Supplementary Figure Legends:

Figure S1. ***LoxP* targeting of *IKKα* and *IKKβ* alleles and Cre mediated IKK deletions**

Mice with IKKα or IKKβ alleles flanked by LoxP recombination sites were generated by Lexicon Genetics Inc. for Boehringer Ingelheim Pharmaceuticals. Schematic maps of IKKα and IKKβ targeting vectors before (B and G) and after homologous recombination (C and H) into their respective loci in ES cells are shown in addition to their Cre recombinase induced deletions (D and I). Modified *IKK* alleles were confirmed with the indicated PCR primer pairs at all steps. Four different IKKα and IKKβ conditional KO strains of mice were generated on a C57Bl/6 background by interbreeding $IKK\alpha^{f/f}$ or $IKK\beta^{f/f}$ homozygous mice with the *rosa(Cre-ESR1/CreER^{T1})*, *rosa(CreER^{T2})* or *MLysCre* strains to produce $IKK\beta^{f/f}$*:CreER^{T1}*, $IKK\alpha^{f/f}$*:CreER^{T2}*, $IKK\alpha^{f/f}$*:MLysCre and* $IKK\beta^{f/f}$*:MLysCre* mice. The *MLysCre* strain expresses Cre recombinase under the control of the macrophage-specific lysozyme promoter. Conditional deletion of IKKα and IKKβ in $IKK\alpha^{f/f}$*:MLysCre and* $IKK\beta^{f/f}$*:MLysCre* mice occurs in mature neutrophils and MΦ (see Fig. 2 in Results and Supplementary Figure S3). The $IKK\beta^{f/f}$*:CreER^{T1}*, $IKK\alpha^{f/f}$*:CreER^{T2}* strains harbor tamoxifen-inducible Cre-recombinases driven by the endogenous mouse *Gt{ROS}26Sor* promoter. The Cre-ER^{T1} or Cre-ER^{T2} proteins are ubiquitously expressed in all tissues of the respective *rosa(Cre-ESR1)* and *rosa(CreER^{T2})* mice; but their Cre recombinase activities are only induced by exposure to 4-OHT. Cre-ESR1/Cre-ER^{T1} encodes a fusion protein consisting of the Cre recombinase fused to a carboxy-terminal ESR-1 domain, a mutant mouse estrogen receptor ligand binding domain refractory to

estrogen but instead activated by the synthetic estrogen derivative 4-orthohydroxytamoxifen (4-OHT) (see Materials and Methods for references). The $CreER^{T2}$ fusion protein contains three site directed mutations in its ER domain (G400V/M543A/L544A) conferring 10X more sensitivity to 4-OHT compared to the mutant ERT1 (G521R) domain in ESR-1 (see Materials and Methods for references). Cre mediated IKK deletion in $IKK\alpha^{f/f}$:$CreER^{T2}$ and $IKK\beta^{f/f}$:$CreER^{T1}$ MEFs was induced by exposure to 100 nM 4-OHT for 36 hr as shown by immunoblotting for IKKα (Panel E) and IKKβ (Panel J) proteins.

Figure S2. **Intra-peritoneal injected HMGB1 recruits Gr-1^{Hi} staining neutrophils in WT but not in *IKK$\alpha^{f/f}$:MLysCre* and *IKK$\beta^{f/f}$:MLysCre* mice.**

A- F: flow cytometric analysis of one representative sample for each experimental condition of IP lavages stained with a FITC-conjugated antibody specific for the neutrophil marker Gr-1. Cells were gated based on size (FSC-height) and high Gr-1 expression. Population gates were drawn to eliminate small debris and doublets. Quadrants were added that focus on the Gr-1^{Hi} cell populations (FL1 10^3). The percentage of Gr-1^{Hi} cells in the parent population was calculated using FlowJo Analysis software version 8.7.3 (TreeStar, Inc, Ashland, OR).

Figure S3: Primary mature neutrophils deficient in either IKKα or IKKβ are defective for HMGB1 chemotaxis in vitro.

A. Boyden chamber migration assays with primary neutrophils (PMNs), obtained from the bone marrows of *WT, IKK$\alpha^{f/f}$:MLysCre and IKK$\beta^{f/f}$:MLysCre* mice, were

performed in 48 well micro-chambers (Neuroprobe Inc.) with 3 micron pore size filters and cells were allowed to migrate for 35 minutes at 37°C. Migration distances were measured by the leading front method (see Materials and Methods for references). HMGB1 was used at 50 ng/ml and C5a at 5 nM. Results are derived from two independent experiments and error bars reflect standard errors of the mean. **B.** Immunoblot for IKKα and IKKβ expression in neutrophils purified from the bone marrows of *WT, IKK$\alpha^{f/f}$:MLysCre and IKK$\beta^{f/f}$:MLysCre* mice. The same blots were re-probed for β-actin as a protein loading reference control. Some residual IKK expression in the respective conditional KO PMNs is due to their 70-80% purity, which was determined as Hi Gr-1 staining by FACS.

Mature neutrophils (PMNs) were isolated from the bone marrow of mouse femurs and tibias (see Materials and Methods for references). Briefly bone marrow cells flushed out by a 27g needle syringe with HBSS-EDTA (HBSS-EDTA (HBSS without calcium, magnesium, phenol red, and sodium bicarbonate; pH 7.2 + 15mM EDTA) were passed through a 70 µm cell strainer (Falcon cat # 352350) and centrifuged at 400 x g for 10 minutes at room temperature. Red blood cells were eliminated by hypotonic lysis and white blood cells (WBCs) were spun down at 400 x g and resuspended in HBSS-EDTA. WBCs were loaded onto a three-layer percoll gradient (78%, 69% and 52%; in that order from the bottom up with 100% percoll being equal to 9 parts percoll and 1 part 10x HBSS). Cells were centrifuged on the gradient at 1500 x g for 30 minutes at room temperature and mature PMNs (Neutrophils) were collected from the 69%/78% interface. Purified PMNs were washed once in 1x HBSS-EDTA and resuspended at 5 million cells per ml in

chemotaxis buffer (1x HBSS + 10 mM HEPES pH 7.4 + 1% BSA) and used for either chemotaxis or stained with PE conjugated anti-Gr1 for FACS analysis to determine PMN purity.

Figure S4: Cytokine gradient formation in Ibidi μ slides

A picture of a μ slide is shown in panel A. The surface of the μ slide channel is coated with 50 μg/ml fibronectin in PBS w/o Ca^{2+}/Mg^{2+} following the supplier's specifications. Next the coated surface is blocked with PBS + 1% BSA at 37°C. Cells are placed into the channel and allowed to adhere to the coated surface in a humidified CO_2 incubator. Once cells have completely adhered to the substratum, the μ slide is placed on the heated and humidified stage of the microscope and the chemoattractant solution is prepared by mixing 70,000-100,000 fluorescent beads (Molecular Probes) to 50 μl of 30 ng/ml HMGB1. The chemoattractant gradient is created by carefully pipetting 40 μl of chemoattractant solution into one channel aperture and then aspirating exactly the same volume of solution from the opposite aperture (the apertures are at the bottom of each well in the μ slide). The fluorescent beads together with the chemoattractant form a gradient shaped like the blue dye in Panel B, in which the concentrations of HMGB1 and beads are higher in the portion of the channel closer to the aperture containing the chemoattractant solution, and by diffusion becomes progressively lower in the central part of the channel thus generating an HMGB1 gradient, as represented in Panel B. Cells at the front of the gradient (see observation area in Panel A) immediately begin to respond and are tracked

by time-lapse microscopy. Directional tracks were defined as those with ending points closer to the higher HMGB1 concentration in the gradient, as compared to their starting points, whist non-directional tracks were the opposite; and indeterminate tracks starting and ending at the same distance from the HMGB1 gradient were considered non-directional tracks. A more detailed description of the use of Ibidi (Integrated BioDiagnostics) μ slides for assessing cell movement in real time microscopy has also been previously described (www.ibidi.de) (also see Materials and Methods).

Figure S5: Effects of IKKβ, IKKα and p52 on Euclidean migration distances at early times and of IKKβ and IKKα on cell velocity at later times during HMGB1 chemotaxis.

A-C: The Euclidean migration distances of WT, IKKβ$^{-/-}$, IKKα$^{-/-}$ and p52$^{-/-}$ MEFs for the first 60 minutes of their response to an HMGB1 gradient were quantified in μ slides by time-lapse microscopy. WT refers to either primary IKKα$^{f/f}$:CreER^{T2} or IKKβ$^{f/f}$:CreER^{T1} MEFs when compared to the IKK KO counterpart. IKKα$^{-/-}$ and IKKβ$^{-/-}$ MEFs were generated from the latter respective WT cells by exposure to 4-OHT for 36 hrs. In the WT vs. p52$^{-/-}$ comparison immortalized WT and classical knock out MEFs were used. Euclidean distance is the straight-line distance between the start and end points of cell movement and is a composite parameter influenced by directionality, velocity and migration efficiency, as determined by the number of turns during cell movement. **D&E:** The velocities of WT, IKKβ$^{-/-}$ and IKKα$^{-/-}$ MEFs in response to an HMGB1 gradient after 180 minutes were quantified in μ slides by

time-lapse microscopy. Bars are mean Euclidean distances or velocities and error bars are standard errors of the mean. *P<0.0001, **P=0.009, ***P=0.005, and #P=0.01.

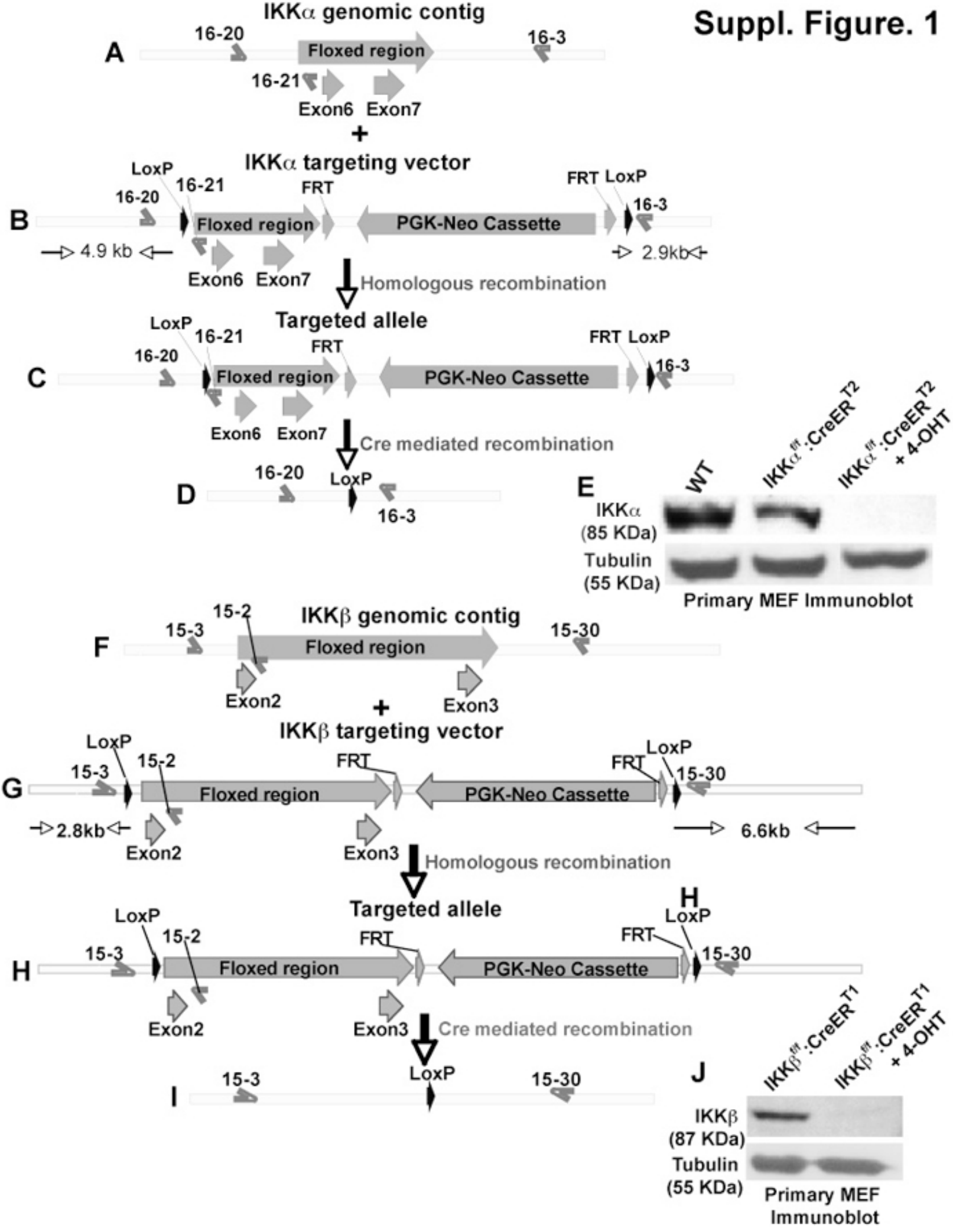
Figure S6: IKKβ is necessary for mature primary macrophages to induce full length RAGE mRNAs

Real time PCR analysis of HMGB1 dependent full length RAGE mRNA expression in mature macrophages derived from the bone marrow progenitors of WT (*IKKβ$^{f/f}$*) and IKKβ conditional knock-out (*IKKβ$^{f/f}$:MLysCre)* mice. Macrophages were exposed to either serum free media or the same media supplemented with 50 ng/ml of HMGB1 for 2 hrs. Results were obtained from four independent experiments.

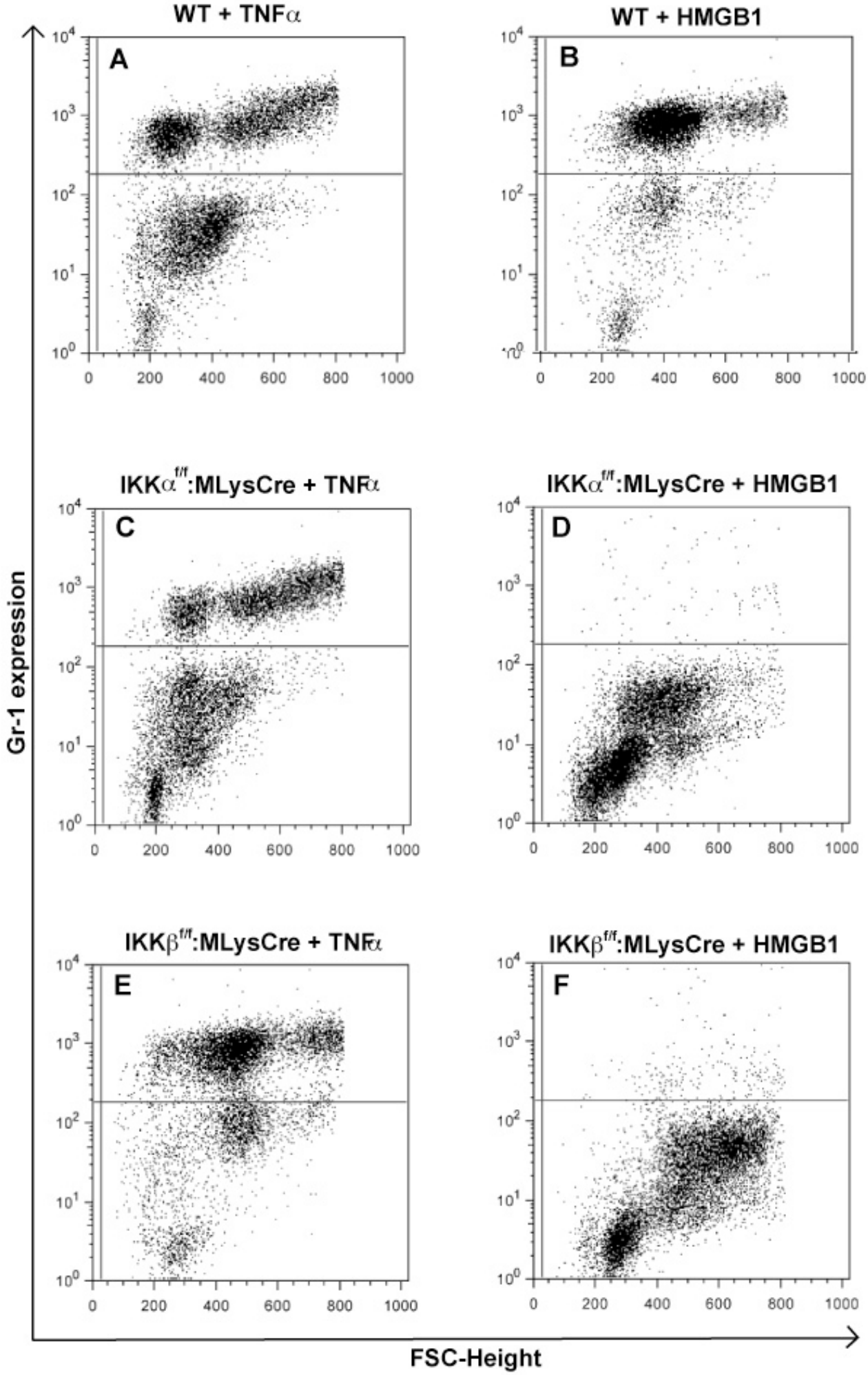
Figure S7: HMGB1 modestly induces p52 nuclear translocation in Wt (but not IKKα$^{-/-}$MEFs) in comparison to the more robust, IKKα dependent effect, of LTβ receptor signaling

Immortalized WT and IKKα$^{-/-}$ MEFs were stimulated with HMGB1 (50 ng/ml) or an agonistic anti-LTβR antibody (10 μg/ml) for up to 8 hrs. Nuclear and cytoplasmic protein fractions were analyzed by immunoblotting for p100/p52. An asterisk denotes a cross-reactive artifact band in the anti-p52 nuclear blots. Immunoblots were stripped and re-probed for Lamin B1 or α-Tubulin as protein reference controls for nuclear and cytoplasmic cell fractions, respectively.

Suppl. Figure. 1



Suppl. Figure 2



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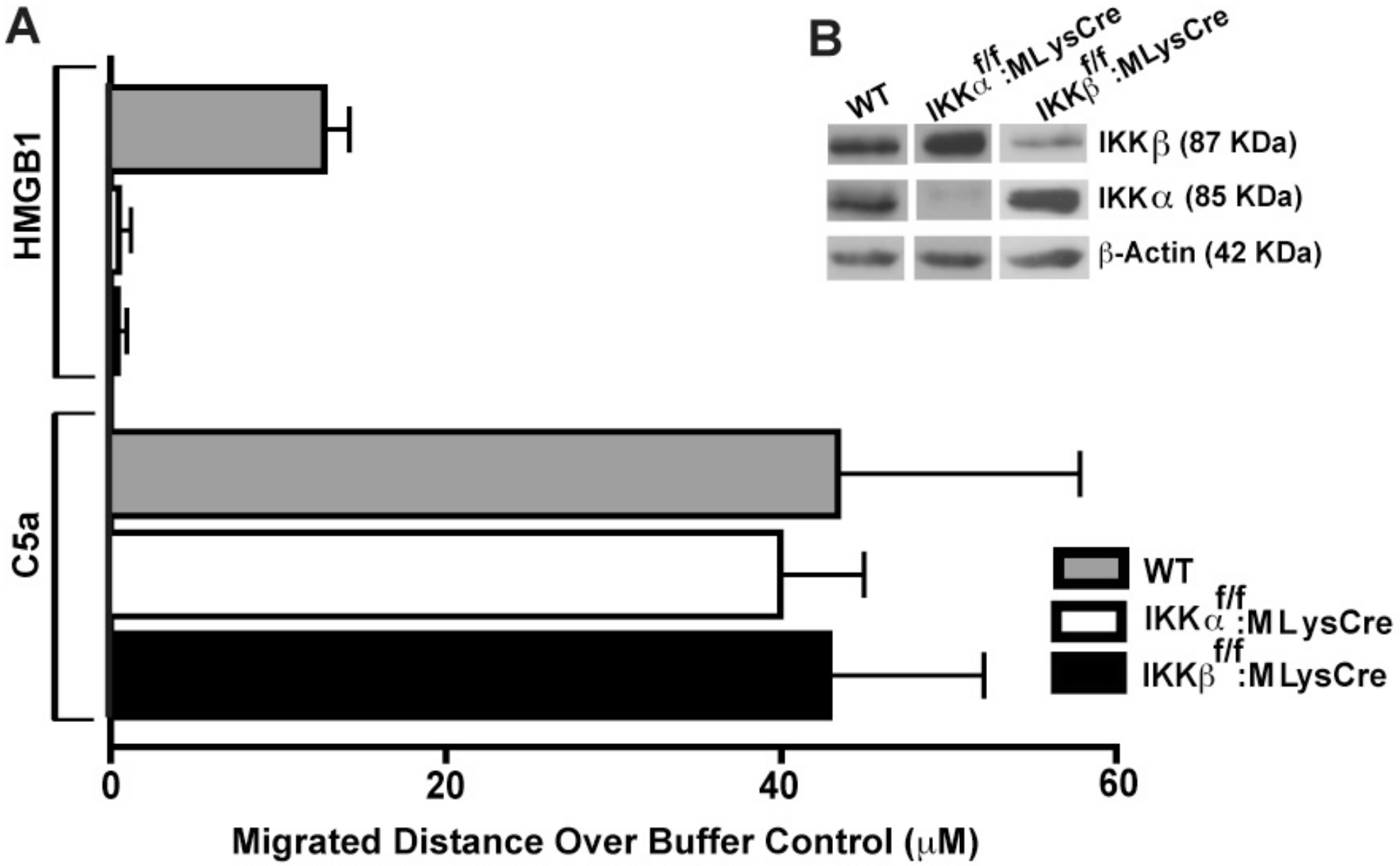

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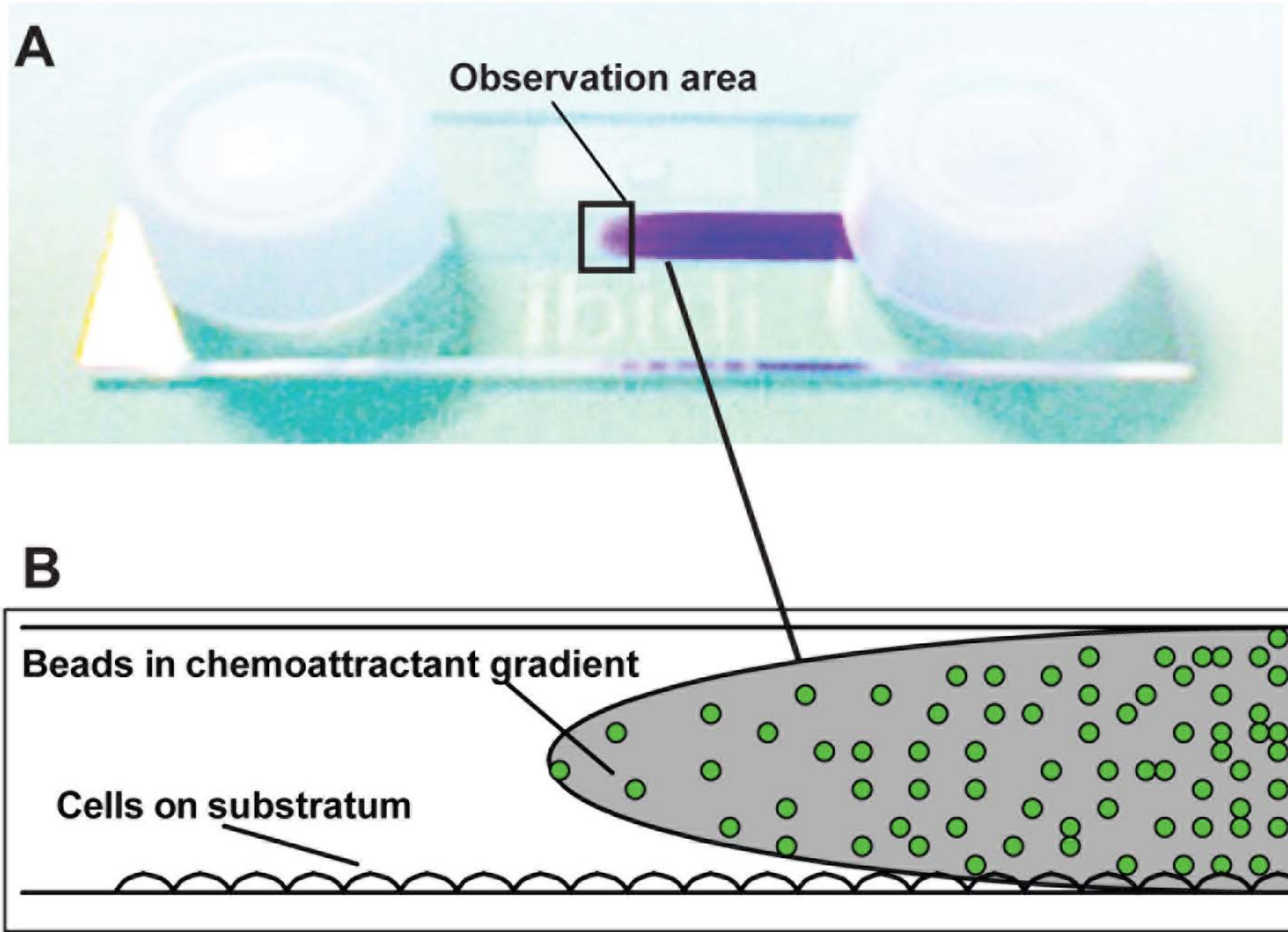

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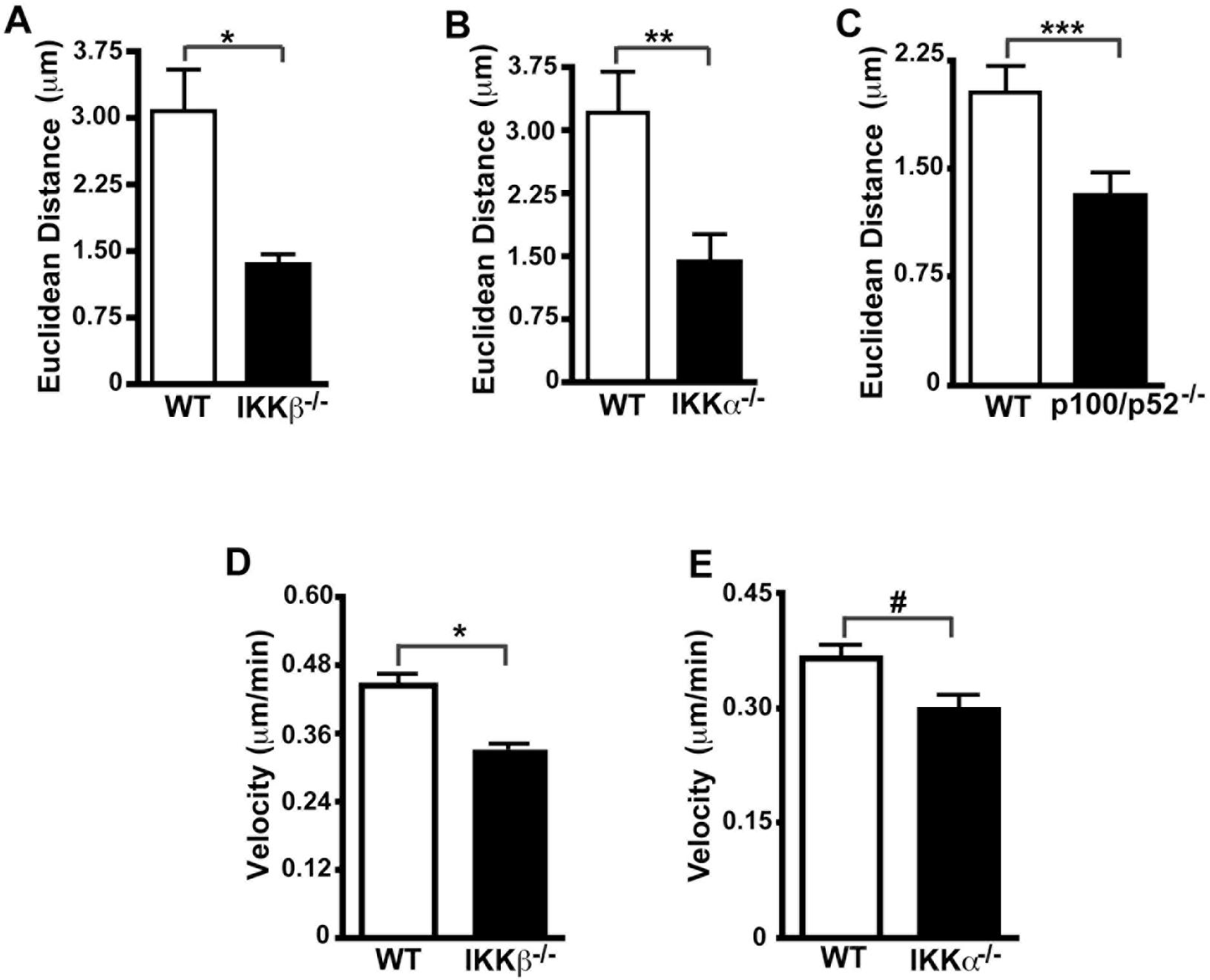
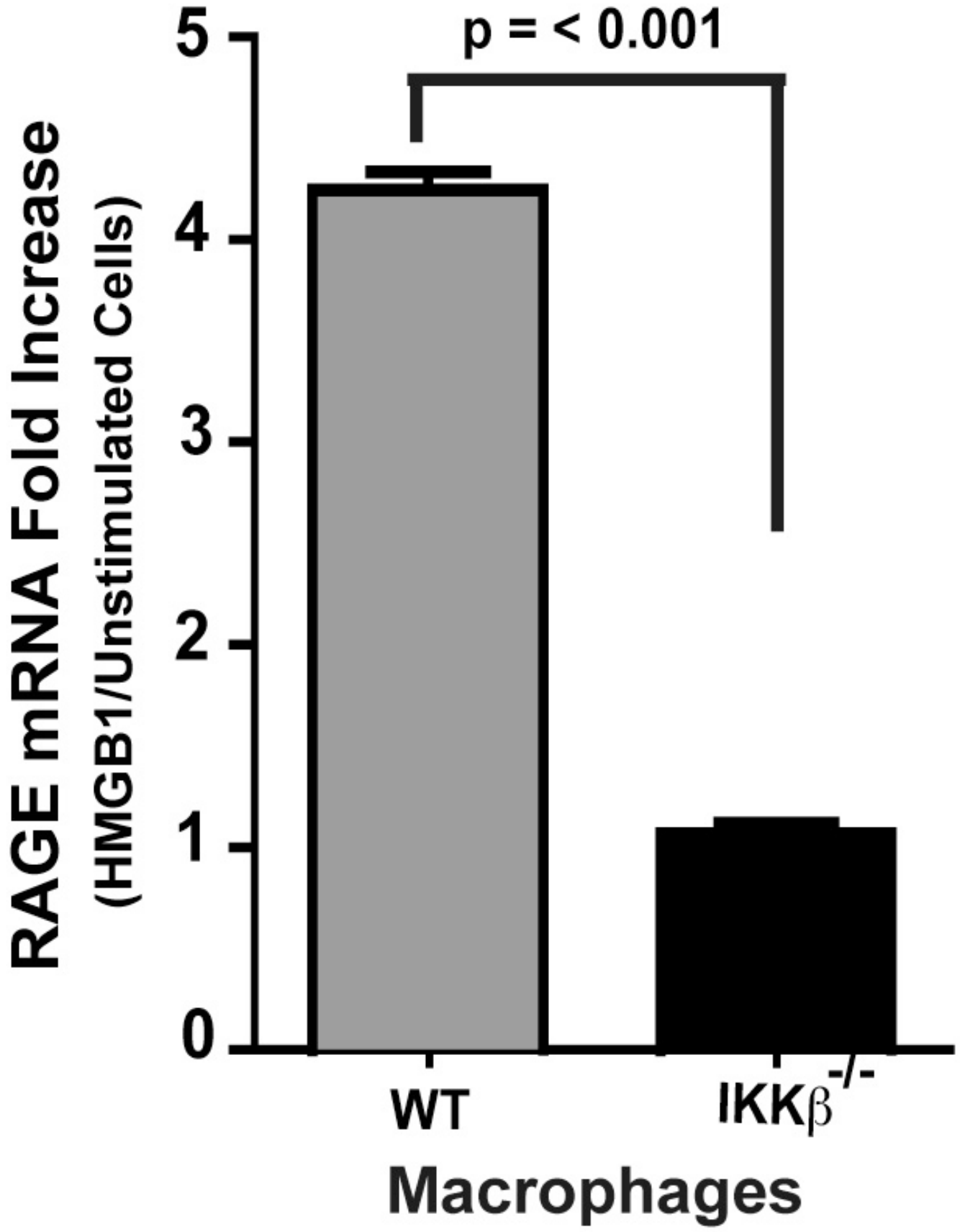


Suppl. Figure 6



Suppl. Figure 7