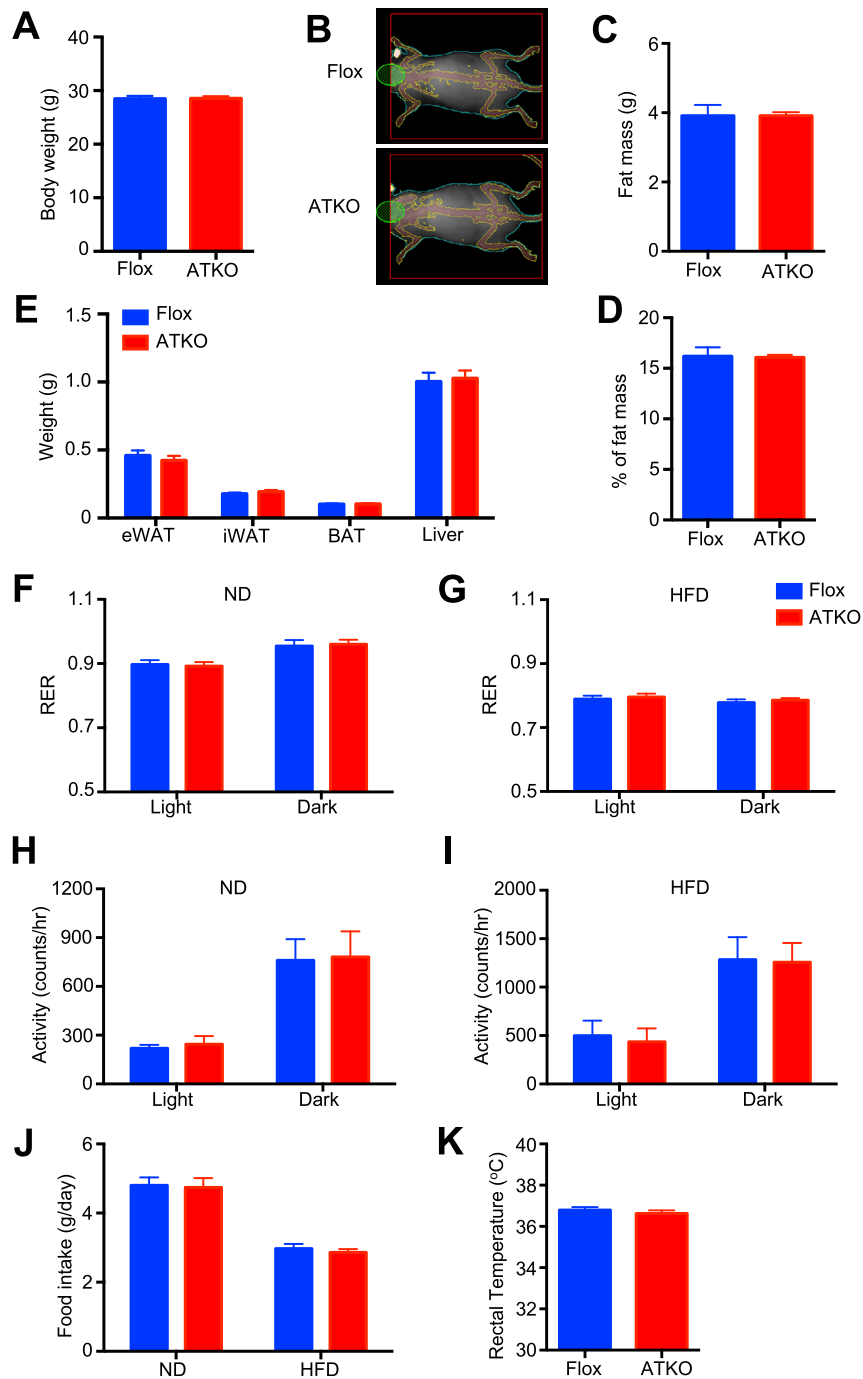


Figure S1. Adipocyte-Specific TBK1 Deficiency Does Not Affect Energy Homeostasis in ND-Fed Mice or Body Temperature in 16 Weeks HFD-Fed Mice, Related to Figure 1

(A) Body weight of ND-fed Flox and ATKO mice. N=11-15.

(B) DEXA imaging of ND-fed Flox and ATKO mice.

(C and D) Fat mass (C) or percentage of fat mass (D) of ND-fed Flox and ATKO mice. N=6.

(E) Tissues weight of ND-fed Flox and ATKO mice. N=11-15.

(F and G) Respiratory exchange rate (RER) of ND (F) or HFD (G)-fed Flox and ATKO mice. N=5-6.

(H and I) Activity of ND (H) or HFD (I)-fed Flox and ATKO mice. N=5-6.

(J) Daily food intake of ND and HFD-fed Flox and ATKO mice. N=5-6.

(K) Rectal temperature of HFD-fed Flox and ATKO mice. N=9. Data are represented as mean \pm SEM. * $p < 0.05$.

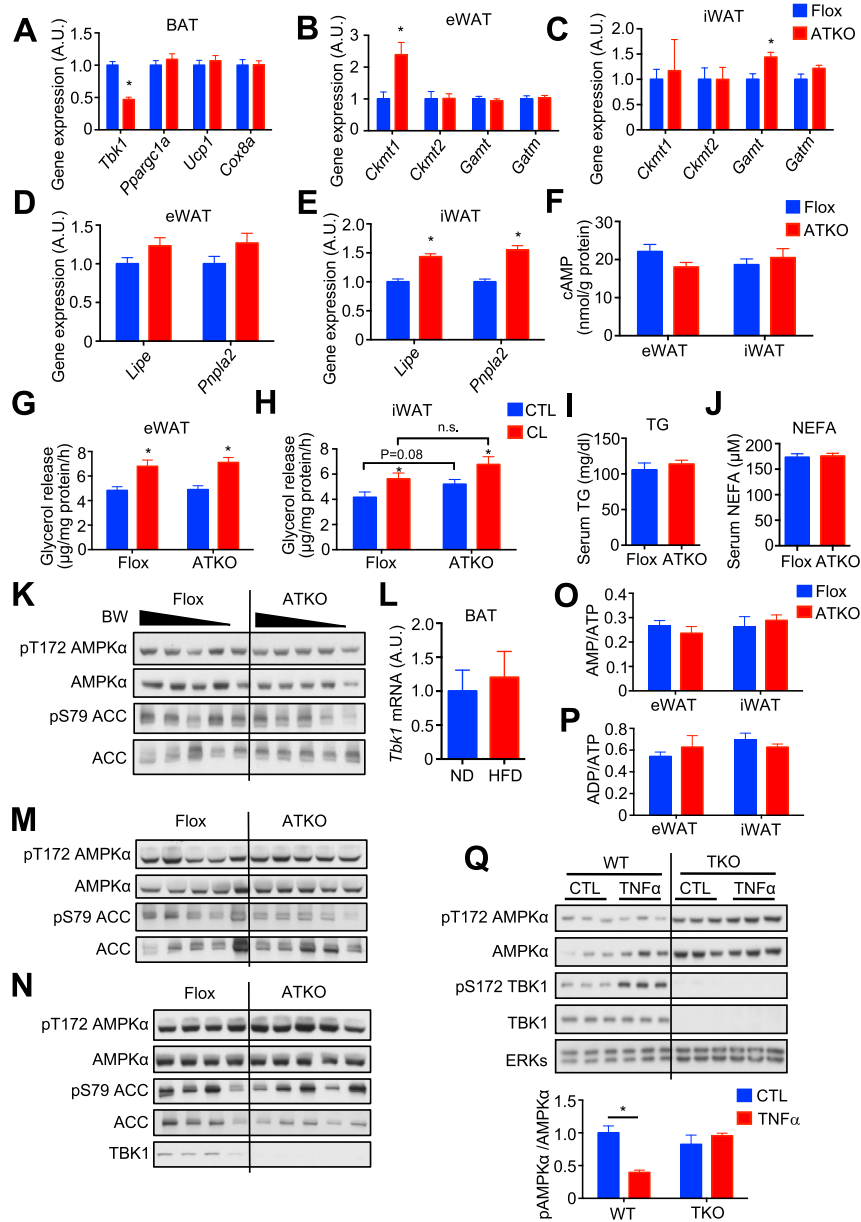


Figure S2. TBK1 Deficiency Increases Expression of Creatine Cycle and Lipase Genes, without Affecting Catecholamine Sensitivity or AMP/ATP in 16 Weeks HFD-Fed Mice, Related to Figure 2

(A) Genes expression in BAT of HFD-fed Flox and ATKO mice. N=9.

(B and C) Expression of creatine cycle genes in eWAT (B) and iWAT (C) of HFD-fed Flox and ATKO mice. N=9.

(D and E) Expression of lipase genes in eWAT (D) and iWAT (E) of HFD-fed Flox and ATKO mice. N=9.

(F) cAMP level in eWAT and iWAT of HFD-fed Flox and ATKO mice. N=4.

(G and H) Glycerol released from explanted eWAT (G) and iWAT (H) of HFD-fed mice in the absence or presence of CL-316,243 (10 μM). N=9.

(I and J) Serum triglyceride (TG) (I) and NEFA. (J) levels in HFD-fed Flox and ATKO mice. N=11-12.

(K) IB analysis of BAT in HFD-fed Flox and ATKO mice.

(L) *Tbk1* expression in BAT of ND or HFD-fed mice. N=4.

(M and N) IB analysis of eWAT (M) and iWAT (N) in ND-fed Flox and ATKO mice.

(O and P) Measurement of AMP/ATP (O) and ADP/ATP (P) ratio in eWAT and iWAT of HFD-fed Flox and ATKO mice. N=5. Data are represented as mean \pm SEM. * $p < 0.05$.

(Q) IB analysis of WT and TKO (TBK1 KO) MEFs treated with Vehicle or TNF α (10 nM). Quantification of pAMPK α /AMPK α ratio (bottom). Data are represented as mean \pm SD. * $p < 0.05$.

A Human PRKAA1 Coverage: 74.78%

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1 MRRLLSSWRKMATAEKQKHDGRVKIGHYILGDTLGVGTFGRVKVVGKHELTGHKVAVKILNR
61 QKIRSLDVVGKIRREIQNLKLFRRHPHIKLYQVISTPDI FMVMEYVSGGELFDYICKNG
121 RLDEKESRRLFQQILSGVDYCHRHMVVHRDLKPENVLLDAHMKIADFGLSNMMSDGEF
181 LRTSCGSPNYAAPEVISGRLYAGPEVDIWSGVIYALLCGTLPFDHDDHVPTLFRKICDG
241 IFYTPQVNLPSVISLKHMLQVDPMKRATIKDIREHEWFKODLPKYLFPEDPSYSSTMI
301 DEALKEVCEKFCSEEEVLSCLYRNHODPLAVAYHLIIDNRIMNEAKDFYLATSPDPS
361 FLDDHHLTRPHERVFPFLVAETPRARHTLDELNPQSKHQGVKAKWHLGI RSQSRPNDI
421 MAEVCRAIKQLDYEWKVVNPYYLRVRRKNPVTSTYSKMSLQLYQVDSRTYLLDFRSIDDE
481 ITEAKSGTATPQRSGSVSNYRSCQRSDDSDAEAGKSSSEVSLTSSVTSILDSSPVDLTPRPG
541 SHTIEFFEMCANLIKILAQ
    
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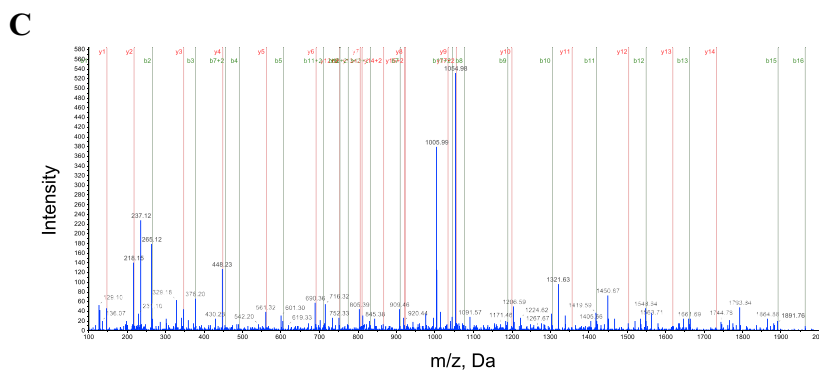
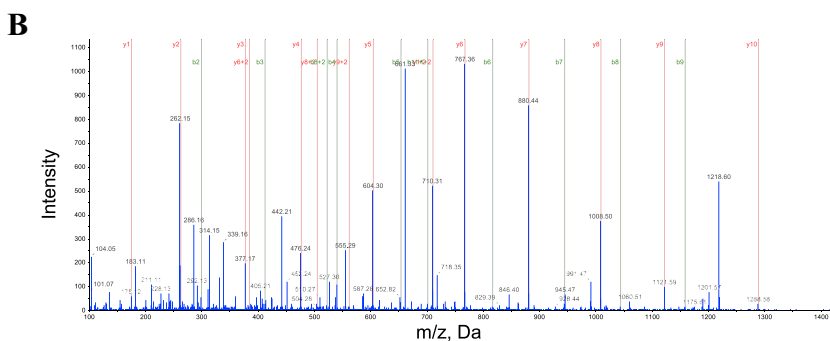


Figure S3. Identification of TBK1-Induced Phosphorylation on AMPK α 1 (PRKAA1) by LC-MS/MS, Related to Figure 3
 (A) Coverage of AMPK α 1 sequence in LC-MS/MS analysis.
 (B and C) MS chromatogram depicting the phosphopeptide showing AMPK α 1 Ser459 (B) and Ser476 (C) phosphorylation (S+80).

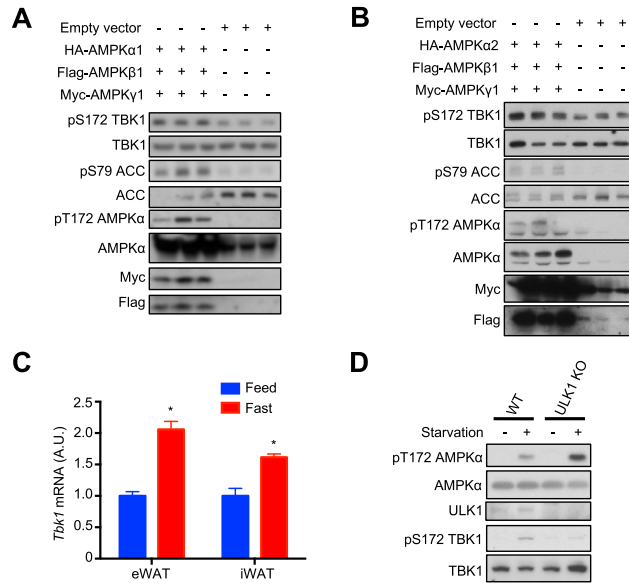


Figure S4. Activation of AMPK Induces TBK1 Activity, Related to Figure 4

(A and B) IB analysis of HEK293T cells overexpressing HA-AMPK α 1 (A) or HA-AMPK α 2 (B) with Flag-AMPK β 1 and Myc-AMPK γ 1.

(C) Expression of *Tbk1* in eWAT and iWAT of C57BL/6J mice fed with ND or fasted for 48hrs. N=4. Data are represented as mean \pm SEM. * $p < 0.05$.

(D) IB analysis of WT and ULK1 KO MEFs glucose-starved for 2hrs or not. Result is a representative experiment that was repeated three times.

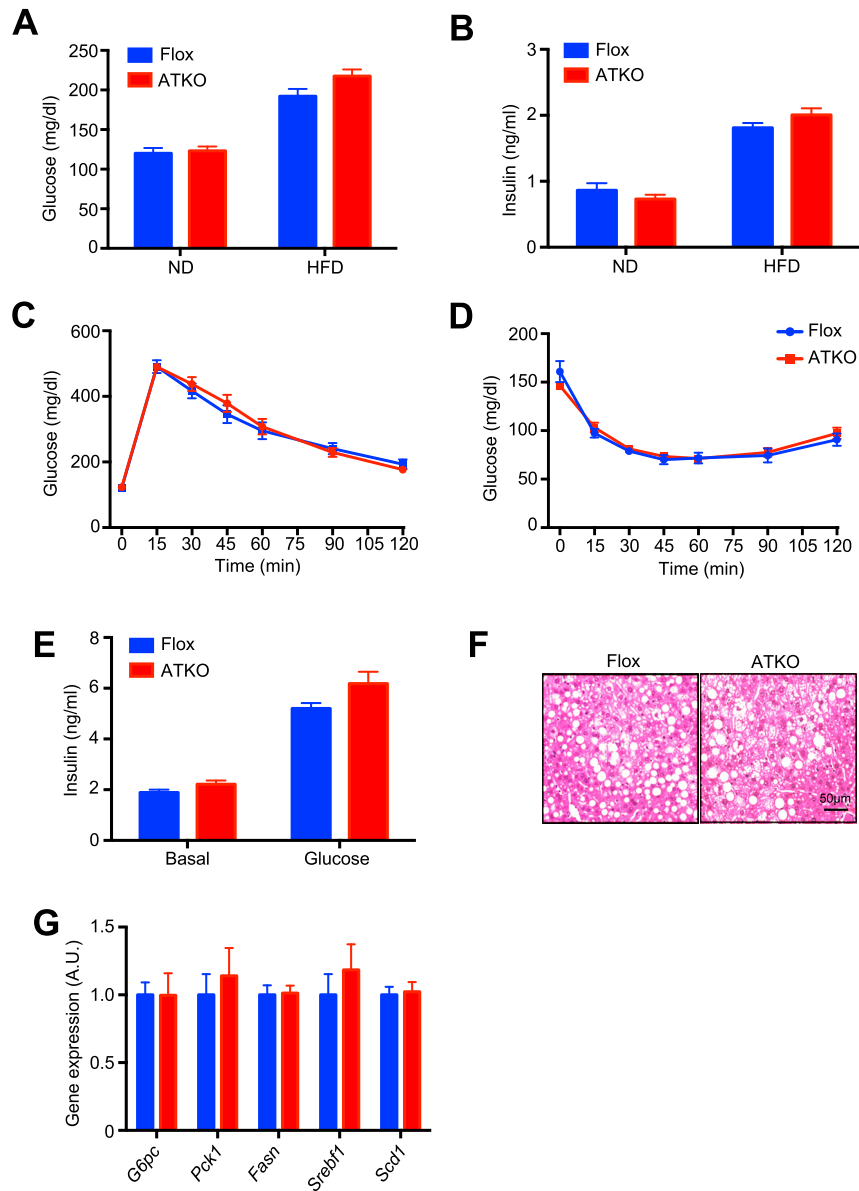


Figure S5. Loss of TBK1 Does Not Affect Glucose Metabolism in ND-Fed Mice or Genes Expression and H&E Staining of Liver in HFD-Fed Mice, Related to Figure 5

(A and B) Fasting blood glucose (A) and insulin (B) levels of 12 weeks ND or HFD-fed Flox and ATKO mice. N=7-11.

(C) Glucose (2g/kg BW) tolerance test on 12 weeks ND-fed Flox and ATKO mice. N=7.

(D) Insulin (1U/kg BW) tolerance test on 12 weeks ND-fed Flox and ATKO mice. N=7-8. Data are represented as mean \pm SEM. * p <0.05.

(E) Blood insulin levels at 0 min or 30 min during GTT on HFD-fed Flox and ATKO mice. N=10-11.

(F) H&E staining of livers in 16 weeks HFD-fed Flox and ATKO mice.

(G) Genes expression in livers of 16 weeks HFD-fed Flox and ATKO mice. N=10-11. Data are represented as mean \pm SEM. * p <0.05.

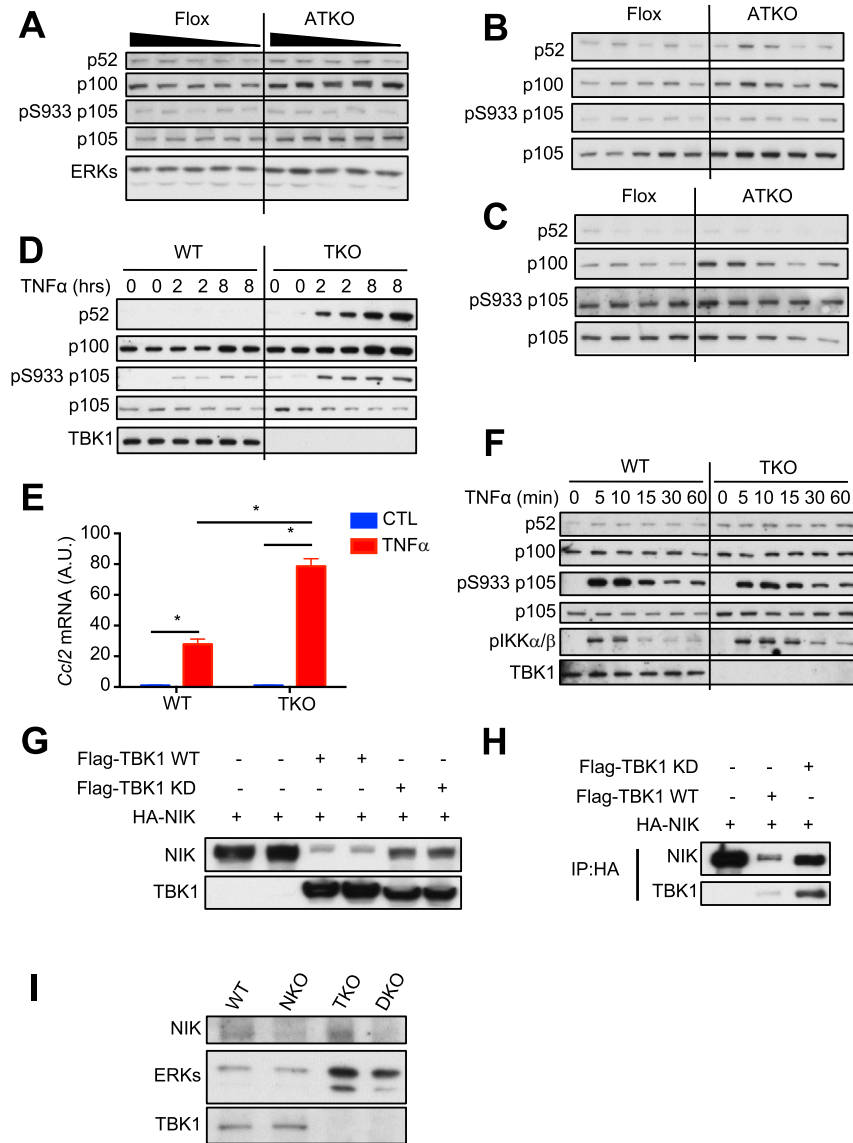


Figure S6. Loss of TBK1 Increases TNF α -Induced NF κ B Activation, Related to Figure 6

(A) IB analysis of BAT from HFD-fed Flox and ATKO mice.
 (B and C) IB analysis of eWAT (B) and iWAT (C) from ND-fed Flox and ATKO mice.
 (D) IB analysis of WT or TKO MEFs treated with TNF α (10nM) for indicated time.
 (E) *Ccl2* expression in WT or TKO MEFs treated with TNF α (5nM) for 16hrs. N=3. Data are represented as mean \pm SD. *p<0.05.
 (F) IB analysis of WT or TKO MEFs treated with TNF α (10nM) for indicated time.
 (G) IB analysis of HEK293T cells overexpressing HA-NIK with Flag-TBK1 WT or Flag-TBK1 KD.
 (H) IB analysis of product from immunoprecipitation with HA-antibody.
 (I) IB analysis of WT, NKO, TKO or DKO MEFs treated with proteasome inhibitor MG132 (20 μ M) for 6hrs.

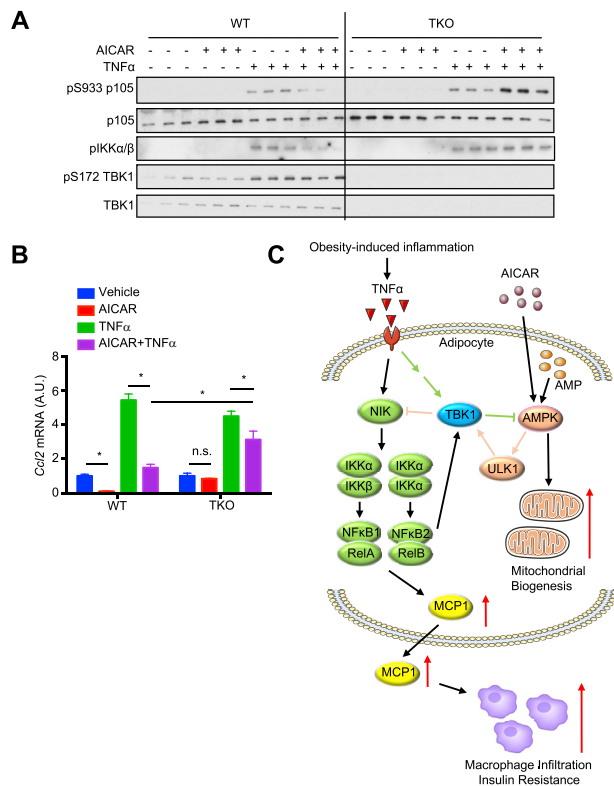


Figure S7. TBK1 Deficiency Attenuates Inhibition of NF κ B Signaling by AMPK, Related to Figure 7

(A) IB analysis of WT and TKO MEFs pretreated with Vehicle or AICAR (500 μ M) for 7hrs, and then treated with TNF α (10nM) for 45min.

(B) *Ccl2* expression in WT and TKO MEFs pretreated with Vehicle or AICAR (500 μ M) for 7hrs, and then treated with TNF α (10nM) for 2hrs. Data are represented as mean \pm SD. * p <0.05.

(C) Proposed model for roles of TBK1 in modulating inflammation and energy metabolism.