Online data supplement belonging to: **NLRP12 Modulates Host Defense through IL-17A-CXCL1 Axis by Cai *et al.***

Immunohistochemistry: Briefly, deparaffinized fixed lung sections were permeabilized with the buffer containing Triton X-100 (0.1%) and then blocked with serum. Lung sections were incubated with anti-NLRP12 (ABGENT) and surface markers including anti-lipocalin Ab for PMNs (R&D), anti-proSPC Ab for type II epithelial cells (Millipore; US Biological) or anti-CD68 Ab for macrophages (BioLegend). For mouse lung sections, we used anti-Gr1 for PMNs (US Biological), anti-proSPC for type II epithelial cells or anti-F4/80 (Biolegend) as surface markers along with anti-NLRP12 Ab (ABGENT). Sections were washed and incubated with Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA). Tissue sections were washed and mounted using Vectashield mounting medium (Vector Laboratories, Inc., CA 94010) containing DAPI stain for nuclear staining. Images were acquired using an Axiocam digital camera (Zeiss, Thornwood, NY) connected to a Zeiss Axioskop 2 Plus research microscope.

Human Macrophages: For monocyte/macrophage differentiation, monocytes were cultured on plates for up to 7 days in RPMI 1640, containing 5% FBS, 1% penicillin-streptomycin, and 100 ng/mL M-CSF. For knockdown experiments, a pre-validated pool of siRNA (a cocktail of 4 siRNAs) for human *NLRP12* was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cells ($0.5x10^6$) were transfected with either 40 nM siRNA or a control siRNA (Santa Cruz Biotechnology Inc) using TransIT-TKO Transfection Reagent from MIRUS (Madison, WI) for 48 hours. Cells were then infected with 1 MOI of *K. pneumoniae* for 6 hours. For cytokine/chemokine assays, supernatants were collected 24 hours post-infection. For immunoblotting experiments, macrophages were washed 3 times with PBS before lysing with Urea/Chaps/Tris buffer supplemented with protease and phosphatase inhibitors.

Mouse macrophages: The lavage fluid was spun at 300x*g* for 10 minutes to pellet alveolar macrophages. Cells were cultured in 12-well culture plates at 37°C with 5% CO_2 at a concentration of $0.5x10^6$ cells per well in 1 ml RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% FBS, 1 mM pyruvate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. After 2 hours of incubation, non-adherent cells were removed with phosphate buffered saline (PBS), and the medium was replaced. Cells were then infected with 1 multiplicity of infection (MOI) of *K. pneumoniae* (ATCC 43816) for designated time points. For cytokine studies, media was collected at 3 and 6 hours following infection. For western blotting, cells were washed three times with PBS and lysed with Urea/Chaps/Tris buffer containing protease and phosphatase inhibitors.

Pneumonia model: *K. pneumoniae* were grown for 8 hours at 37°C in 50 mL tryptic soy broth (TSB) with shaking at 225 rpm. Bacteria were harvested by centrifuging the culture at 1200x*g* for 2 min and washed twice in sterile saline. The cells were resuspended in an isotonic saline at a concentration of 10^3 CFUs/50 µL/mouse. After anesthesia, *K. pneumoniae* suspension (10^3 CFUs in 50 µL) in 0.9% saline was inoculated via the intratracheal (intrapulmonary) route. The CFUs were enumerated by serially diluting the suspension of initial inoculums and subsequently plating 20 µL aliquots of each dilution onto a tryptic soy agar (TSA) plate and a MacConkey agar plate. Similarly, for counting bacterial CFUs in lungs and spleen, tissues were homogenized in PBS for 15 and 30 sec respectively, and 20 µL of homogenates were plated in 10-fold serial dilutions onto TSA and MacConkey agar plates. The survival of $NLRP12^{-/-}$ and WT mice was monitored for up to 15 days following inoculation with *K. pneumoniae*.

BALF collection. The animals were euthanized, and the trachea was exposed and subsequently cannulated with a 20-gauge catheter as described earlier [1-4]. BAL fluid was collected 4 times by instilling 0.8 mL of PBS containing heparin and dextrose. Total leukocytes in BAL fluid were enumerated by counting on a hemocytometer, whereas BAL differential leukocyte cell counts

were determined by standard light microscopy. The remaining (2 mL) of the undiluted cell-free BAL fluid was passed through a 0.22 μm filter and used for the determination of cytokine/chemokine levels.

Lung isolation. Following infection, the whole (non-lavaged) lungs were excised and snap frozen. For long-term storage, these lung tissues were stored at -70°C and used for cytokine/chemokine determination, western blots, and MPO activity assay. Lung tissue was briefly homogenized in 2 mL PBS supplemented with 0.1% triton X-100 and complete protease inhibitor (1 tablet/50 mL media), and the resulting homogenates were centrifuged at 12,000x*g*/20 min. The supernatants were harvested, passed through a 0.22 μm filter, and used as required.

MPO activity. The lung homogenates were resuspended in 50 mM potassium phosphate buffer (pH 6.0) supplemented with 0.5% hexadecyltrimethylammonium bromide (HTAB), as described in previous publications [1-9]. Samples were then sonicated, incubated at 60°C for 2 hours, and assayed for MPO activity in a hydrogen peroxide/*O*-dianisidine buffer at 460 nm. Absorbance was measured at 460 nm using a spectrophotometer. The increase activity was calculated between 0 sec and 90 sec.

Cytokine/chemokine determination. Cytokines/chemokines were determined by sandwich ELISA as described earlier [1-9]. The minimum detection limit of the assay was 2 pg/mL of protein. For mouse lungs, TNF-α, IL-6, LIX, MIP-2, IL-23, IL-17A, IL-1β, and IL-18 concentrations were normalized to the total protein concentration in the samples measured. Results are expressed as pg/mg of total protein for lung tissue and in pg/mL for BALF.

Semiquantitative histology. The lungs were perfused from the right ventricle of heart with an isotonic saline, 24 and 48 hours post-infection, and harvested. For hematoxylin and eosin staining, lungs were fixed in 4% phosphate-buffered formalin, processed in paraffin blocks, and

cut into fine sections (5 µm in thickness). Semiquantative histology was performed by a Veterinary Pathologist in a blinded fashion according to the following scoring scale: 0, no inflammatory cells (macrophages or neutrophils) are present in section; 1, <5% of section is infiltrated by inflammatory cells; 2, 5–10% of section is infiltrated by inflammatory cells; and 3, >10% of section is infiltrated by inflammatory cells, as indicated in our earlier publications [2, 3].

Th1/Th2/Th17 differentiation. Th1/Th2/Th17 differentiation has been performed as previously described [10-12] . Cells were washed and resuspended in PBS followed by blocking with Fc receptor blocking reagent. Cells were surface stained with anti-CD4 and intracellular with anti-IFN-γ, -IL-4 or -IL-17A. Flowjo software was used for data analysis.

Bone marrow (BM) transplantation. BM chimera experiments were performed as described in earlier publications [1, 5, 9]. BM was flushed from tibias and femurs from donor mice, and a total of 8 x 10^6 BM cells were injected into the tail veins of lethally irradiated (two 525-rad doses separated by 3 hours) recipient mice. Reconstituted mice were treated with 0.2% neomycin sulfate for the first 2 weeks post-transplantation. Experiments were performed 8 weeks after BM reconstitution. We found that more than 75-85% of blood leukocytes were derived from donor marrow at the time the mice were used for experiments (6–8 weeks post-transplantation).

Pyroptosis. Lung or spleen digests from C57BL/6 (WT) or *Nlrp12*$^{-/-}$ mice challenged with *K. pneumoniae* for 24 or 48 h were used to determine cells undergoing pyroptosis as outlined in previous publication [13]. Briefly, lung and spleens cell suspensions were passed through a 0.70 µ filter. Following 2 PBS washings, cells were FcR blocked and aliquoted for surface staining with conjugated PerCP anti-mouse Gr-1/Ly6G or EMR1 and APC anti-mouse CCR2 or CXCR2. Red blood cells (RBCs) were lysed by adding NH_4Cl lysing buffer. Cells were resuspended in 1x binding buffer containing 5 µl of Annexin V-FITC and 5 µl of PI according to the manufacturer's protocol (Annexin V apoptosis detection kit from BD Pharmingen). The cell suspension was

vortexed and incubated for 15 mins in the dark at room temperature. A total of 100 µl 1x binding buffer was added, and cells were analyzed by flow cytometry. CCR2 or CXCR2 positive Gr-1/Ly6G (neutrophils) and EMR1 (macrophages) that were positive for Annexin V-FITC and negative for PI are shown in histograms.

Adoptive transfer of $CD4^+$ T cells and macrophages. Splenic $CD4^+$ T cells were isolated and made single cell suspensions. Cell suspensions were washed, RBCs were lysed and CD4+ T cells were isolated by negative selection from single cell suspension using the EasySep cell separation procedure (StemCell Technologies, Vancouver, Canada). Resulting cell preparations were resuspended to a final density of 0.5×10^6 cells per 50 µL PBS for i.t. administration. BM from femur and tibia was flushed and marrow was passed through a 21G needle 4-6 times to dissociate the cells. RBCs were lysed using 1X RBC lysis buffer. Cell suspension was washed with PBS twice and cells were resuspended in DMEM +5% FBS+P/S containing 2 million cells/ml with M-CSF 25 ng/ml and seeded for 6-7 days. Fresh BMDM growth medium was added on day 3 and 5, and the formation of mature BMDM was evaluated after 7 days using flow cytometry analysis to detect cells expressing CD11b and F4/80. Resulting cell preparations were resuspended to a final density of 0.5×10^6 cells per 50 µL PBS for i.t. administration.

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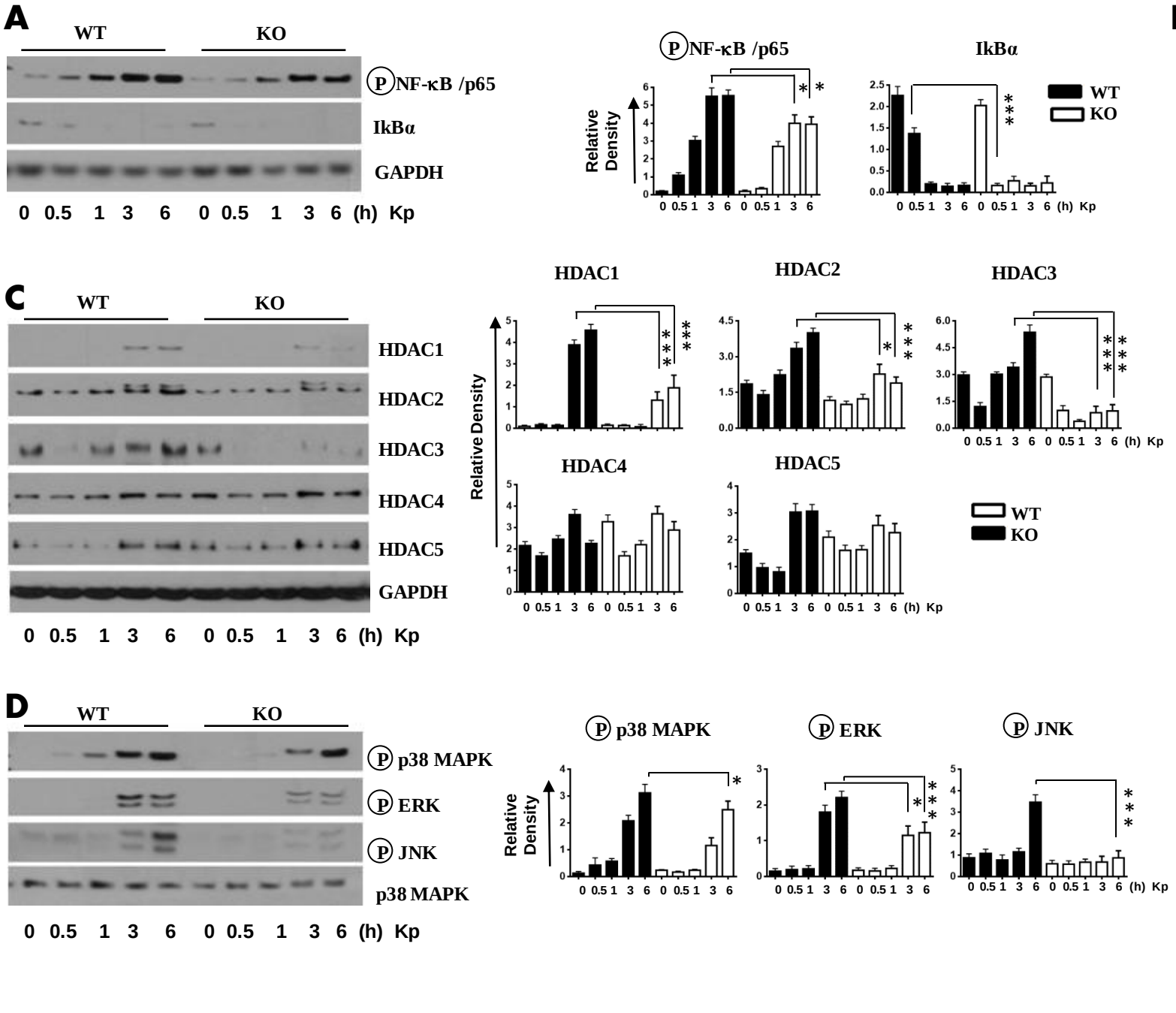
FIGURE LEGENDS

Figure 1. NLRP12 is important for NF-κB activation, cytokine/chemokine production, HDAC expression and MAPK activation in human macrophages following *K. pneumoniae* infection. A. Cell lysates obtained from mouse alveolar macrophages (0.5×10^6 cells/ml) challenged with 1 MOI of *K. pneumoniae* were used for the assessment of NF-κB activation by Western blotting. Representative blots are shown from 3 experiments with identical results. Immunoreactive bands were quantified by densitometry and normalized to GAPDH. $p<0.05$; **, $p<0.01$;***, $p<0.001$, *Nlrp12*$^{-/-}$ versus WT macrophages. Data are expressed as mean ± SE (*n* = 4 mice per group). **B**. Mouse alveolar macrophages were infected with 1 MOI of *K. pneumoniae* for different time periods, and macrophage supernatants were used for cytokine/chemokine measurement. Mean ± SE were obtained from three independent experiments. $p<0.05$; **, $p<0.01$;***, $p<0.001$, *Nlrp12*$^{-/-}$ versus WT macrophages. **C-D**. Cell lysates obtained from mouse alveolar macrophages infected with 1 MOI of *K. pneumoniae* were used for the measurement of HDAC expression–and MAPK activation by immunoblotting. Representative blots are shown from 3 experiments with identical results. Immunoreactive bands were quantified by densitometry and normalized to GAPDH (for HDAC expression) or to p38 MAPK (for MAPK activation). $p<0.05$; **, $p<0.01$;***, $p<0.001$, *Nlrp12*$^{-/-}$ versus WT macrophages. Data are expressed as mean ± SE (*n* = 4-6 mice per group). Error bars represent SE.

Figure 2. NLRP12 is required for activation of NF-κB, expression of HDAC and activation of MAPK *in vivo* after *K. pneumoniae* infection. **A**. WT and *Nlrp12*$^{-/-}$ mice *were* infected with 1×10^3 CFUs of *K. pneumoniae*/mouse i.t., and lungs were obtained 24 h and 48 h post-infection. Nuclear fractions obtained from lung homogenates were used for NF-κB binding using ELISA. The values are means ± SE. Values significantly different between the *K. pneumoniae*- and saline-treated groups are indicated by asterisks ($P < 0.05$; 4-6 mice/group). $OD_{450\ nm}$, optical

density at 450 nm. **B-E**. *Nlrp12*$^{+/+}$ and *Nlrp12*$^{-/-}$ mice *were* infected with 1 × 10^3 CFUs of *K. pneumoniae*/mouse i.t. and lungs were obtained 24 h and 48 h post-infection. Lung homogenates were used for activation of NF-κB and expression of HDAC by Western blotting. Representative blot is shown from three blots/experiments with identical results. *Protein* immunoblot bands were quantified by densitometry and normalized to GAPDH. Data are expressed as means ± SE (*n* = 4). **F-G**. WT and *Nlrp12*$^{-/-}$ mice *were* infected with 1 × 10^3 CFUs of *K. pneumoniae*/mouse i.t., and lungs were obtained 24 and 48 h post-infection. Lung homogenates were used to determine activation of MAPKs by western blotting. A representative blot is shown from three blots/experiments with identical results. *E. The intensity of* immunoreactive bands was quantified by densitometry and normalized to total p38. $p<0.05$; **, $p<0.01$;***, $p<0.001$, *Nlrp12*$^{-/-}$ versus WT macrophages. Data are expressed as mean ± SE (*n* = 4-8 mice per group). Error bars represent SE.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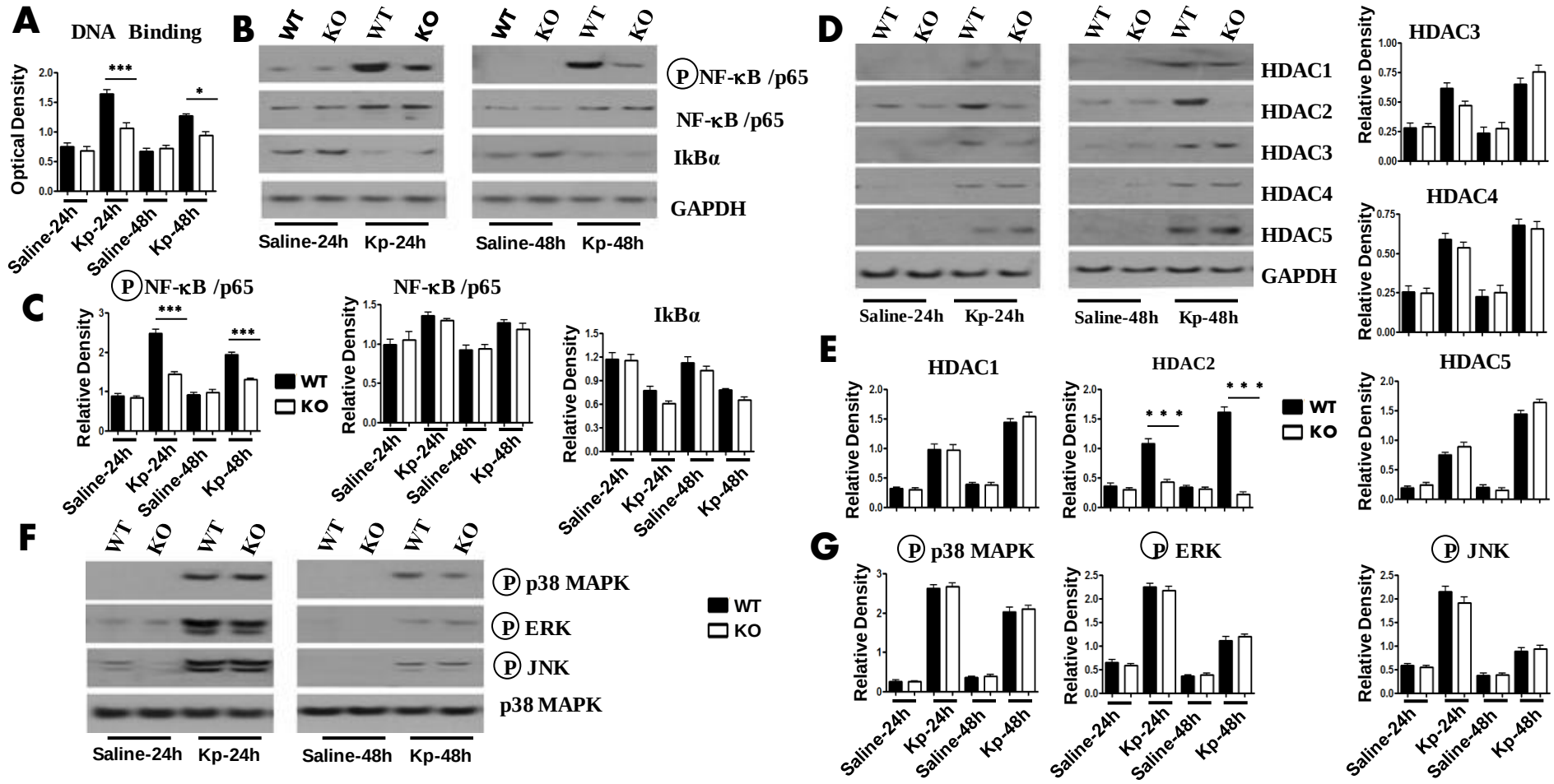
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