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IKK β Is Essential for Adipocyte Survival and Adaptive Adipose Remodeling in Obesity

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IKK β kinase β (IKK β), a central coordinator of inflammatory responses through activation of nuclear factor- κ B (NF- κ B), has been implicated as a critical molecular link between inflammation and metabolic disorders; however, the role of adipocyte IKK β in obesity and related metabolic disorders remains elusive. Here we report an essential role of IKK β in the regulation of adipose remodeling and adipocyte survival in diet-induced obesity. Targeted deletion of IKK β in adipocytes does not affect body weight, food intake, and energy expenditure but results in an exaggerated diabetic phenotype when challenged with a high-fat diet (HFD). IKK β -deficient mice have multiple histopathologies in visceral adipose tissue, including increased adipocyte death, amplified macrophage infiltration, and defective adaptive adipose remodeling. Deficiency of IKK β also leads to increased adipose lipolysis, elevated plasma free fatty acid (FFA) levels, and impaired insulin signaling. Mechanistic studies demonstrated that IKK β is a key adipocyte survival factor and that IKK β protects murine and human adipocytes from HFD- or FFA-elicited cell death through NF- κ B-dependent upregulation of antiapoptotic proteins and NF- κ B-independent inactivation of proapoptotic BAD protein. Our findings establish IKK β as critical for adipocyte survival and adaptive adipose remodeling in obesity.

It is generally accepted that obesity is associated with a state of chronic low-grade inflammation that is a major contributor to insulin resistance and type 2 diabetes, yet the detailed molecular mechanisms remain elusive (1–3).

IKK β kinase β (IKK β), a central coordinator of inflammatory responses through activation of nuclear factor- κ B (NF- κ B) (4–6), has been implicated as a key molecular link between obesity, inflammation, and metabolic disorders (7,8). It is known that overnutrition can activate IKK β in vitro and in vivo (3,9,10) and that diet-induced insulin resistance has been associated with the activation of IKK β /NF- κ B in multiple tissues (9,11–15). Moreover, heterozygous deletion of IKK β or inhibiting IKK β with salicylates protected mice against insulin resistance triggered by high-fat (HF) diet (HFD) or obesity (11,16). Deletion of IKK β in the liver improved diet-induced insulin resistance, and deficiency of IKK β in myeloid cells rendered global insulin sensitivity upon HF feeding (12). By contrast, constitutive activation of IKK β in the liver caused systemic insulin resistance (13).

Compared with other tissues and cell types, the role of adipose IKK β signaling in obesity and metabolic disorders remains incompletely understood. Obesity is associated with elevated activity of IKK β and another key stress/inflammatory kinase, c-Jun amino-terminal kinase (JNK), in adipose tissue (3,9,17). The contribution of JNK signaling to obesity-associated insulin resistance has been well-established (3,17). Although the role of IKK β in inflammatory factor-mediated adipocyte insulin resistance has been confirmed by in vitro studies (9,18), in vivo studies using transgenic or knockout mice have generated inconsistent results. For example, overexpression of NF- κ B subunit p65 or a constitutive active form of IKK β in adipose tissue increased systemic and tissue inflammation but also resulted in improved insulin sensitivity (19,20).

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A very recent study showed that the adipocyte IKK β also plays an important role in inflammation resolution by inducing interleukin 13 (IL-13) expression in adipose tissue (21). We previously demonstrated that IKK β functions in adipocyte precursor cells to regulate adipogenesis and adipose tissue development (6). Deficiency of IKK β in adipocyte precursor cells inhibited adipocyte differentiation and protected mice from diet-induced obesity and metabolic disorders (6). These findings suggest that the functions of IKK β signaling in adipose tissue are complex and that further studies are needed to define the role of adipocyte IKK β in obesity and insulin resistance.

The adipocyte is subject to constant turnover once formed, and obesity is associated with a higher adipocyte turnover due to an elevated rate of adipocyte death (22,23). In addition to mediating inflammatory responses, IKK β is also a key cell survival factor (4,5) but the functions of IKK β in adipocyte survival has not been thoroughly investigated under the condition of obesity. We demonstrate here that IKK β plays an essential role in the regulation of adipocyte survival in diet-induced obesity and is required for adaptive adipose remodeling in response to nutrient changes.

RESEARCH DESIGN AND METHODS

Animals

Adipocyte-specific IKK β -knockout (IKK $\beta^{\Delta Ad}$) mice were generated by crossing IKK $\beta^{E/F}$ mice (6,24) with Adipoq-Cre transgenic mice (25). IKK $\beta^{E/F}$ mice were also mated with aP2-Cre mice to generate IKK $\beta^{\Delta aP2}$ mice. IKK β -deficient mice and IKK $\beta^{E/F}$ control littermates (4 weeks old) were fed a normal chow diet (ND) or a Western-type HFD (42% kcal from fat; Harlan Teklad) for 12 weeks until they were killed at 16 weeks of age (6). For the HFD-to-ND (HFD/ND) conversion diet experiment, mice were fed the HFD for 24 weeks then switched to the ND for 6 weeks. All animals were housed in an animal facility under a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee.

Metabolic and Histological Analyses

Body weight was measured weekly, and body composition was measured by MRI (Echo MRI). Food intake, total activity, oxygen consumption, and carbon dioxide production were measured with a LabMaster system (6). Intraperitoneal glucose tolerance test and insulin tolerance test were performed as described previously (6). Ex vivo lipolysis was performed by using a Lipolysis Assay Kit (ZenBio) following the manufacturer's protocol. Immunohistochemical staining of adipose tissue sections was performed following standard protocols (6). TUNEL staining for adipose tissue sections was performed using the In Situ Cell Death Detection Kit (Roche Applied Science).

MRI Analysis and Data Segmentation

HFD-fed IKK $\beta^{\Delta Ad}$ and IKK $\beta^{E/F}$ littermates were imaged on a 7T Cliniscan MRI (Bruker, Ettlingen, Germany). The

fat and water images were generated from the in and out of phase images using Siemens Syngo software. FSL software was used for image analysis, and fat volume was calculated following standard protocols (26,27).

Adipose Stromal Vascular Cell Isolation and Analyses

Adipose stromal vascular (SV) cells and adipocytes were isolated as previously described (6). Flow cytometry analysis was performed using freshly isolated SV cells labeled with Alexa Fluor 488-F4/80 (Serotec) and PE-CD11b (BD Biosciences) antibodies. For the Cre-mediated IKK β deletion assay, fully differentiated SV cells from IKK $\beta^{E/F}$ mice were infected with lentivirus expressing Cre or LacZ as the control. Cells were treated with a 0.5 mmol/L free fatty acid (FFA) mixture containing myristic, lauric, arachidonic, oleic, and linoleic acids (Sigma-Aldrich) or vehicle in the presence of 1% FFA-free BSA (9,10).

3T3-L1 Cell Differentiation and Short Interfering RNA Knockdown

3T3-L1 cells were first differentiated into mature adipocytes as previously described (6). Differentiated 3T3-L1 cells were transfected with control short interfering (si)RNA or siRNA targeting the mouse IKK β or BAD gene sequences by electroporation using the Amaxa Cell Line Nucleofector Kit V (Lonza) (6). Cells were treated with 0.5 mmol/L FFA mixture or vehicle for Western blot analysis.

Quantitative PCR and Western Blot Analyses

Quantitative PCR and Western blot analyses were performed as described before (6,28,29). The primer sets used in this study are listed in Supplementary Table 1. Anti-phosphorylated (phospho)-BAD (Ser26) antibody was purchased from Abiocode, anti-actin antibody was purchased Sigma-Aldrich, and all of the other antibodies were purchased from Cell Signaling.

Human Subjects and Adult-Derived Human Adipose Stem Cell Isolation

Human adipocytes were derived from the differentiation of adult-derived human adipose stem cells (ADHASCs) (30,31). The adipose tissue was from a collagenase digestion of the lipoaspirate of young and healthy subjects undergoing liposuction of subcutaneous fat. Differentiation medium was used to induce differentiation, as previously described (30,31).

Statistical Analysis

Data are presented as the mean \pm SEM. Individual pairwise comparisons were analyzed by two-sample, two-tailed Student *t* test in which *P* < 0.05 was regarded as significant.

RESULTS

Adipocyte IKK β Deficiency Does Not Affect Body Weight and Energy Expenditure but Results in an Exaggerated Diabetic Phenotype When Challenged With an HFD

To investigate the functions of adipocyte IKK β in vivo, we generated IKK $\beta^{\Delta Ad}$ mice by crossing mice carrying

loxP-flanked IKK β alleles (IKK $\beta^{F/F}$) (6,24) with adiponectin (Adipoq)-Cre transgenic mice (25,32). As expected, IKK β mRNA levels were significantly reduced in white adipose tissue (WAT), including subcutaneous WAT (subWAT) and epididymal WAT (epiWAT), and in brown adipose tissue (BAT), but not in other major tissues or cell types such as liver and macrophages of IKK $\beta^{\Delta Ad}$ mice (Fig. 1A). Western blot analysis also confirmed the deletion of IKK β in WAT but not in liver or macrophages (Fig. 1B). Further, Adipoq-Cre was only present in mature adipocytes but not in adipose SV cells of IKK $\beta^{\Delta Ad}$ mice, resulting in decreased IKK β

mRNA levels in adipocytes only (Supplementary Fig. 1A and B). Consistently, adipose SV cells of IKK $\beta^{\Delta Ad}$ mice had similar adipogenic potential compared with those of IKK $\beta^{F/F}$ mice (Supplementary Fig. 1C). Taken together, these results validate the specific deletion of IKK β in adipocytes of IKK $\beta^{\Delta Ad}$ mice.

To assess the effect of deficiency of adipocyte IKK β on obesity, 4-week-old male IKK $\beta^{\Delta Ad}$ and IKK $\beta^{F/F}$ littermates were fed the ND or HFD. Body weight was significantly increased after HF feeding for both IKK $\beta^{\Delta Ad}$ and IKK $\beta^{F/F}$ mice, but deficiency of adipocyte IKK β did not affect the diet-induced weight gain (Fig. 1C).

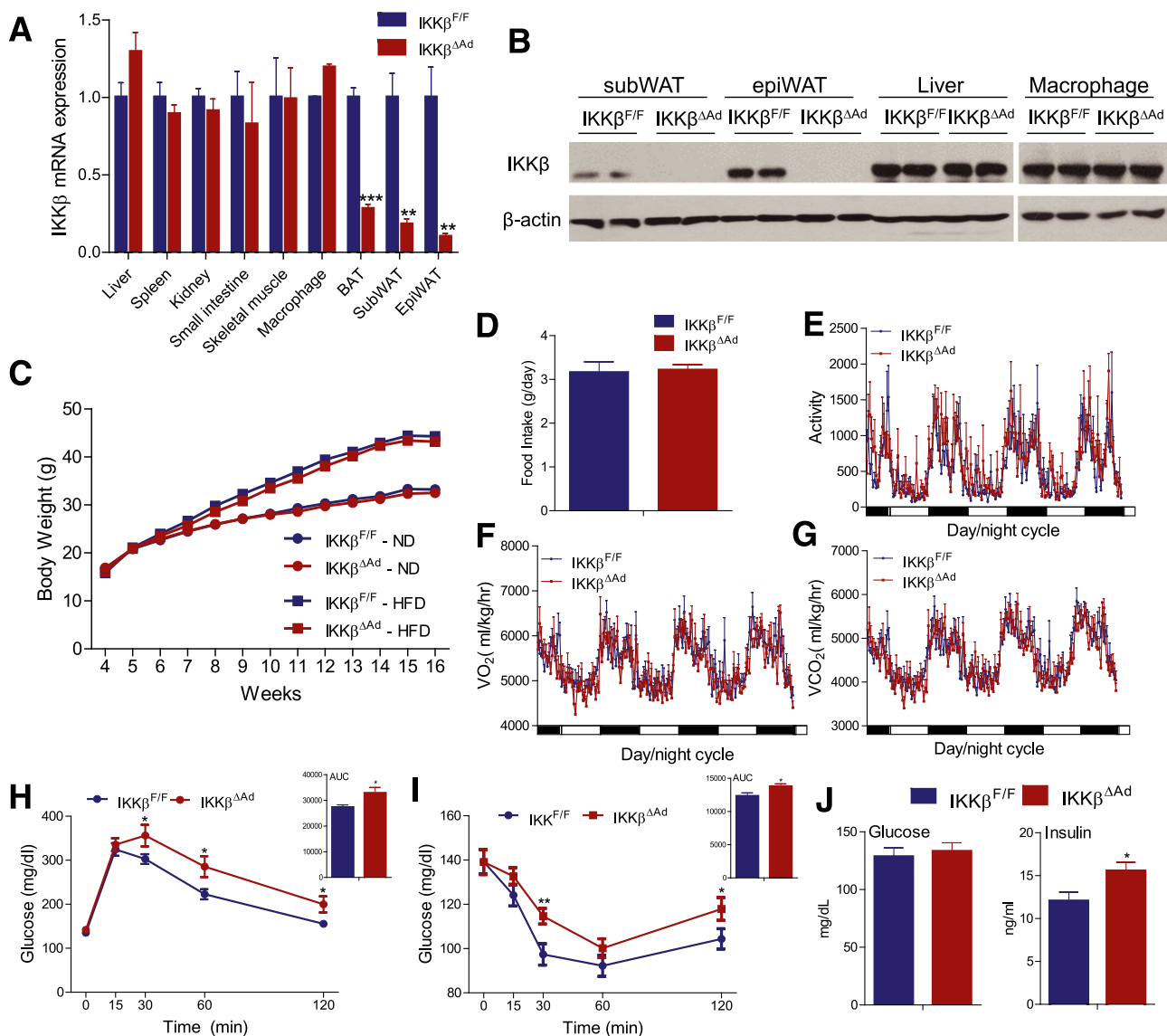


Figure 1—Deficiency of adipocyte IKK β does not affect diet-induced weight gain but results in an exaggerated diabetic phenotype when challenged with an HFD. **A:** IKK β mRNA expression levels in major organs of IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice were analyzed by quantitative PCR ($n = 3-7$). **B:** Western blot analysis of IKK β protein levels in subWAT, epiWAT, liver, and macrophages of IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice. **C:** Growth curves of ND- or HFD-fed male IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice ($n = 19-28$). Food intake (**D**), total activity (**E**), VO $_2$ (**F**), and VCO $_2$ (**G**) of 16-week-old male IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice fed the HFD for 12 weeks ($n = 5$). Food intake was averaged from 4-day measurements. Glucose tolerance test (GTT) and the area under the curve (AUC) of GTT (**H**), insulin tolerance test (ITT), and AUC of ITT (**I**), and fasting glucose and insulin levels (**J**) in HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice ($n = 10-17$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

IKK $\beta^{\Delta Ad}$ mice were also phenotypically normal in food intake, oxygen consumption, and carbon dioxide production compared with IKK $\beta^{F/F}$ mice (Fig. 1D–G). Unexpectedly, HFD-fed IKK $\beta^{\Delta Ad}$ mice developed an exaggerated diabetic phenotype. Upon glucose and insulin tolerance testing, IKK $\beta^{\Delta Ad}$ mice had worse glucose tolerance (Fig. 1H) and showed a decreased hypoglycemic response to the injected insulin (Fig. 1I). Further, deficiency of adipocyte IKK β also elevated fasting serum insulin concentrations (Fig. 1J), suggesting worse insulin sensitivity.

Loss of Adipocyte IKK β Causes Partial Lipodystrophy and Defects in Adaptive Adipose Remodeling in Response to Dietary Changes

Intriguingly, we found that visceral (vis) adipose pads including epididymal (epi) and retroperitoneal fat pads of IKK $\beta^{\Delta Ad}$ mice failed to expand properly in response to HF feeding (Fig. 2A and B). MRI analyses confirmed that IKK $\beta^{\Delta Ad}$ mice had a significantly decreased volume of visWAT but a similar subWAT volume compared with IKK $\beta^{F/F}$ mice after HF feeding (Fig. 2C and D). Histological analyses showed that visWAT of HFD-fed IKK $\beta^{\Delta Ad}$ mice exhibited

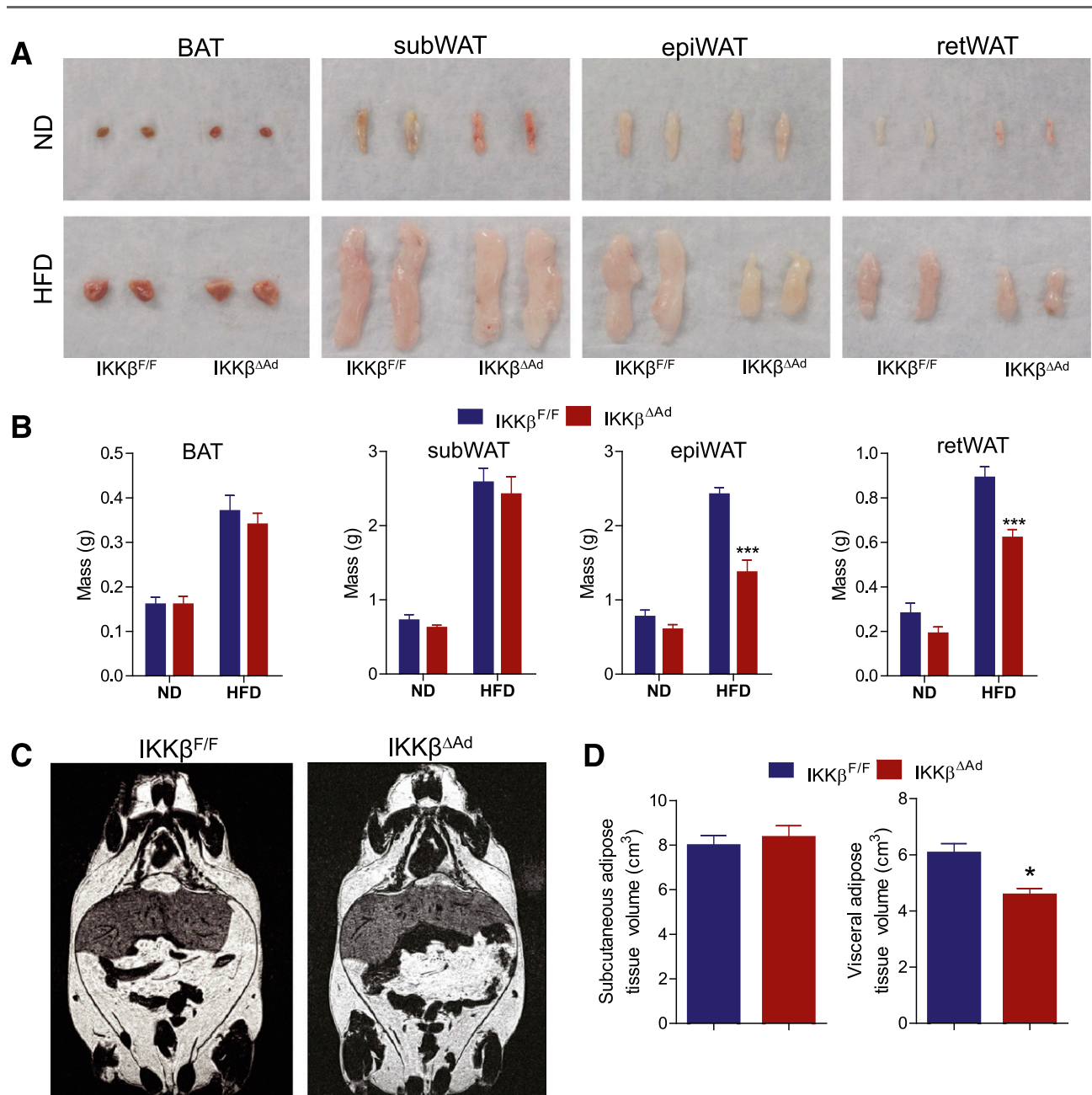


Figure 2—Visceral adipose tissue of adipocyte IKK β -deficient mice fails to expand properly in response to HF feeding. Representative photographs (A) and weight (B) of fat pads from ND- or HFD-fed male IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice ($n = 7-10$). Representative coronal section MRIs (C) and visceral and subcutaneous adipose tissue volume (D) of HFD-fed male IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice ($n = 5-6$). ret, retroperitoneal. * $P < 0.05$; *** $P < 0.001$.

a form of lipodystrophy with striking structure abnormalities and marked heterogeneity in adipocyte sizes (Fig. 3A). Massively increased collagen deposition in epiWAT of HFD-fed IKK $\beta^{\Delta Ad}$ mice was confirmed by Masson's trichrome stain (Fig. 3B). Quantification of adipocyte sizes demonstrated the aberrant adipocyte size distribution in IKK β -deficient mice (Fig. 3C). These histopathological phenotypes suggest that visWAT of IKK $\beta^{\Delta Ad}$ mice failed to execute the appropriate adipose remodeling program in response to HFD stress.

Because adipose tissue needs to expand and contract properly in response to nutrient status, we then tested whether adipocyte IKK β plays a role in adipose tissue contraction capacity on withdrawal from the HFD. HFD-fed IKK $\beta^{\Delta Ad}$ and control littermates were switched to the ND for 6 weeks. The control and IKK $\beta^{\Delta Ad}$ mice both had similar decreased body weight in response to the HFD/ND change (Fig. 4A). Although subWAT was similar in IKK $\beta^{\Delta Ad}$ and IKK $\beta^{F/F}$ mice, IKK $\beta^{\Delta Ad}$ mice had much smaller and discolored epiWAT (Fig. 4B and C). Histological examination revealed a normal subWAT phenotype but severe degeneration of architecture and integrity in epiWAT of IKK $\beta^{\Delta Ad}$, indicative of defective adipose remodeling (Fig. 4D). Immunofluorescence staining confirmed the loss of perilipin, an adipocyte marker, for many cells in epiWAT of IKK $\beta^{\Delta Ad}$ mice, suggesting increased adipocyte death (Fig. 4E). Further, unusual granuloma-like structures were also abundant in epiWAT of HFD/ND-fed IKK $\beta^{\Delta Ad}$ mice (Fig. 4C and D), which were also observed in other mouse models of lipodystrophy (33). Taken together, the results from HFD-fed and HFD/ND-fed mice demonstrate the requirement of IKK β in adaptive adipose tissue remodeling in response to dietary changes.

IKK β Protects Adipocytes From HFD-Induced Cell Death

Obesity is associated with increased adipocyte death (23,34,35), and IKK β is a well-established cell survival factor (4,5). Indeed, further analysis of WAT with TUNEL staining showed increased number of apoptotic (TUNEL-positive) cells in epiWAT of HFD-fed IKK $\beta^{\Delta Ad}$ mice (Fig. 5A). Immunostaining of perilipin demonstrated that IKK $\beta^{\Delta Ad}$ mice had many adipocytes that were devoid of perilipin labeling (Fig. 5B). Western blot analysis showed that HF feeding activated IKK β /NF- κ B signaling in epiWAT of IKK $\beta^{F/F}$ mice, as indicated by increased IKK β expression and phosphorylation of the IKK β substrates I κ B α and p65 (Fig. 5C). In addition, JNK was also activated by HF feeding in epiWAT of IKK $\beta^{F/F}$ mice and further enhanced in that of IKK $\beta^{\Delta Ad}$ mice (Fig. 5C). Although JNK signaling induces cell death, activation of NF- κ B has been well known to inhibit JNK activation through upregulation of its target genes (36,37). Several NF- κ B-regulated antiapoptotic proteins, including X-linked inhibitor of apoptosis (XIAP) and Bcl2, were increased in epiWAT of HFD-fed control mice but were repressed in that of IKK $\beta^{\Delta Ad}$ mice (Fig. 5C). In addition to the NF- κ B-dependent mechanism, IKK β can also inhibit cell

death in an NF- κ B-independent manner by phosphorylating BAD, a BH3-only proapoptotic protein, to prime it for inactivation (38). Using a specific antibody against phospho-BAD (serine-26) (38), we also found that deficiency of IKK β abolished HFD-elicited BAD phosphorylation without affecting total BAD protein levels (Fig. 5C). Consistently, the levels of activated caspase-3 and cleaved poly(ADP-ribose) polymerase (PARP) proteins, indicators of cell apoptosis, were markedly increased in epiWAT of HFD-fed IKK $\beta^{\Delta Ad}$ mice (Fig. 5C). Collectively, these results suggest that IKK β protects visceral adipocytes from HFD-induced cell death.

IKK β -Deficient Mice Have Increased Adipose Tissue Macrophage Infiltration and Elevated Lipolysis in Visceral Adipose Depots

Adipocyte death has been implied as one of initial events that leads to adipose tissue macrophage (ATM) infiltration (33–35,39). Indeed, ATM infiltration was substantially increased in epiWAT of IKK $\beta^{\Delta Ad}$ upon the HFD challenge (Fig. 6A). Flow cytometric analyses confirmed that the F4/80⁺CD11b⁺ macrophage population significantly increased in IKK $\beta^{\Delta Ad}$ mice (Fig. 6B). Consistently, the mRNA levels of macrophage markers and several key proinflammatory cytokines, including tumor necrosis factor- α (TNF- α), MCP-1, and IL-2, generally considered M1 macrophage markers, were significantly elevated in epiWAT of IKK $\beta^{\Delta Ad}$ mice (Fig. 6C). In addition, we also noticed the increased expression of IL-10 and Arg 1, markers for anti-inflammatory M2 macrophages, coincident with the increased adipocyte death. Interestingly, the expression levels of IL-13, another anti-inflammatory cytokine, were also significantly increased in epiWAT of IKK $\beta^{\Delta Ad}$ mice (Fig. 6C). By contrast, deficiency of IKK β did not affect the adipocyte apoptotic signaling in subWAT (Supplementary Fig. 2) or the ATM accumulation in subWAT (Fig. 6A). The expression levels of macrophage markers and most proinflammatory cytokines in subWAT were also comparable to that of IKK $\beta^{F/F}$ mice (Fig. 6C).

Increased ATM number has been associated with elevated adipose lipolysis (40), and some proinflammatory cytokines, such as TNF- α , can also stimulate basal lipolysis in adipocytes (23,41). We found that epiWAT of IKK $\beta^{\Delta Ad}$ mice also had significantly increased basal lipolysis but similar stimulated lipolysis compared with that of IKK $\beta^{F/F}$ mice (Fig. 6D). Consistently, IKK $\beta^{\Delta Ad}$ mice had elevated plasma FFA levels as well as increased hepatic cholesterol and triglyceride concentrations (Fig. 6E–G). It is well established that increased plasma FFA levels and ectopic lipid accumulation in the liver impair insulin signaling and are highly responsible for insulin resistance. Next, HFD-fed mice IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice were injected with insulin before being killed, and phosphorylation of Akt was analyzed in the liver and WAT. IKK $\beta^{\Delta Ad}$ mice had decreased phosphorylation of Akt in response to insulin in WAT and in the liver (Fig. 6H), indicative of impaired insulin signaling in these mice.

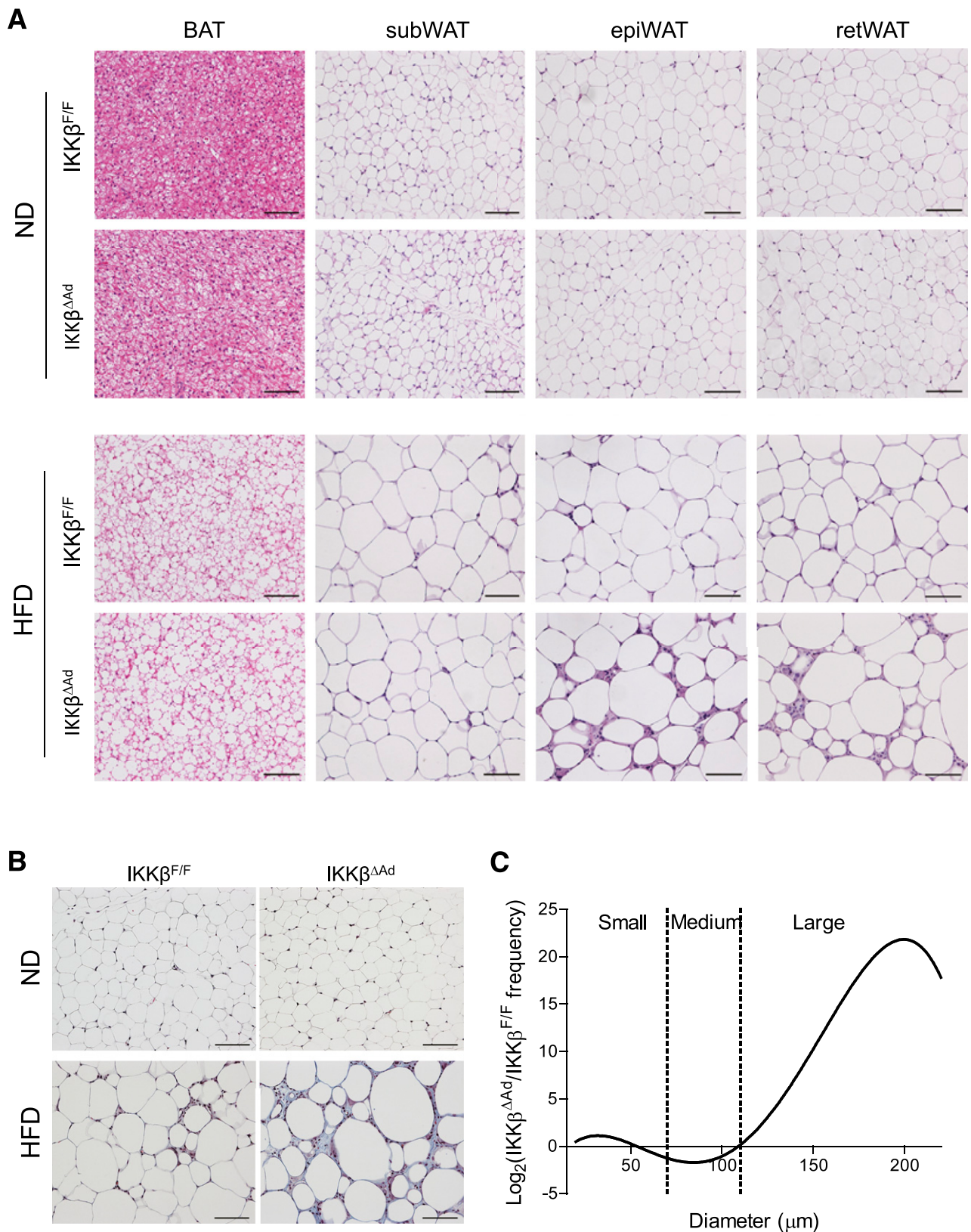


Figure 3—Deficiency of adipocyte IKK β causes impaired visceral adipose tissue expansion when challenged with an HFD. **A**: Representative hematoxylin and eosin staining of BAT, subWAT, epiWAT, and retroperitoneal (ret)WAT of ND- or HFD-fed male IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice. Scale bar, 100 μ m. **B**: Trichrome staining for collagen deposition in epiWAT from ND- or HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice. Scale bar, 100 μ m. **C**: Quantitation of adipocyte cross-sectional sizes from HFD-fed mice, expressed as the ratio of the numbers of cells from IKK $\beta^{\Delta Ad}$ and IKK $\beta^{F/F}$ mice defined as small (15–70 μ m), medium (70–110 μ m), and large (>110 μ m).

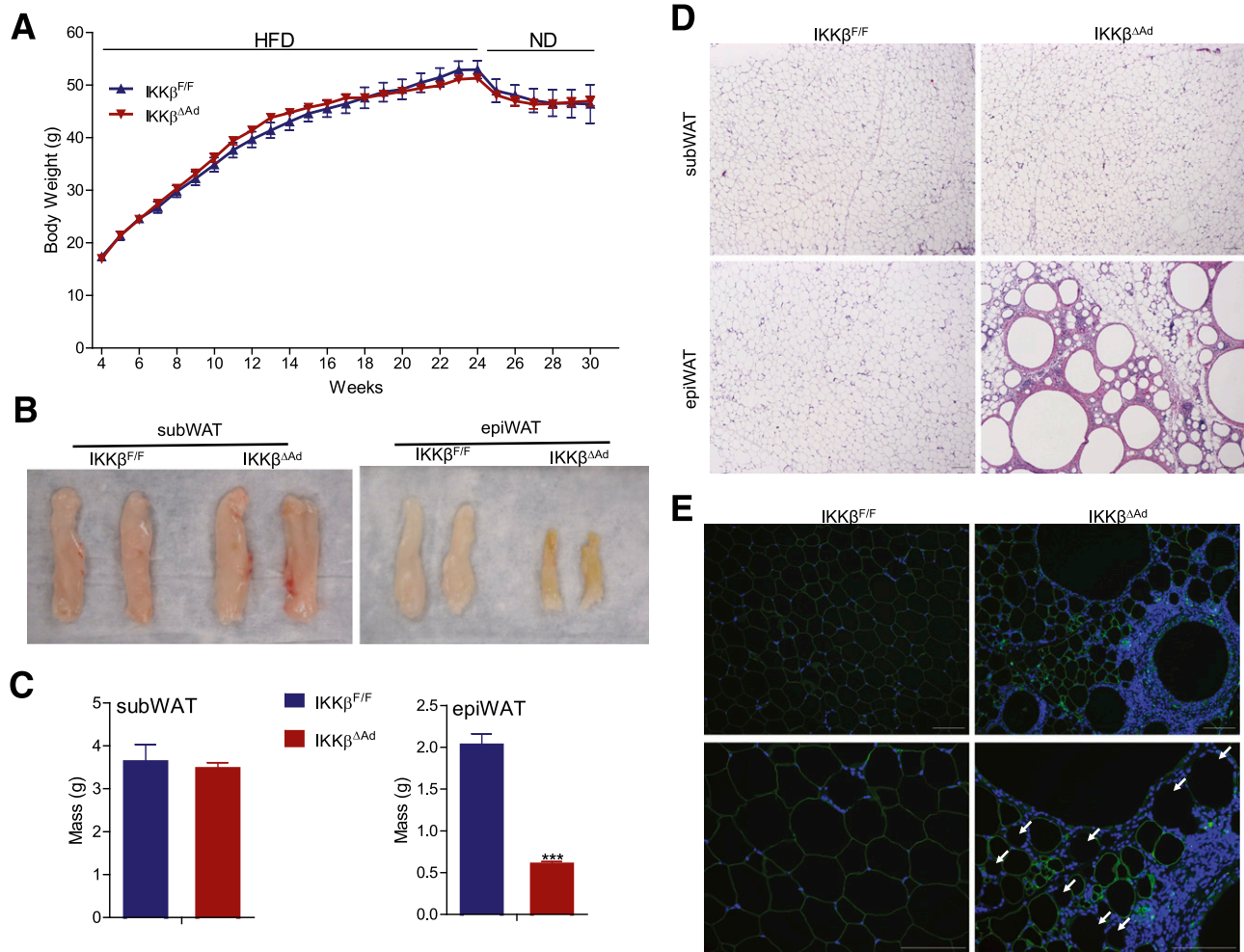


Figure 4—Loss of adipocyte IKK β results in severe defects in adipose tissue remodeling in response to dietary changes. **A**: Growth curves of HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice switched to the ND for 6 weeks ($n = 6-10$). Representative photographs (**B**), weight (**C**), and hematoxylin and eosin staining (**D**) of subWAT and epiWAT from HFD/ND-fed male IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice ($n = 3-7$). *** $P < 0.001$. **E**: Representative immunofluorescence staining for perilipin (green) of epiWAT from IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice fed HFD/ND. Bottom panels represent the magnification of areas in the top panels. The nuclei were stained with DAPI (blue), and the perilipin-negative adipocytes are indicated by arrows. Scale bar, 100 μm .

aP2-Cre-Mediated IKK β Deletion Also Results in Defects in Adipose Remodeling and Accentuated Inflammatory Responses After HF Feeding

To further confirm the role of IKK β in adipose tissue remodeling and adipocyte survival, we also used aP2-Cre transgenic mice to generate IKK $\beta^{\Delta aP2}$ mice, another adipose IKK β -deficient mouse model. As expected, the aP2-Cre line also led to effective deletion of IKK β in WAT of IKK $\beta^{\Delta aP2}$ mice (Fig. 7A). Consistent with previous reports demonstrating that the aP2 promoter is expressed by other cell types, including macrophages (42,43), IKK β expression levels were also decreased in macrophages of IKK $\beta^{\Delta aP2}$ mice (Fig. 7A). Similar to IKK $\beta^{\Delta Ad}$ mice, IKK $\beta^{\Delta aP2}$ mice displayed no significant phenotype under standard laboratory conditions and had similar body weight gain as littermate controls when challenged with the HFD (Fig. 7B). HFD-fed IKK $\beta^{\Delta aP2}$ mice also had decreased size

and weight of epiWAT but comparable BAT and subWAT compared with control mice (Fig. 7C and D). Although IKK $\beta^{\Delta aP2}$ mice had IKK β deficiency in adipocytes and macrophages, the epiWAT of IKK $\beta^{\Delta aP2}$ mice exhibited the same histopathologies as that of IKK $\beta^{\Delta Ad}$ mice, including aberrant adipose tissue expansion, increased heterogeneity in adipocyte sizes, and increased ATM accumulation (Fig. 7E and F). Therefore, these results confirmed that deficiency of IKK β in adipocytes caused the defects in adipose remodeling and increased inflammatory responses after HFD feeding.

IKK β -BAD Axis Is Required for Adipocyte Survival in Response to FFAs In Vitro

We next used cultured 3T3-L1 adipocytes as a model system to further investigate the role of IKK β in adipocyte survival in response to overnutrition. Differentiated 3T3-L1 cells were introduced with control siRNA or siRNA

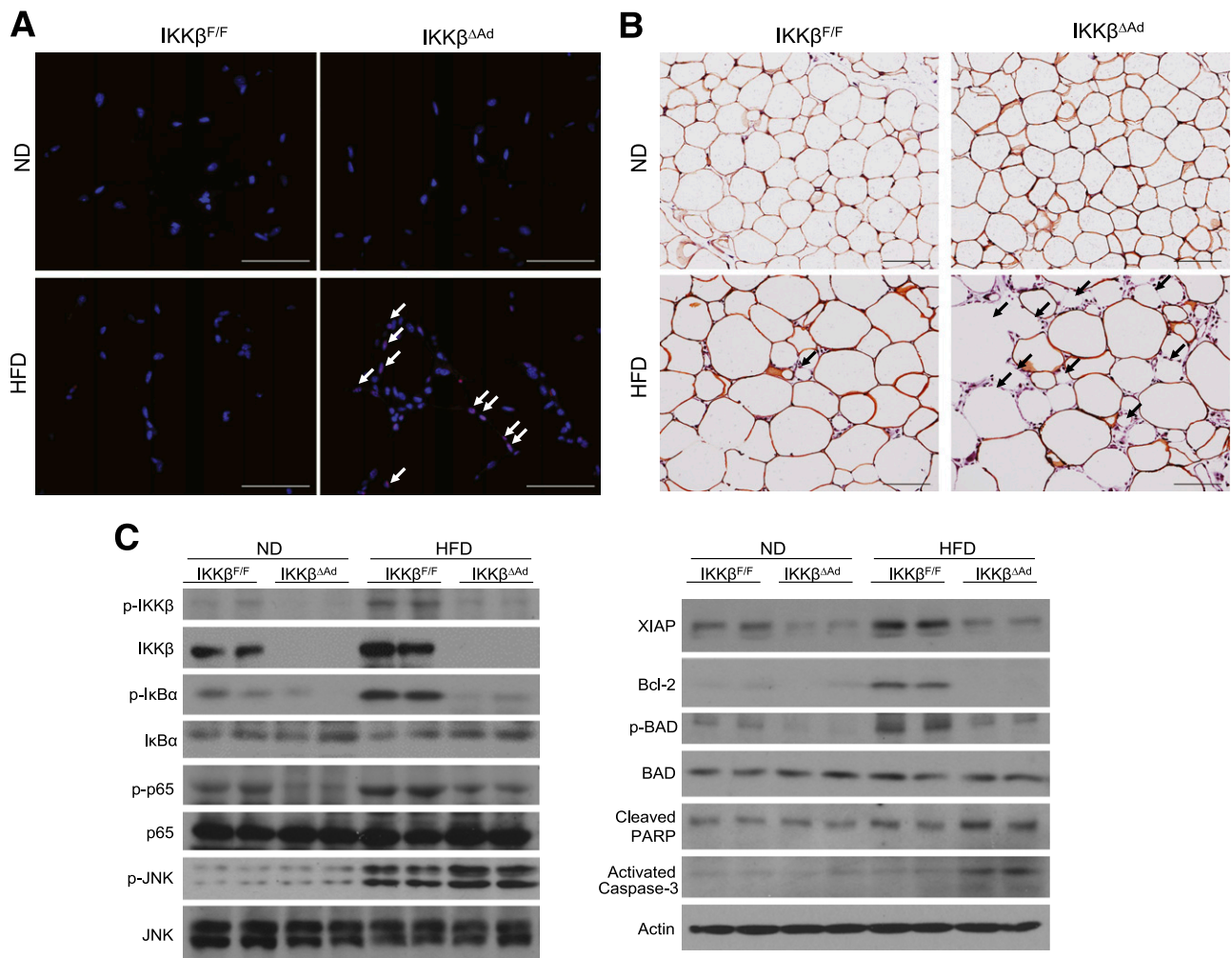


Figure 5—IKK β protects adipocytes from HFD-induced cell death. **A**: Representative TUNEL staining of epiWAT sections from ND- or HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice. The nuclei were stained with DAPI (blue), and the TUNEL-positive cells were indicated by arrows. Scale bar, 50 μ m. **B**: Representative immunohistochemistry for perilipin of epiWAT sections from ND- or HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice. The nuclei were stained with hematoxylin (blue), and the perilipin-negative adipocytes were indicated by arrows. Scale bar, 100 μ m. **C**: Western blot analysis with the indicated total and phosphorylated (p) antibodies in epiWAT from ND or HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice.

against IKK β (Fig. 8A) and then treated with a mixture of FFAs that activate IKK β and induce adipocyte inflammation (9,10,18). FFA treatment activated IKK β and increased phospho-I κ B α and phospho-p65 levels in control but not in siIKK β 3T3-L1 adipocytes (Fig. 8A). The levels of NF- κ B-regulated antiapoptotic proteins, Bcl2 and XIAP, were suppressed by IKK β knockdown, and FFA-elicited JNK activation was further enhanced in IKK β -deficient cells (Fig. 8A). FFAs also induced IKK β -mediated BAD phosphorylation in control but not in siIKK β adipocytes (Fig. 8A). As a consequence, reduction of IKK β expression increased FFA-triggered cell death as indicated by elevated levels of activated caspase-3 and cleaved PARP proteins (Fig. 8A) and increased TUNEL staining (Supplementary Fig. 3).

To determine the importance of the NF- κ B-independent mechanism in mediating adipocyte survival, we also

used siRNA to knockdown BAD in control or siIKK β 3T3-L1 cells (Fig. 8B). Knockdown of BAD did not affect control adipocytes that had intact IKK β /NF- κ B signaling but protected siIKK β 3T3-L1 cells from FFA-induced cell death (Fig. 8B). In addition to genetic approaches, we also used a highly selective IKK β inhibitor, BMS-345541 (6,44), and found that BMS-345541 treatment efficiently blocked IKK β -mediated I κ B α phosphorylation elicited by FFAs (Fig. 8C). BMS-345541-treated cells were more susceptible from FFA-induced cell death, as indicated by increased cleaved PARP and caspase-3 levels (Fig. 8C). However, knockdown of BAD protected BMS-345541-treated cells against FFA-induced cell death (Fig. 8C). Collectively, these results suggest that the newly identified IKK β -BAD axis plays a critical role in protecting adipocytes from FFA-induced cell death.

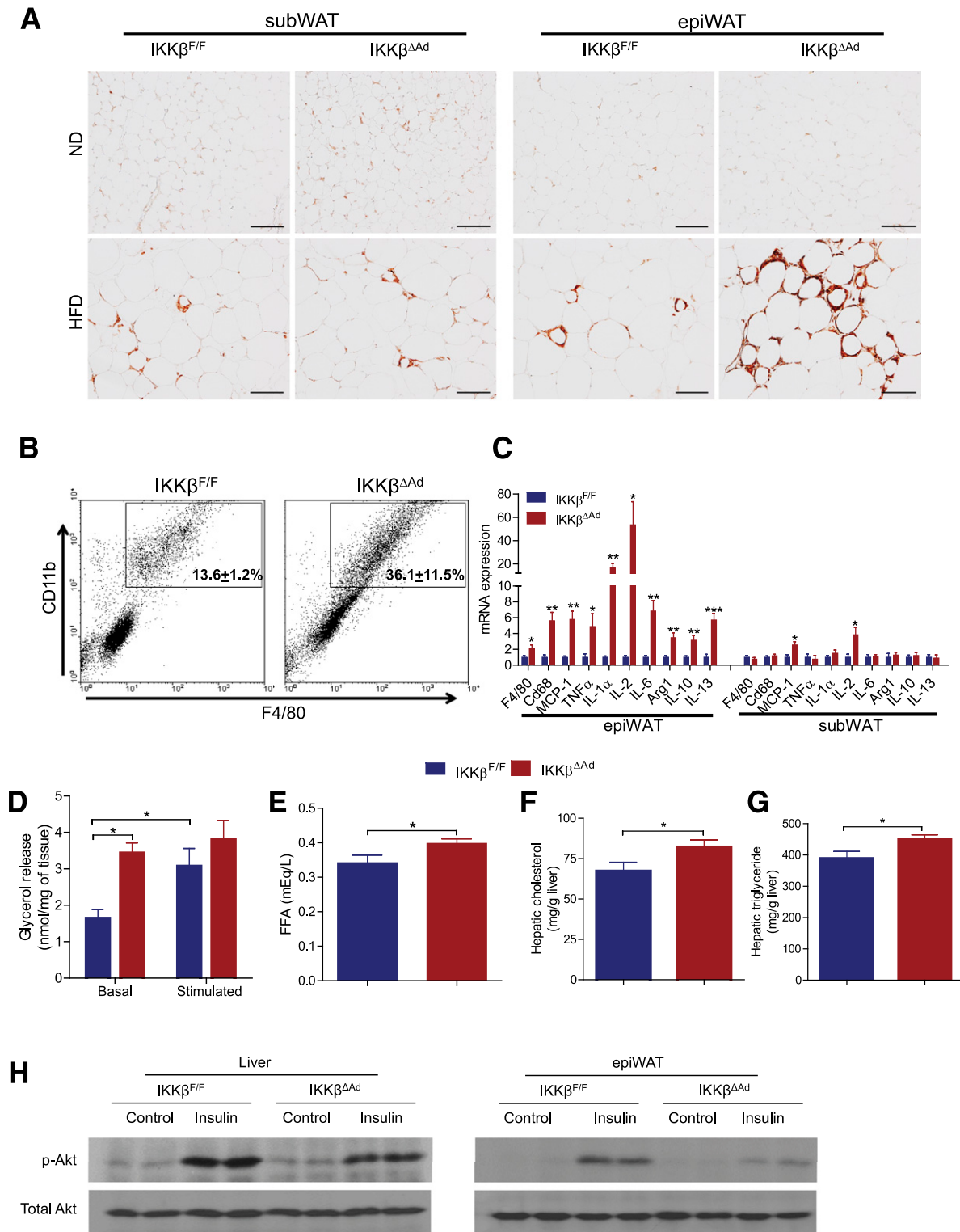


Figure 6—Deficiency of adipocyte IKK β increases macrophage infiltration and lipolysis in visceral adipose tissue and impairs insulin signaling in adipose tissue and liver. **A**: Representative immunohistochemistry for the macrophage marker F4/80 in subWAT and epiWAT from ND- or HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice. Scale bar, 100 μ m. **B**: Adipose SV cells isolated from epiWAT of HFD-fed mice were examined for expression of macrophage cell makers F4/80 and CD11b with flow cytometry. The percentages of F4/80 $^{+}$ CD11b $^{+}$ cells are as indicated in the flow profiles ($n = 3-5$). $P < 0.05$. **C**: Expression levels of macrophage markers and inflammatory genes in epiWAT and subWAT of HFD-fed mice were analyzed by quantitative PCR ($n = 6$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **D**: Glycerol release in epiWAT from HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice under basal conditions or stimulated with 1 μ mol/L isoproterenol ($n = 9-12$). * $P < 0.05$. Plasma FFA concentration (**E**) and hepatic cholesterol (**F**) and triglyceride (**G**) levels in HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice ($n = 9-13$). * $P < 0.05$. **H**: Western blot analysis of phospho(p)-Akt (ser473) and total Akt levels in liver and epiWAT of HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta Ad}$ mice injected with saline or 0.35 units/kg body weight insulin.

IKK β Protects Primary Murine and Human Adipocytes From FFA-Induced Cell Death

Lastly, we investigated the effect of IKK β -deficiency on primary murine and human adipocyte survival. Adipose SV cells were isolated from IKK $\beta^{E/F}$ mice, differentiated into mature adipocytes, and then infected with lentivirus expressing Cre to acutely delete IKK β (Fig. 8D). Deficiency of IKK β enhanced FFA-elicited JNK activation (Fig. 8D) but abolished FFA-induced BAD phosphorylation (Fig. 8E). Accordingly, FFA treatment increased activated caspase-3 and cleaved PARP levels in IKK β -deficient cells but not in control cells (Fig. 8E).

ADHASCs were isolated from healthy subjects (30,31). ADHASCs can efficiently differentiate into mature adipocytes *in vitro* (Supplementary Fig. 4). Differentiated mature adipocytes were treated with FFAs in the presence or absence of BMS-345541, the IKK β inhibitor. Consistently, BMS-345541 inhibited FFA-elicited NF- κ B activation and IKK β -mediated BAD phosphorylation in human adipocytes (Fig. 8F). Control human adipocytes were protected from FFA-induced cell death but BMS-345541-treated cells had elevated cleaved PARP and caspase-3 levels upon FFA treatment (Fig. 8F). Taken together, these results demonstrated an essential role of IKK β in protecting both murine and human adipocytes from FFA-induced cell death.

DISCUSSION

In response to stresses linked to inflammation, cells are often forced to choose to die or survive. For example, in the immune system, TNF- α can signal both cell death and survival by activating JNK and IKK β signaling, respectively (36,45). Previous studies have demonstrated that activation of NF- κ B promotes cell survival by inducing expression of genes encoding inhibitor of JNK signaling (36,37). In addition to NF- κ B-dependent functions, IKK β can also inhibit TNF- α -induced apoptosis independently of NF- κ B activation by phosphorylating and inactivating BAD, a key proapoptotic protein (38). Similar to TNF- α , FFAs can activate both JNK and IKK β signaling in adipocytes (9,10). Although much attention has been focused on the role of IKK β in linking inflammation to insulin resistance, the function of IKK β in the regulation of adipocyte survival has not been thoroughly investigated. In the current study, we revealed an essential role of IKK β in promoting adipocyte survival in diet-induced obesity. Under standard laboratory conditions, deficiency of adipocyte IKK β did not affect adipose tissue development or metabolic function. However, when the mice were challenged with the HFD, IKK β and JNK signaling were both activated in control mice, and ablation of IKK β resulted in enhanced JNK activation and increased adipocyte death in IKK $\beta^{\Delta Ad}$ mice. We also demonstrated that IKK β can phosphorylate BAD in adipocytes and that knockdown of BAD protected IKK β -deficient adipocytes from FFA-induced cell death, suggesting the importance of the IKK β -BAD axis in mediating adipocyte survival. IKK β is

therefore a key adipocyte survival factor in obesity and can protect adipocytes from HFD- or FFA-elicited cell death through activation of the NF- κ B-mediated survival factors and inactivation of the proapoptotic protein BAD (Fig. 8G).

Several groups have previously studied the role of adipose IKK β /NF- κ B signaling by overexpressing p65 or a constitutively active form of IKK β in adipose tissue (19,20). Surprisingly, these transgenic mice had increased adipose tissue inflammation but were resistant to diet-induced obesity and insulin resistance, which may be partially explained by the increased energy expenditure (19,20). However, we found that deletion of endogenous IKK β in adipocytes did not affect energy expenditure or diet-induced weight gain. While our work was in its final stages, Kwon et al. (21) also reported that deficiency of adipocyte IKK β led to visceral adipose tissue inflammation without affecting body weight or energy expenditure under HF conditions. It is plausible that endogenous IKK β in adipocytes does not affect energy metabolism at basal levels but that robustly constitutive activation of IKK β /NF- κ B signaling may cause increased energy expenditure. Future studies are required to determine the detailed mechanisms through which IKK β /NF- κ B signaling regulates energy homeostasis under basal and HF feeding conditions. Although the histopathologies in visWAT of our IKK $\beta^{\Delta Ad}$ mice were consistent with those Kwon et al. reported (21), they attributed the increased inflammation phenotype to the decreased IL-13 expression of IKK β -deficient mice (21). NF- κ B signaling also plays an important role in inflammation resolution by regulating anti-inflammatory cytokines such as IL-10 and IL-13. However, IL-13 is one of many cytokines regulated by NF- κ B signaling, and we found that the expression levels of both proinflammatory and anti-inflammatory cytokines, including MCP-1, TNF- α , IL-10, and IL-13, were all affected by IKK β deficiency in the epiWAT of HFD-fed mice. Further, both IL-10 and IL-13 gene expression was significantly increased rather than decreased in the epiWAT of IKK $\beta^{\Delta Ad}$ mice. Our mechanistic studies then suggest that the histopathologies in IKK $\beta^{\Delta Ad}$ mice are likely due to the loss of protection against cell death in response to HFD stress. Thus, adipose IKK β has other important functions beyond the scope of inflammation, and more studies are required to further investigate its role in obesity and insulin resistance.

The mechanisms triggering ATM recruitment remain incompletely understood (23,46,47), and adipocyte death has been proposed as a key mechanism that leads to macrophage infiltration into adipose tissue and metabolic disorders (33–35,39). For example, Alkhoury et al. (39) demonstrated that adipocyte death was a link between obesity and insulin resistance. They also showed that genetic inactivation of a proapoptotic molecule can reduce adipocyte death, prevent ATM infiltration, and protect mice against insulin resistance. Consistent with their

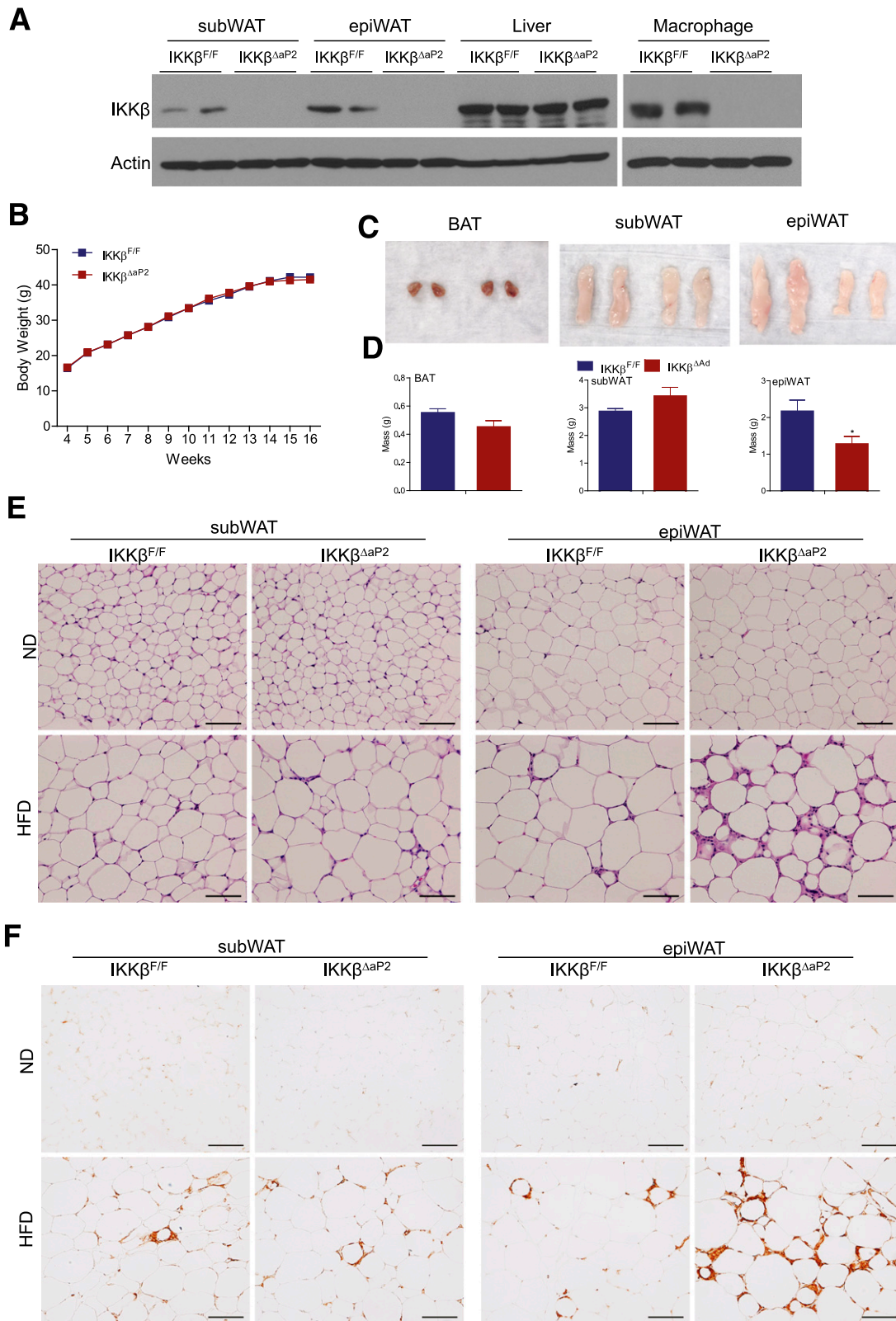


Figure 7—aP2-Cre-mediated IKK β deletion results in similar defects in adipose remodeling and accentuated inflammatory responses after HF feeding. **A**: Western blot analysis of IKK β protein levels in subWAT, epiWAT, liver, and macrophages of IKK $\beta^{F/F}$ and IKK $\beta^{\Delta aP2}$ mice. **B**: Growth curves of HFD-fed male IKK $\beta^{F/F}$ and IKK $\beta^{\Delta aP2}$ mice ($n = 24$ – 38). Representative photographs (**C**) and weight of fat pads (**D**) of HFD-fed male IKK $\beta^{F/F}$ and IKK $\beta^{\Delta aP2}$ mice ($n = 5$ – 7). * $P < 0.05$. **E**: Representative hematoxylin and eosin staining of subWAT and epiWAT of ND- or HFD-fed male IKK $\beta^{F/F}$ and IKK $\beta^{\Delta aP2}$ mice. Scale bar, 100 μ m. **F**: Representative immunohistochemistry for the macrophage marker F4/80 in subWAT and epiWAT from ND- or HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta aP2}$ mice. Scale bar, 100 μ m.

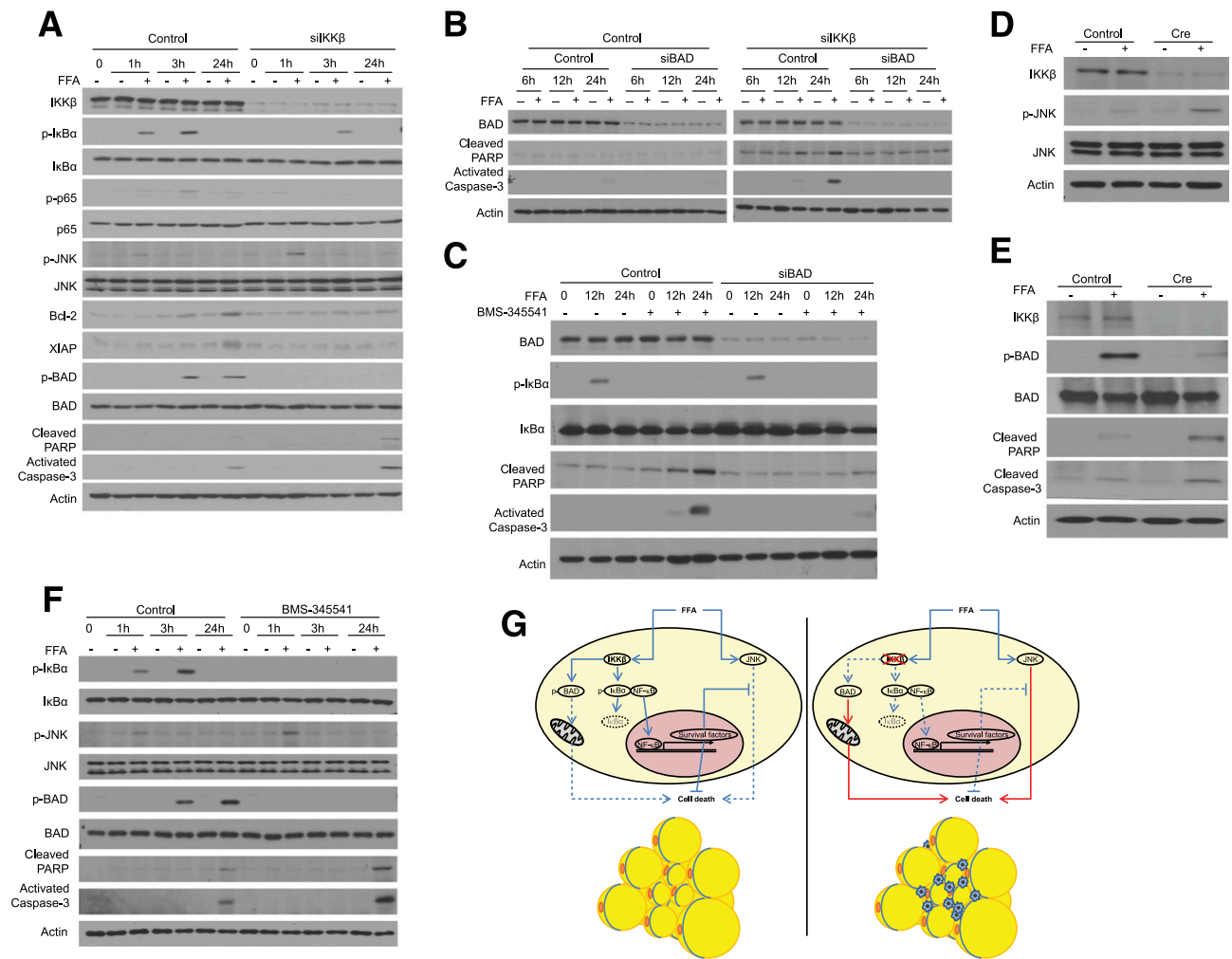


Figure 8—IKKβ protects murine and human adipocytes from FFA-induced cell death. **A**: 3T3-L1 cells were differentiated into adipocytes and then introduced with control siRNA or siRNA against IKKβ. Western blot analysis with the indicated total and phosphorylated (p) antibodies in control or siIKKβ 3T3-L1 adipocytes treated with vehicle or FFAs for the indicated times. **B**: 3T3-L1 adipocytes were introduced with control siRNA or siRNA against IKKβ and/or BAD. Western blot analysis with the indicated antibodies in control, siBAD, siIKKβ, or siIKKβ/siBAD 3T3-L1 adipocytes treated with vehicle control or FFAs. **C**: 3T3-L1 adipocytes were introduced with control siRNA or siRNA against BAD. Western blot analysis with the indicated antibodies in control or siBAD 3T3-L1 adipocytes treated with vehicle control or FFAs in the absence or presence of 5 μmol/L IKKβ inhibitor, BMS-345541. Adipose SV cells isolated from IKKβ^{F/F} mice were differentiated into adipocytes and then infected with control lentivirus or lentivirus expressing Cre. Western blot analysis with the indicated antibodies in cells treated with vehicle control or FFAs for 3 h (**D**) or 24 h (**E**). **F**: ADHASCs were differentiated into adipocytes and then treated with FFAs in the presence or absence of 5 μmol/L IKKβ inhibitor, BMS-345541. Western blot analysis with the indicated antibodies in cells treated with control, FFAs and/or BMS-345541. **G**: Schematic representation of the mechanisms through which IKKβ protects adipocytes from cell death in diet-induced obesity. IKKβ inhibits FFA-induced adipocyte death through NF-κB-dependent upregulation of antiapoptotic proteins and NF-κB-independent inactivation of proapoptotic BAD protein.

observations, we found that the increased adipocyte death in visWAT of IKKβ^{ΔAd} mice was associated with massive macrophage infiltration and elevated levels of proinflammatory cytokines. Increased ATM number has also been associated with elevated adipose lipolysis and circulating FFA levels (40). Indeed, deficiency of IKKβ increased adipose lipolysis and plasma FFA levels, leading to ectopic hepatic lipid accumulation and impaired insulin signaling. That ATM accumulation in IKKβ^{ΔAd} mice is driven by increased lipolysis is also possible, and whether IKKβ can directly regulate adipocyte lipolysis would be an interesting study in the future.

It is intriguing that deficiency of adipocyte IKKβ promoted HFD-induced adipocyte death and ATM infiltration in visceral but not in subcutaneous fat. The regional differences between different fat depots have been well-recognized (48) and there is a major ontogenetic difference between visceral and subcutaneous fat because they have different developmental origins (49). HF feeding also rapidly and specifically activates adipogenesis in visceral but not in subcutaneous adipose tissue in mice (50). Consistently, adipocyte death rate was much higher in visWAT after long-term HF feeding (34). It is plausible that visceral adipocytes are more

susceptible to HFD-induced cell death and that ablation of IKK β therefore has detectable deleterious effects on visceral adipocytes compared with subcutaneous adipocytes. Future studies will be required to determine the detailed mechanisms that account for the different effects of IKK β ablation on visceral versus subcutaneous fat.

In summary, we have revealed a pivotal role of IKK β in adipocyte survival and adipose remodeling in obesity. IKK β promotes adipocyte survival through NF- κ B-dependent and NF- κ B-independent mechanisms. Deficiency of IKK β in adipocytes causes defects in adaptive adipose remodeling in response to dietary change, leading to an exaggerated diabetic phenotype coupled to partial lipodystrophy in visceral adipose tissue. IKK β is therefore a key adipocyte survival factor and is required for adaptive adipose remodeling in obesity.

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References

- Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 2006;444:840–846
- Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. *Nature* 2006;444:875–880
- Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. *Nat Rev Immunol* 2008;8:923–934
- Hayden MS, Ghosh S. Shared principles in NF- κ B signaling. *Cell* 2008;132:344–362
- Karin M. Nuclear factor- κ B in cancer development and progression. *Nature* 2006;441:431–436
- Sui Y, Park SH, Xu J, et al. IKK β links vascular inflammation to obesity and atherosclerosis. *J Exp Med* 2014;211:869–886
- Baker RG, Hayden MS, Ghosh S. NF- κ B, inflammation, and metabolic disease. *Cell Metab* 2011;13:11–22
- Solinas G, Karin M. JNK1 and IKK β : molecular links between obesity and metabolic dysfunction. *FASEB J* 2010;24:2596–2611
- Jiao P, Ma J, Feng B, et al. FFA-induced adipocyte inflammation and insulin resistance: involvement of ER stress and IKK β pathways. *Obesity (Silver Spring)* 2011;19:483–491
- Nguyen MT, Satoh H, Favellyukis S, et al. JNK and tumor necrosis factor- α mediate free fatty acid-induced insulin resistance in 3T3-L1 adipocytes. *J Biol Chem* 2005;280:35361–35371
- Yuan M, Konstantopoulos N, Lee J, et al. Reversal of obesity- and diet-induced insulin resistance with salicylates or targeted disruption of Ikk β . *Science* 2001;293:1673–1677
- Arkan MC, Hevener AL, Greten FR, et al. IKK- β links inflammation to obesity-induced insulin resistance. *Nat Med* 2005;11:191–198
- Cai D, Yuan M, Frantz DF, et al. Local and systemic insulin resistance resulting from hepatic activation of IKK- β and NF- κ B. *Nat Med* 2005;11:183–190
- Zhang X, Zhang G, Zhang H, Karin M, Bai H, Cai D. Hypothalamic IKK β /NF- κ B and ER stress link overnutrition to energy imbalance and obesity. *Cell* 2008;135:61–73
- Chiang SH, Bazuine M, Lumeng CN, et al. The protein kinase IKK β regulates energy balance in obese mice. *Cell* 2009;138:961–975
- Kim JK, Kim YJ, Fillmore JJ, et al. Prevention of fat-induced insulin resistance by salicylate. *J Clin Invest* 2001;108:437–446
- Hirosumi J, Tuncman G, Chang L, et al. A central role for JNK in obesity and insulin resistance. *Nature* 2002;420:333–336
- Jiao P, Chen Q, Shah S, et al. Obesity-related upregulation of monocyte chemotactic factors in adipocytes: involvement of nuclear factor- κ B and c-Jun NH2-terminal kinase pathways. *Diabetes* 2009;58:104–115
- Tang T, Zhang J, Yin J, et al. Uncoupling of inflammation and insulin resistance by NF- κ B in transgenic mice through elevated energy expenditure. *J Biol Chem* 2010;285:4637–4644
- Jiao P, Feng B, Ma J, et al. Constitutive activation of IKK β in adipose tissue prevents diet-induced obesity in mice. *Endocrinology* 2012;153:154–165
- Kwon H, Laurent S, Tang Y, Zong H, Vemulapalli P, Pessin JE. Adipocyte-specific IKK β signaling suppresses adipose tissue inflammation through an IL-13-dependent paracrine feedback pathway. *Cell Reports* 2014;9:1574–1583
- Sun K, Kusminski CM, Scherer PE. Adipose tissue remodeling and obesity. *J Clin Invest* 2011;121:2094–2101
- Rosen ED, Spiegelman BM. What we talk about when we talk about fat. *Cell* 2014;156:20–44
- Li ZW, Omori SA, Labuda T, Karin M, Rickert RC. IKK β is required for peripheral B cell survival and proliferation. *J Immunol* 2003;170:4630–4637
- Eguchi J, Wang X, Yu S, et al. Transcriptional control of adipose lipid handling by IRF4. *Cell Metab* 2011;13:249–259
- Jenkinson M, Beckmann CF, Behrens TE, Woolrich MW, Smith SM. *Fsl Neuroimage* 2012;62:782–790
- Hu HH, Nayak KS, Goran MI. Assessment of abdominal adipose tissue and organ fat content by magnetic resonance imaging. *Obesity Rev* 2011;12:e504–e515
- Park SH, Sui Y, Gizard F, et al. Myeloid-specific I κ B kinase β deficiency decreases atherosclerosis in low-density lipoprotein receptor-deficient mice. *Arterioscler Thromb Vasc Biol* 2012;32:2869–2876
- Sui Y, Helsley RN, Park SH, Song X, Liu Z, Zhou C. Intestinal pregnane X receptor links xenobiotic exposure and hypercholesterolemia. *Mol Endocrinol* 2015;29:765–776
- Rasouli N, Yao-Borengasser A, Varma V, et al. Association of scavenger receptors in adipose tissue with insulin resistance in nondiabetic humans. *Arterioscler Thromb Vasc Biol* 2009;29:1328–1335
- Finlin BS, Zhu B, Starnes CP, McGehee RE Jr, Peterson CA, Kern PA. Regulation of thrombospondin-1 expression in alternatively activated macrophages and adipocytes: role of cellular cross talk and omega-3 fatty acids. *J Nutr Biochem* 2013;24:1571–1579

32. Lee KY, Russell SJ, Ussar S, et al. Lessons on conditional gene targeting in mouse adipose tissue. *Diabetes* 2013;62:864–874
33. Pajvani UB, Trujillo ME, Combs TP, et al. Fat apoptosis through targeted activation of caspase 8: a new mouse model of inducible and reversible lipodystrophy. *Nat Med* 2005;11:797–803
34. Strissel KJ, Stancheva Z, Miyoshi H, et al. Adipocyte death, adipose tissue remodeling, and obesity complications. *Diabetes* 2007;56:2910–2918
35. Cinti S, Mitchell G, Barbatelli G, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 2005;46:2347–2355
36. Tang G, Minemoto Y, Dibling B, et al. Inhibition of JNK activation through NF- κ B target genes. *Nature* 2001;414:313–317
37. De Smaele E, Zazzeroni F, Papa S, et al. Induction of gadd45beta by NF- κ B downregulates pro-apoptotic JNK signalling. *Nature* 2001;414:308–313
38. Yan J, Xiang J, Lin Y, et al. Inactivation of BAD by IKK inhibits TNF α -induced apoptosis independently of NF- κ B activation. *Cell* 2013;152:304–315
39. Alkhoury N, Gornicka A, Berk MP, et al. Adipocyte apoptosis, a link between obesity, insulin resistance, and hepatic steatosis. *J Biol Chem* 2010;285:3428–3438
40. Kosteli A, Sugaru E, Haemmerle G, et al. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. *J Clin Invest* 2010;120:3466–3479
41. Yang X, Zhang X, Heckmann BL, Lu X, Liu J. Relative contribution of adipose triglyceride lipase and hormone-sensitive lipase to tumor necrosis factor- α (TNF- α)-induced lipolysis in adipocytes. *J Biol Chem* 2011;286:40477–40485
42. Lee YH, Petkova AP, Granneman JG. Identification of an adipogenic niche for adipose tissue remodeling and restoration. *Cell Metab* 2013;18:355–367
43. Kang S, Kong X, Rosen ED. Adipocyte-specific transgenic and knockout models. *Methods Enzymol* 2014;537:1–16
44. Burke JR, Pattoli MA, Gregor KR, et al. BMS-345541 is a highly selective inhibitor of I kappa B kinase that binds at an allosteric site of the enzyme and blocks NF- κ B-dependent transcription in mice. *J Biol Chem* 2003;278:1450–1456
45. Kyriakis JM. Life-or-death decisions. *Nature* 2001;414:265–266
46. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;112:1796–1808
47. Xu H, Barnes GT, Yang Q, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003;112:1821–1830
48. Tchkonina T, Thomou T, Zhu Y, et al. Mechanisms and metabolic implications of regional differences among fat depots. *Cell Metab* 2013;17:644–656
49. Chau YY, Bandiera R, Serrels A, et al. Visceral and subcutaneous fat have different origins and evidence supports a mesothelial source. *Nat Cell Biol* 2014;16:367–375
50. Jeffery E, Church CD, Holtrup B, Colman L, Rodeheffer MS. Rapid depot-specific activation of adipocyte precursor cells at the onset of obesity. *Nat Cell Biol* 2015;17:376–385