EXTENDED EXPERIMENTAL PROCEDURES

Reagents

Inhibitors of palmitate metabolism and second messenger signaling were obtained from Sigma (St. Louis, MO) and included myriocin (Cat. No. M1177), cycloserine (Cat. No. C6880), fumonisin B1 (Cat. No. F1147), and bromoenol lactone (Cat. No. B1552). Bromoenol lactone (BEL) is an inhibitor of phosphatidic acid phosphohydrolase (PAP) (Balsinde and Dennis, 1996). Blocking antibody for human TLR4 (Cat. No. 312808) was from BioLegend (San Diego, CA) and human IL-1R antagonist (Cat. No. CRI134A) was purchased from Cell Sciences (Canton, MA). Calphostin C (Cat. No. 208725), an inhibitor of DAG-sensitive PKC isoforms (Kobayashi et al., 1989), was from EMD Chemicals (Gibbstown, NJ). Antibodies for Western analysis included α-pAkt (Ser473, Cat. No. 9271; Cell Signaling Technology), α-Bcl10 (Cat. No. sc-5611; Santa Cruz Biotechnology), α-pIκB-α (Ser32/36, Cat. No. 9246; Cell Signaling Technology), α-GAPDH (Cat. No. sc-32233; Santa Cruz), α-RelA (Cat. No. sc-8008; Santa Cruz), α-pRelA (Ser536, Cat. No. 3031; Cell Signaling Technology), α-JNK (Cat. No. 9252; Cell Signaling Technology), α-pJNK (Cat. No. 4668; Cell Signaling Technology), α-PKCζ (Cat. No. 9372; Cell Signaling Technology), α-PKCδ (Cat. No. 2058; Cell Signaling Technology), α-IKKβ (Cat. No. sc-7329; Santa Cruz) and α-HDAC1 (Cat. No. sc-81598; Santa Cruz). IKK-2 Inhibitor VI, a potent and selective inhibitor of IKKβ (Baxter et al., 2004), was from EMDBiosciences/Calbiochem. TPA was from Sigma (Cat. No. P8139).

RNA Interference

HepG2 cells were reverse transfected with 10nM human Bcl10 ON-TARGETplus SMARTpool siRNA or non-targeting pool (Dharmacon) using Lipofectamine RNAiMAX (Invitrogen). ON-TARGETplus SMARTpools for CARMA3, IKKβ, and MALT1 were used between 20-40 nM. For Figure S3A, cells were transfected with 50nM of a single human Bcl10 siRNA (Invitrogen;

Stealth™), or scrambled control siRNA. Rat H4IIE cells were reverse transfected with 20nM rat Bcl10 ON-TARGETplus SMARTpool siRNA or non-targeting pool. Cells were allowed to recover for 72 hours prior to treatments.

Cell Culture and Western Analysis

Cultured cells were treated with either 500 µM palmitate/BSA, 1-10 ng/ml IL-1β (R&D Systems; Cat. No. 201-LB), 1-2 µg/ml LPS (Sigma; Cat. No. L5293), 1 ng/ml TNFα (Sigma; Cat. No. T6674), or 1 µM Ang II (Sigma; Cat. No. A9525) under serum free conditions. In experiments designed to test palmitate-dependent inhibition of insulin signaling, cells were serum starved overnight prior to the addition of 500 µM palmitate/BSA and 100 nM insulin (Invitrogen; Cat. No. 12585-014), so as to reduce basal levels of pAkt. Nuclear extracts were prepared with the NE-PER kit (Thermo). Immunoblotting was performed as described (McAllister-Lucas et al., 2007).

Bioinformatics

Data on TLR4 expression were obtained from BioGPS and consist of Affymetrix gene expression profiles obtained from the MOE430 Gene Atlas Data set (Lattin et al., 2008). Similar patterns were observed, but are not shown, when querying the human U133A Data set (Su et al., 2004).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Palmitate, but not LPS, activates NF-kB in hepatocytes, Related to Figure 1.

(A) Dose response for palmitate-dependent NF-κB activation in HepG2 cells. HepG2 cells were treated for 3 hours with increasing doses of palmitate/BSA (FFA), and NF-κB activation was assessed by Western blotting for p-IκBα. Activation consistently occurs at levels of approximately 250 μM or more.

(B) TLR4 expression across tissues and cells. TLR4 expression data were culled from publicly available databases, accessed through BioGPS (http://biogps.gnf.org). Data are from the murine Affymetrix MOE430 Gene Atlas (Lattin et al. 2008). Similar patterns were observed with the human Affymetrix U133A array (Su et al. 2004).

(C) Lack of NF-κB response in HepG2 cells following treatment with the classic TLR4 agonist, LPS. Cells were treated with up to 2 μg/ml LPS for the indicated times, with IL-1β treatment serving as a control. The Western blot was overexposed in an attempt to uncover any potential LPS response.

Figure S2. Ceramide production is not required for palmitate-dependent NF-κB activation in hepatocytes, Related to Figure 2.

HepG2 cells were treated for 3 hours with 500 μM palmitate in the presence or absence of 10 μM myriocin, 1 mM cycloserine, or 50 μM Fumonisin B1. None of these inhibitors, which target distinct enzymatic steps in the pathway of ceramide synthesis, had any impact on the NF-κB response, as measured by IκBα phosphorylation.

Figure S3. Bcl10 is required for FFA-dependent NF-κB activation, but not JNK activation, in hepatocytes, Related to Figure 3.

(A) Bcl10 knockdown abrogates palmitate-dependent NF-κB activation. HepG2 cells were depleted of Bcl10 using a single siRNA (STEALTH™; Invitrogen) and assayed for canonical NF-κB activation up to 4 hours following treatment with 500 µM palmitate (FFA). The experimental approach used was similar to that employed in Figures 3A and S3B, except in those cases knockdown was accomplished with an independently designed and produced siRNA pool (ON-TARGETplus SMARTpool™; Thermo/Dharmacon).

(B) HepG2 cells were transfected with control or Bcl10 siRNAs and subsequently treated with 500 µM palmitate for the indicate times. Phosphorylation of JNK1/2 was used as a measure of JNK activation. While palmitate treatment induced JNK, Bcl10 knockdown had a minimal affect on the response. In contrast, IκB phosphorylation was almost entirely blocked by Bcl10 knockdown.

(C) Wild-type (WT) mice were placed on a 60% high fat diet (HFD) for up to 5 days. Mice were sacrificed on each day of the time course, and evaluated for hepatic NF-κB activation using phosphorylation of the RelA subunit of NF-κB as a marker of sustained activation. Maximal NF-κB activation occurred following approximately 3 days of high fat feeding.

(D) WT and *Bcl10*$^{-/-}$ mice were sacrificed following 3 days on either normal diet (ND) or HFD, and liver samples were analyzed for JNK activation. Phosphorylation of JNK was highly variable and did not show a clear relationship to either diet or Bcl10 status.

Figure S4. Role for Bcl10 in the cross-inhibition of insulin action, Related to Figure 4.

(A) Dose response for palmitate-dependent inhibition of insulin signaling in HepG2 cells. HepG2 cells were pre-treated for 8 hours with increasing doses of palmitate/BSA (FFA), and insulin-dependent Akt activation was assessed by Western blotting for p-Akt. Inhibition of

insulin signaling was notable at palmitate doses above 250 µM, correlating closely with the dose required for palmitate-dependent NF-κB activation.

(B) Lack of an effect of ceramide synthesis inhibitors on palmitate/BSA (FFA) -dependent inhibition of insulin signaling in HepG2 cells. Cells were pretreated with FFA for 8 hours, with or without inhibitors (10 µM myriocin, 1 mM cycloserine, or 50 µM Fumonisin B1). Insulin was then added for 5 minutes and Akt activation was assessed as in (A).

(C) Weight gain for mice on HFD. Weight gain, expressed as percent gain from baseline, following either one week on a 60% HFD or six weeks on a 45% HFD. No statistical difference was seen in weight gain between the WT and *Bcl10*$^{-/-}$ strains.

(D) Model for FFA-dependent insulin resistance in liver. We propose a model for hepatic insulin resistance during high fat feeding/obesity that takes into account both (1) acute effects of excess fat as a pathologic dietary nutrient and (2) chronic effects that involve the participation of inflammatory cells, particularly macrophages. In the acute post-prandial state, excessive FFAs delivered to hepatocytes through the portal circulation undergo metabolism in hepatocytes to produce DAG, activating conventional/novel PKC isoforms. These PKCs then activate NF-κB through a pathway that critically depends on Bcl10. NF-κB activation may then cause direct inhibition of insulin signaling, leading to acute insulin resistance. In addition, NF-κB activation induces expression of pro-inflammatory cytokines (eg, TNFα and IL-6) which may act on neighboring kupffer cells to enhance expression of pro-IL-1b, thereby "priming" (1) the inflammasome. In this way, continued exposure to excess FFAs potentially allows kupffer cells, and perhaps macrophages at distant sites, to "trigger" (2) the inflammasome since FFAs appear to represent "danger" signals. Paracrine signaling by IL-1β likely provides additional inhibitory signals within hepatocytes, adding to the insulin resistance.

SUPPLEMENTAL REFERENCES

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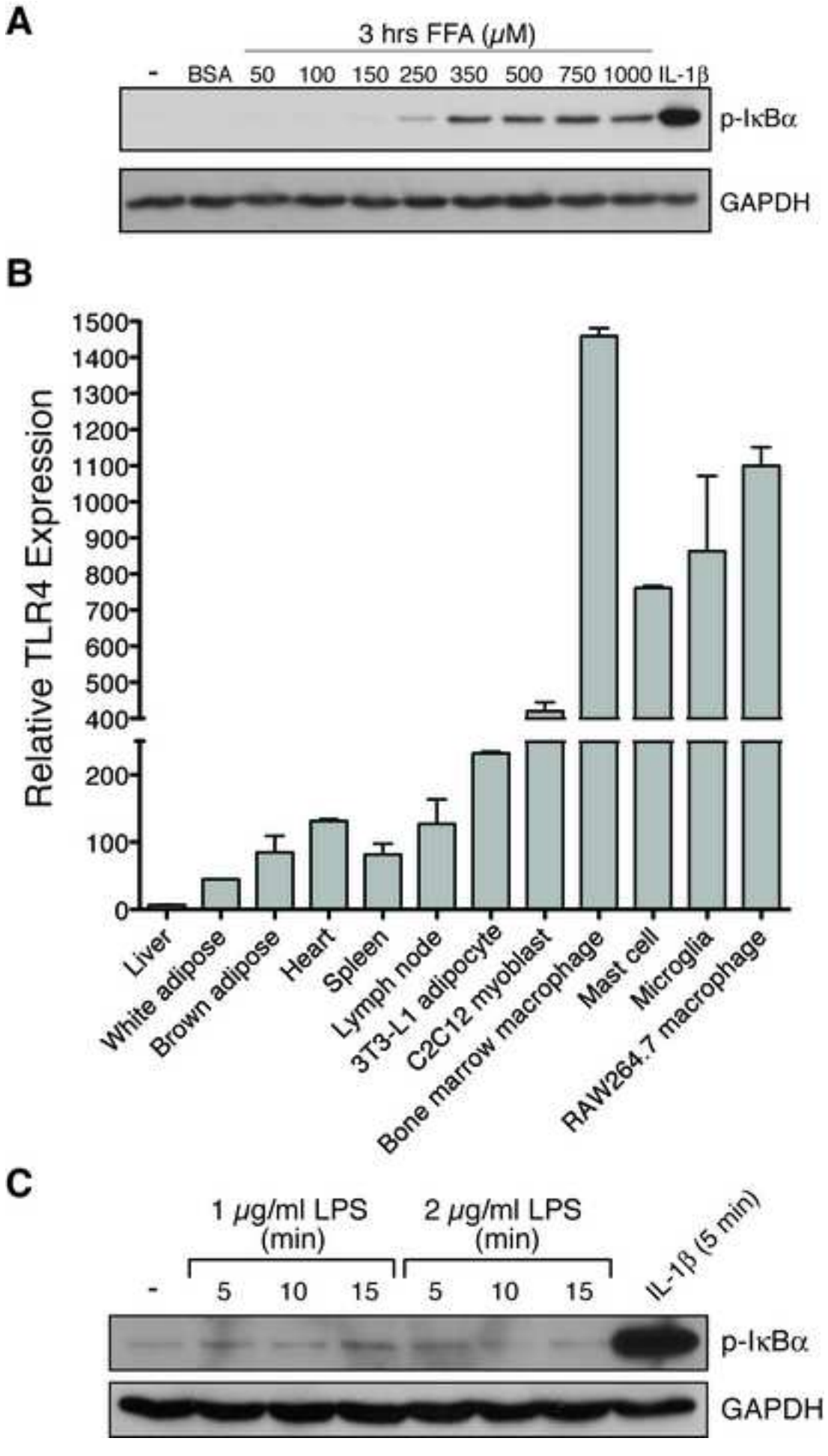
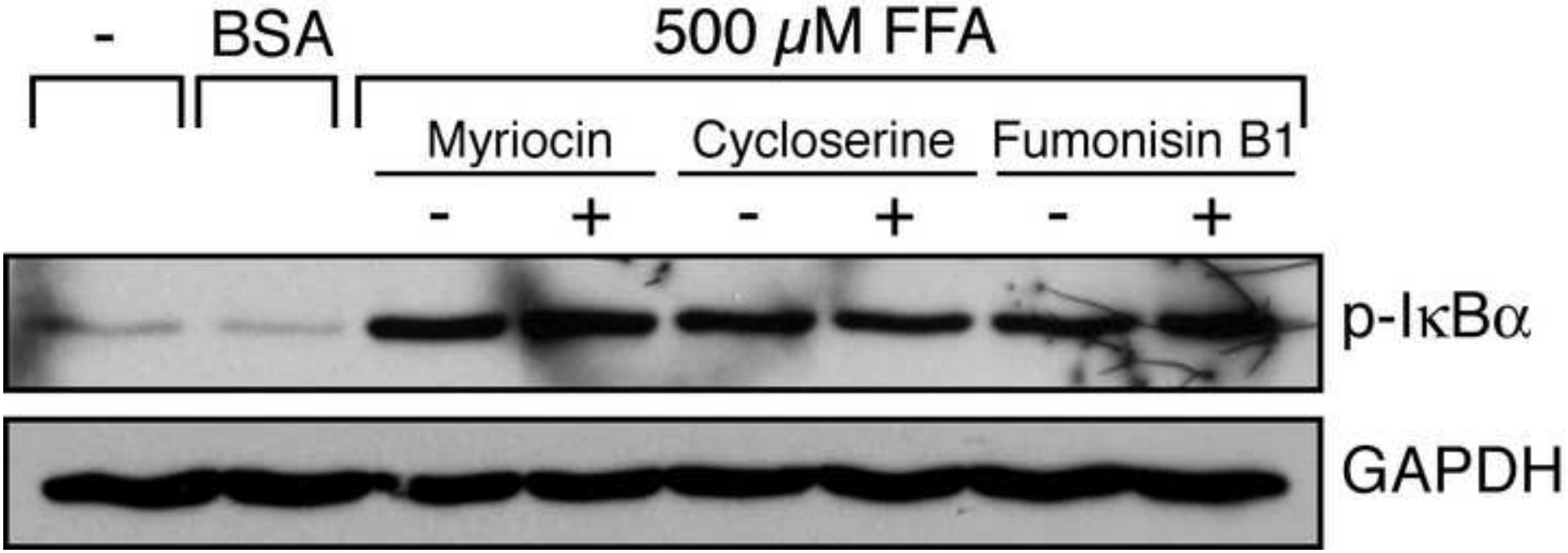
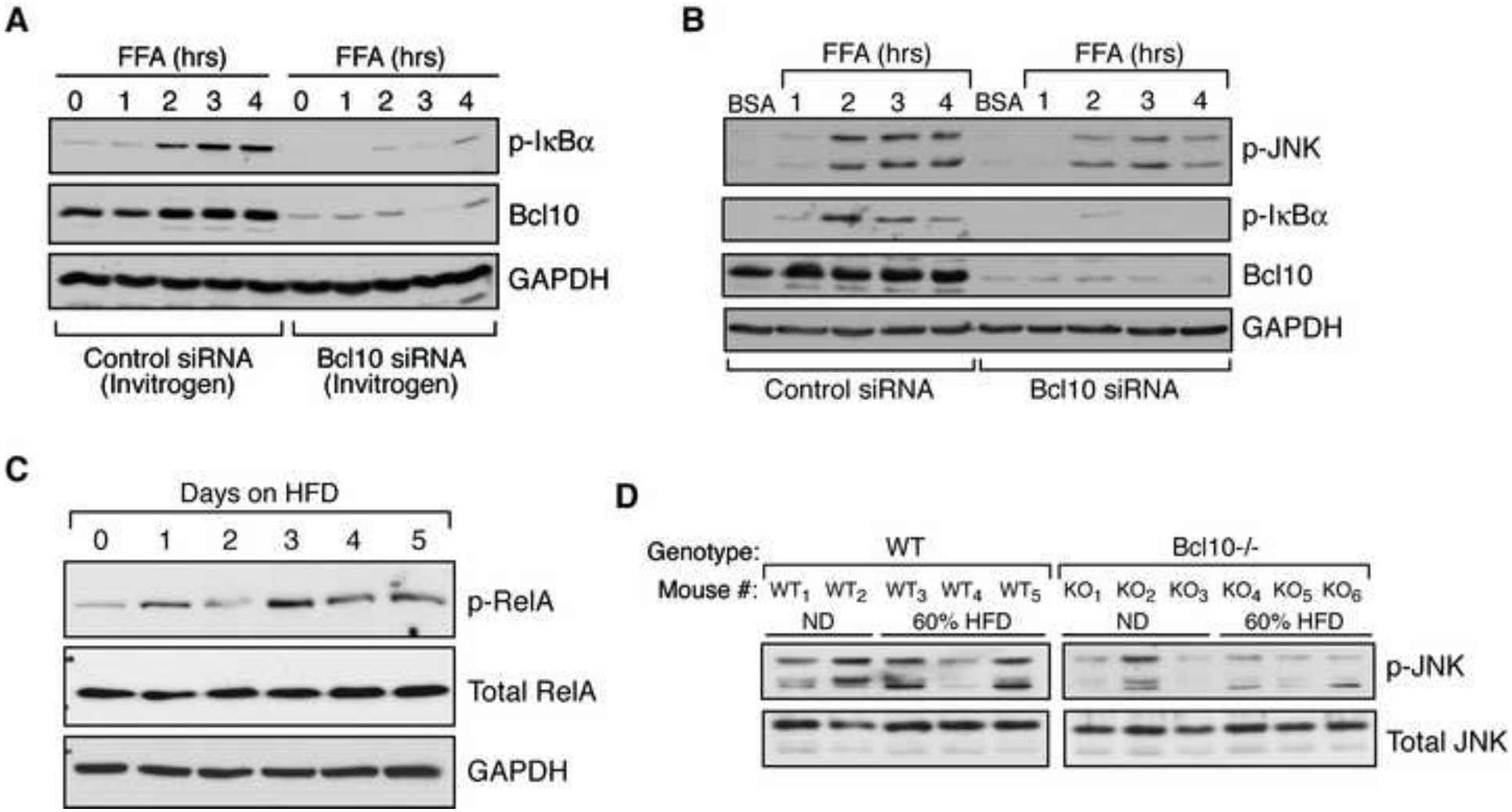
Supplemental Figure S1



Supplemental Figure S2



Supplemental Figure S3



Supplemental Figure S4