ONLINE SUPPLEMENT

Detailed Methods

Materials. We obtained acetylated-human LDL (acLDL) from Biomedical Technologies; compound 58035, LPS, zymosan, Bay 11-7082, SB203580, SP600125 and LY294002 from Sigma; U0126 from Cayman Chemical. The concentrations of inhibitors used in the present study were chosen from previous studies [1-3]. Sources of antibodies are as follows: acetyl-lysine, Mek1/2, phospho-Mek1/2, p38, phospho-p38, Erk1/2, phospho-Erk1/2, Jnk1/2, phospho-Jnk1/2, nucleophosmin, Akt, phospho-Akt (Ser-473 and Thr-308), phospho-GSK3β and GSK3β from Cell Signaling; TnfR1, FoxO1, p105/50, p65, and α-tubulin from Santa Cruz Biotechnology; SR-A from TransGenic; TLR-4 from Invitrogen; and actin from EMD4bioscience.

Immunocytochemsitry. We cultured peritoneal macrophages in Lab-Tek II Chamber Slide System (Nalgene Nunc International), fixed them with 4% paraformaldehyde and performed FoxO1 immunocytochemstry using three antisera as described [4]. FITC-conjugated anti-rabbit IgG (Jackson ImmunoResearch) were used as secondary antibodies. We measured apoptosis by Alexa 488-labeled annexin V and propidium iodide staining (Vybrant Apoptosis Assay kit, Invitrogen). For each condition, we randomly selected four separate fields, counted > 200 positive cells and plotted them as percentage of total cells.

Quantitative RT-PCR and ELISA. We extracted RNA using TRIzol (Invitrogen), synthesized cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and performed quantitative RT-PCR using DyNAmo HS SYBR Green qPCR Kit (Finnzymes) in a Chromo4 Real-Time PCR Detection System (Bio-Rad). Primer sequences are available on request. We used ELISA to measure protein levels of Mcp-1 (Invitrogen), Tnf-α, and Il-6 (BD Bioscience).

Adenoviruses. We have previously described adenoviruses encoding LacZ, GFP-tagged FoxO1-WT and FoxO1-KR, HA-tagged FoxO1-ADA, and nuclear factor-κB (Nf-κB)-luciferase [4-7]. We transduced macrophages at MOI 500, 18- to 24-h prior to experiments.

Immunoprecipitation and Immunoblotting. We lysed cells in buffer containing 2% SDS, 50mM Tris-HCl, 5mM EDTA, and protease/phosphatase inhibitors (Thermo Scientific), sonicated the extract and clarified it by centrifugation, followed by separation of nuclear

and cytoplasmic fractions using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). We performed immunoblotting followed by ECL detection (GE Lifescience), and quantified band intensity using NIH ImageJ 1.41 and normalization with gel loading controls. We carried out immunoprecipitation as described [7, 8].

Luciferase assays. We measured luciferase activity in peritoneal macrophages transfected with Nf-κB reporter construct using Dual Luciferase reporter Assay System (Promega). We normalized reporter activity by protein concentration or cotransfected *Renilla* luciferase, as appropriate.

Statistical analysis. We show data as mean ± SE. We determined statistically significant differences ($P < 0.05$) using unpaired *t*-test or ANOVA with Dunn's *post hoc* test.

SUPPLEMENTAL FIGURE LEGENDS

Suppl. Figure I. Zymosan- and LPS-induced inflammatory cytokine expression in macrophages. Quantitative time course analyses of Mcp-1, Il-6, and Tnf-α in *Foxo1*$^{+/+}$ (+/+, open circles) and *Foxo1*$^{KR/KR}$ macrophages (KR/KR, closed circles) loaded with (A) zymosan (100μg/ml) and (B) LPS (10ng/ml) (n=4). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ *vs*. KR/KR.

Suppl. Figure II. FC–induced ER stress and apoptosis in macrophages. (A) *Bip* and *Chop* mRNA levels in *Foxo1*$^{+/+}$ (+/+) and *Foxo1*$^{KR/KR}$ (KR/KR) macrophages loaded with FC for the indicated times. (B) Annexin V (green) and propidium iodide (red) staining in macrophages loaded with FC or thapsigargin (Tg, 2μM) for 24 h. (C) Percentage of positive cells. (D) SR-A, TLR-4, TnfR1, and α-tubulin western blots in WT (+/+) and FoxO1-KR (KR/KR) macrophages. ns: not significant.

Suppl. Figure III. FC–induced inflammatory cytokine expression in response to Mapk or Nf-κB inhibitors. *Mcp-1*, *Tnf-α*, and *Il-6* mRNA in *Foxo1*$^{+/+}$ macrophages pretreated with IκBα phosphorylation inhibitor Bay 11-7085 (Bay, 2μM), Mek1/2 phosphorylation inhibitor U0126 (U, 10μM), p38 phosphorylation inhibitor SB203580 (SB, 20μM), and Jnk1/2 phosphorylation inhibitor SP600125 (SP, 10μM) for 1h followed by FC loading for 8h (n=5). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ between the indicated groups.

Suppl. Figure IV. FC activates Akt and Mapk in macrophages transduced with adenovirus encoding FoxO1 mutants. (A) Representative immunoblots and (B-E) quantification of phospho-Akt Ser-473 (B), phospho-Akt Thr-308 (C), phospho-Mek1/2 (D), and phospho-Erk1/2 (E) in *Foxo1*$^{+/+}$ macrophages transduced with adenovirus (AdV) encoding LacZ, FoxO1-WT, FoxO1-KR, or FoxO1-ADA for 24h followed by FC loading for the indicated times. Band intensity was quantified from three independent experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ *vs*. LacZ-transduced macrophages.

Suppl. Figure V. *IκBs* mRNA levels in macrophages. (A) *IκBα*, *IκBβ*, and *IκBε* mRNA levels in *Foxo1*$^{+/+}$ (+/+) and *Foxo1*$^{KR/KR}$ (KR/KR) macrophages loaded with FC for 8h (n=5), or (B) in WT macrophages transduced with adenovirus (AdV) encoding LacZ, FoxO1-WT, FoxO1-KR, or FoxO1-ADA for 24h (n=5). * $P < 0.05$.

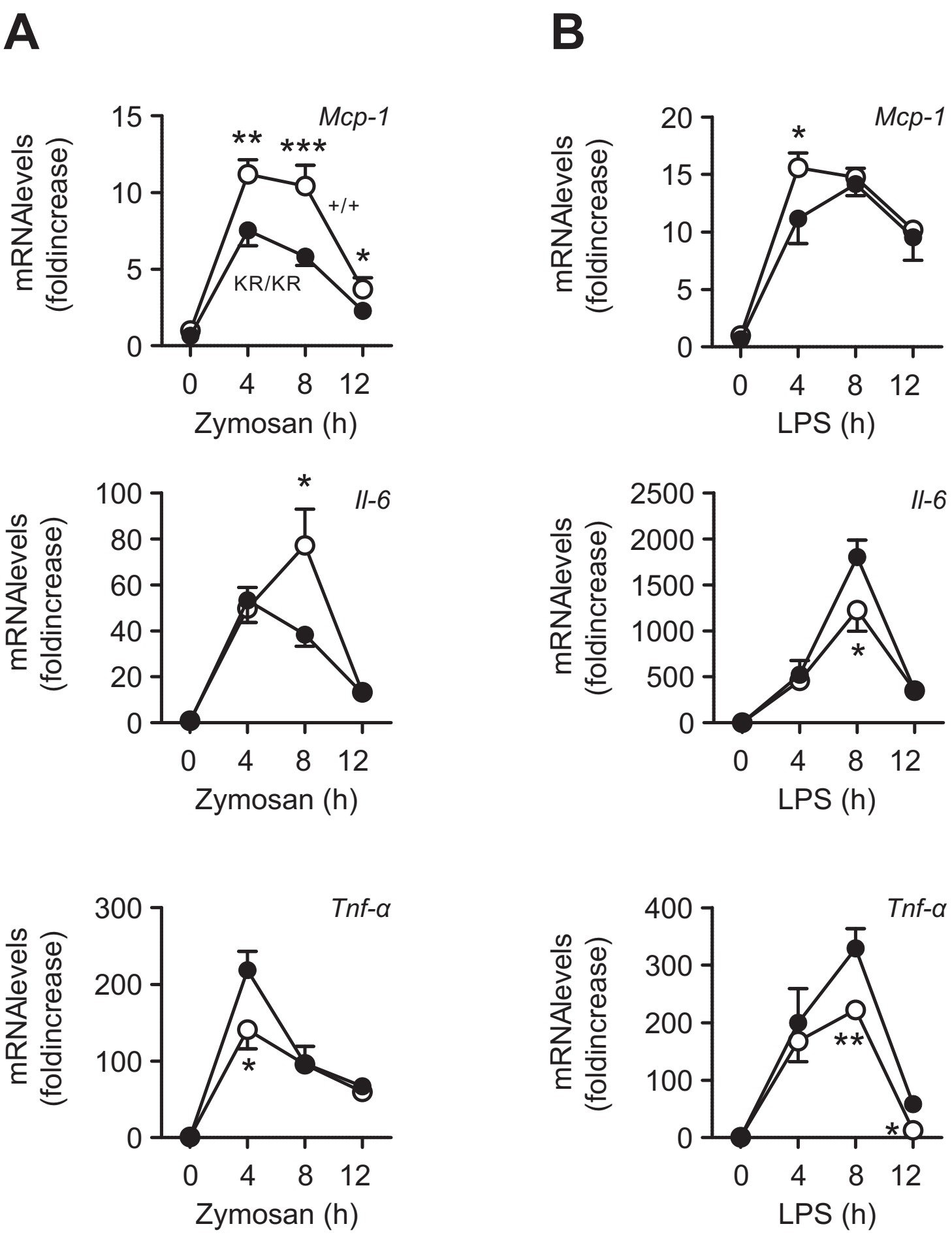
Suppl. Figure VI. Model of FoxO1 function in macrophages. (A) FoxO1

deacetylation (KR mutant) inhibits FC-induced Akt phosphorylation. This leads to (*i*) decreased IκBα phosphorylation and Nf-κB activity, and (*ii*) increased p105 and reduced Mek/Erk activity. These changes decrease inflammation, without increasing apoptosis. (B) FoxO1 dephosphorylation (FoxO1-ADA) increases FC-induced Akt phosphorylation, and increases IκBε expression, leading to Nf-κB inhibition, lower inflammation and increased apoptosis. ↑: increased, →: not changed, ↓: decreased.

SUPPLEMENTAL REFERENCES

1. Tsuchiya K, Yoshimoto T, Hirono Y, Tateno T, Sugiyama T, Hirata Y. Angiotensin ii induces monocyte chemoattractant protein-1 expression via a nuclear factor-kappab-dependent pathway in rat preadipocytes. *Am J Physiol Endocrinol Metab*. 2006;291:E771-778
2. Martinez-Moczygemba M, Huston DP, Lei JT. Jak kinases control il-5 receptor ubiquitination, degradation, and internalization. *J Leukoc Biol*. 2007;81:1137-1148
3. Li Y, Schwabe RF, DeVries-Seimon T, Yao PM, Gerbod-Giannone MC, Tall AR, Davis RJ, Flavell R, Brenner DA, Tabas I. Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: Model of nf-kappab- and map kinase-dependent inflammation in advanced atherosclerosis. *J. Biol. Chem.* 2005;280:21763-21772
4. Kitamura YI, Kitamura T, Kruse JP, Raum JC, Stein R, Gu W, Accili D. Foxo1 protects against pancreatic beta cell failure through neurod and mafa induction. *Cell Metab*. 2005;2:153-163
5. Kitamura T, Kitamura YI, Funahashi Y, Shawber CJ, Castrillon DH, Kollipara R, DePinho RA, Kitajewski J, Accili D. A foxo/notch pathway controls myogenic differentiation and fiber type specification. *J Clin Invest*. 2007;117:2477-2485
6. Matsumoto M, Han S, Kitamura T, Accili D. Dual role of transcription factor foxo1 in controlling hepatic insulin sensitivity and lipid metabolism. *J Clin Invest*. 2006;116:2464-2472
7. Senokuchi T, Liang CP, Seimon TA, Han S, Matsumoto M, Banks AS, Paik JH, DePinho RA, Accili D, Tabas I, Tall AR. Forkhead transcription factors (foxos) promote apoptosis of insulin-resistant macrophages during cholesterol-induced endoplasmic reticulum stress. *Diabetes*. 2008;57:2967-2976
8. Tanaka J, Qiang L, Banks AS, Welch CL, Matsumoto M, Kitamura T, Ido-Kitamura Y, DePinho RA, Accili D. Foxo1 links hyperglycemia to ldl oxidation and endothelial nitric oxide synthase dysfunction in vascular endothelial cells. *Diabetes*. 2009;58:2344-2354

Suppl. Figure I



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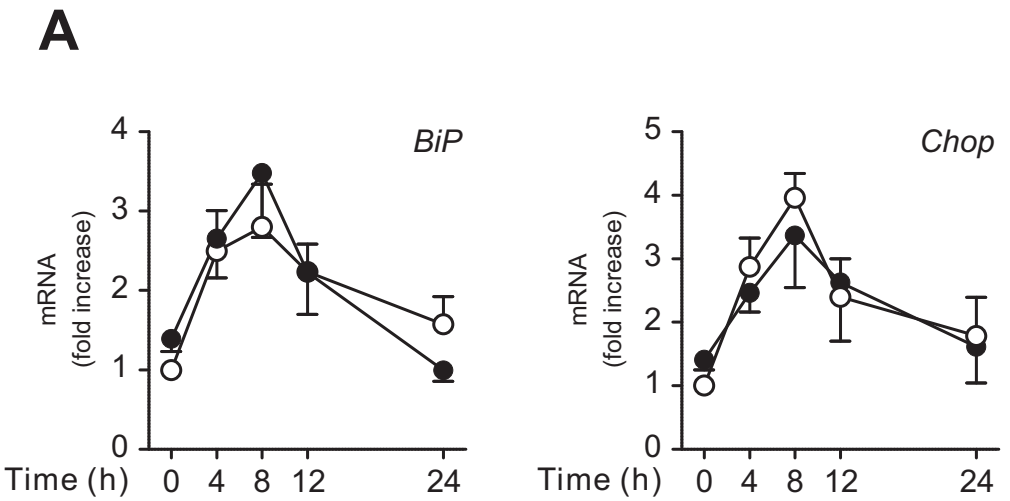

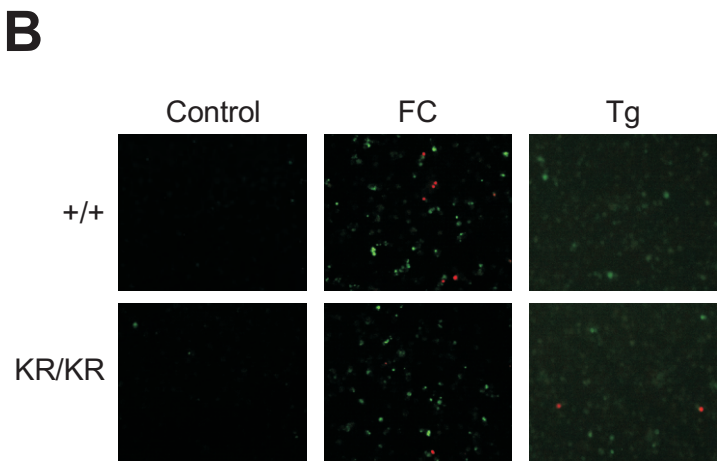

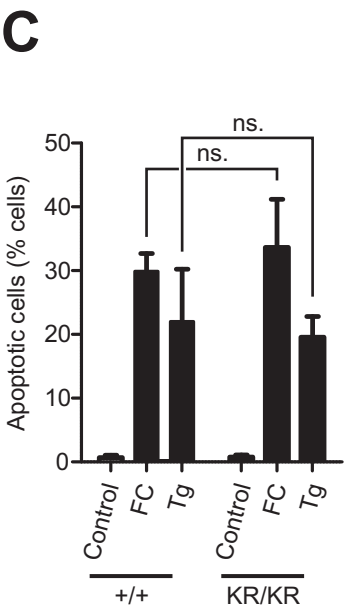

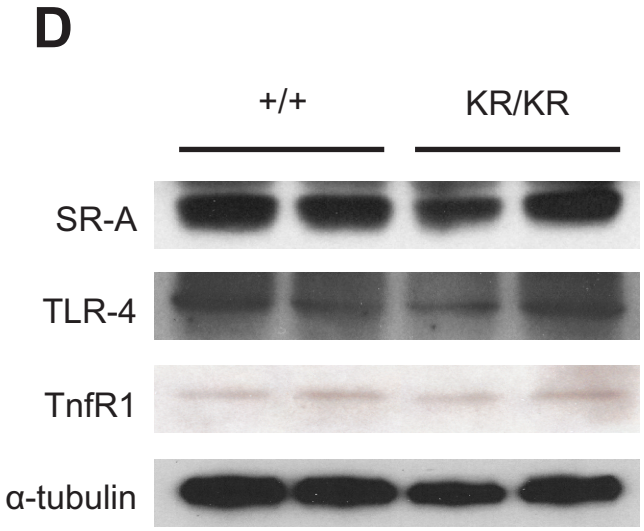

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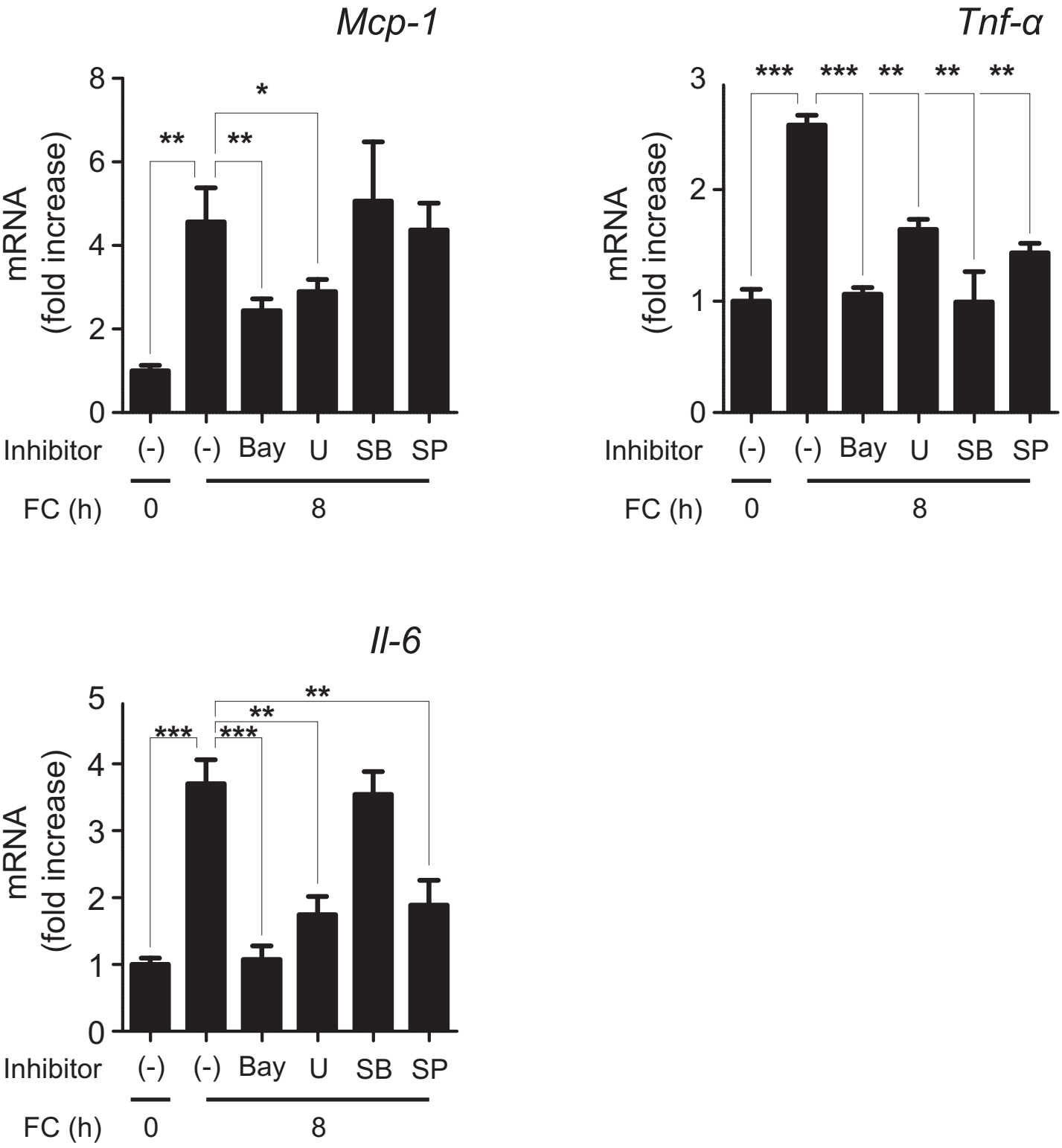

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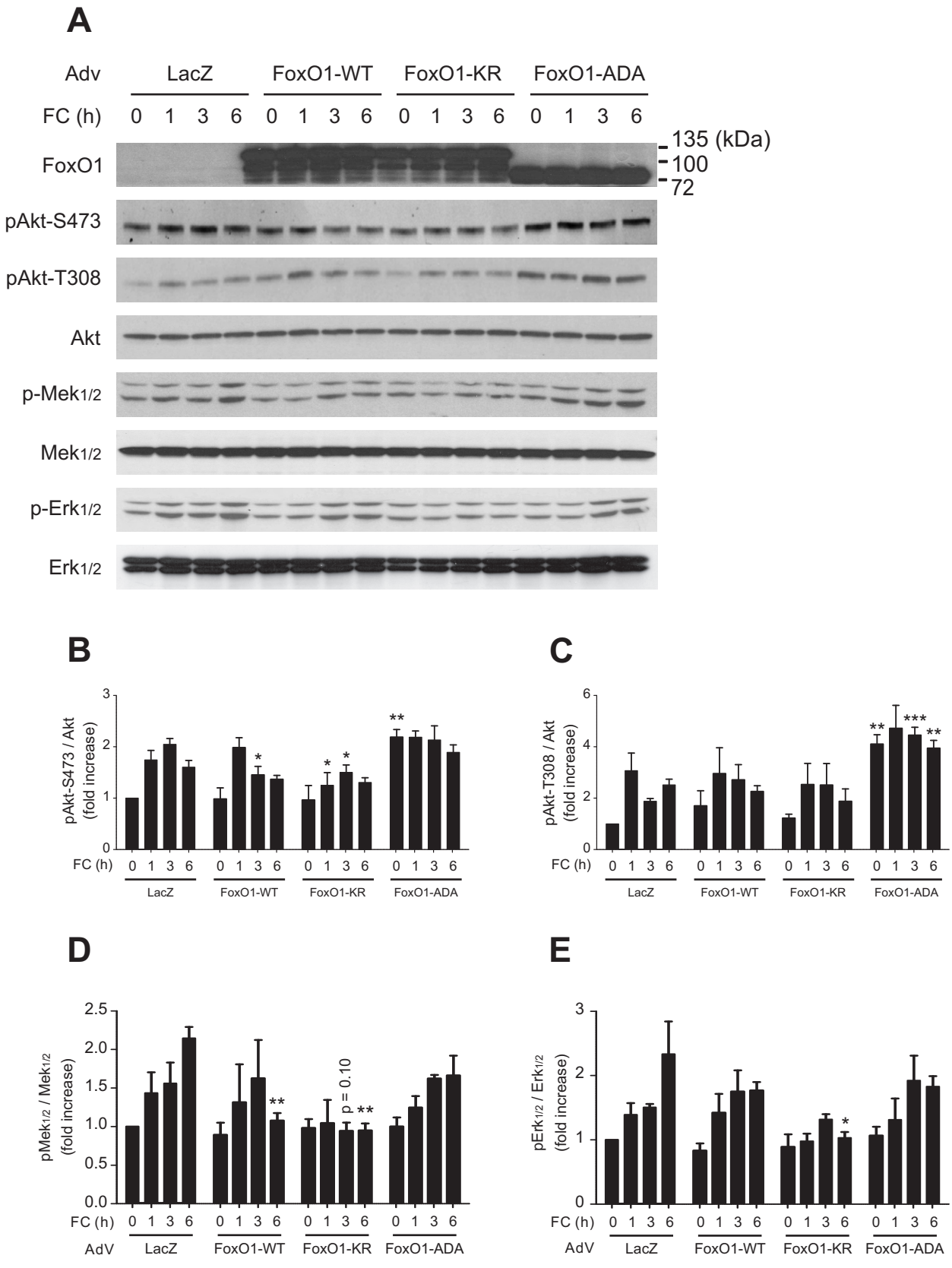

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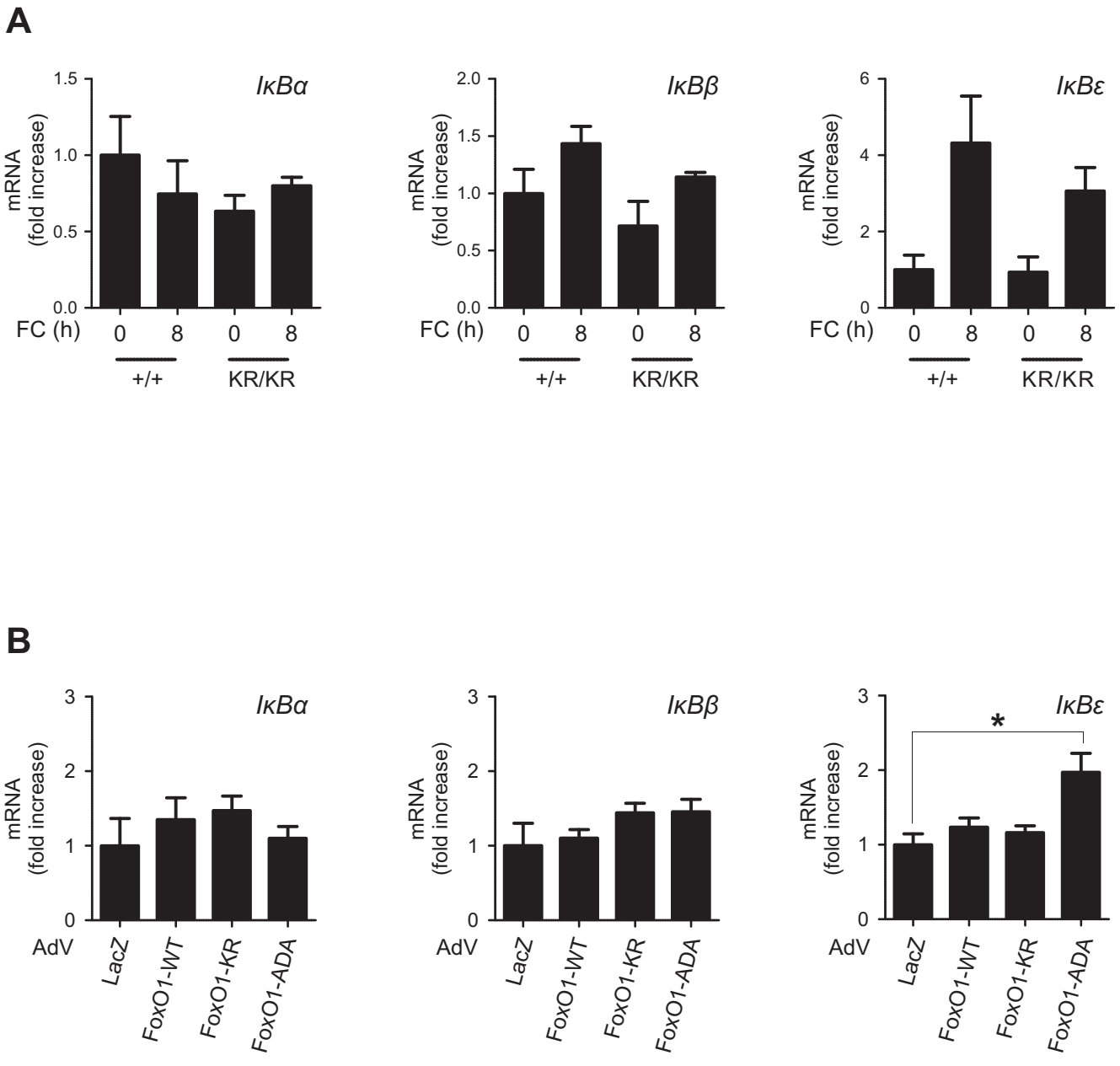

Suppl. Figure VI