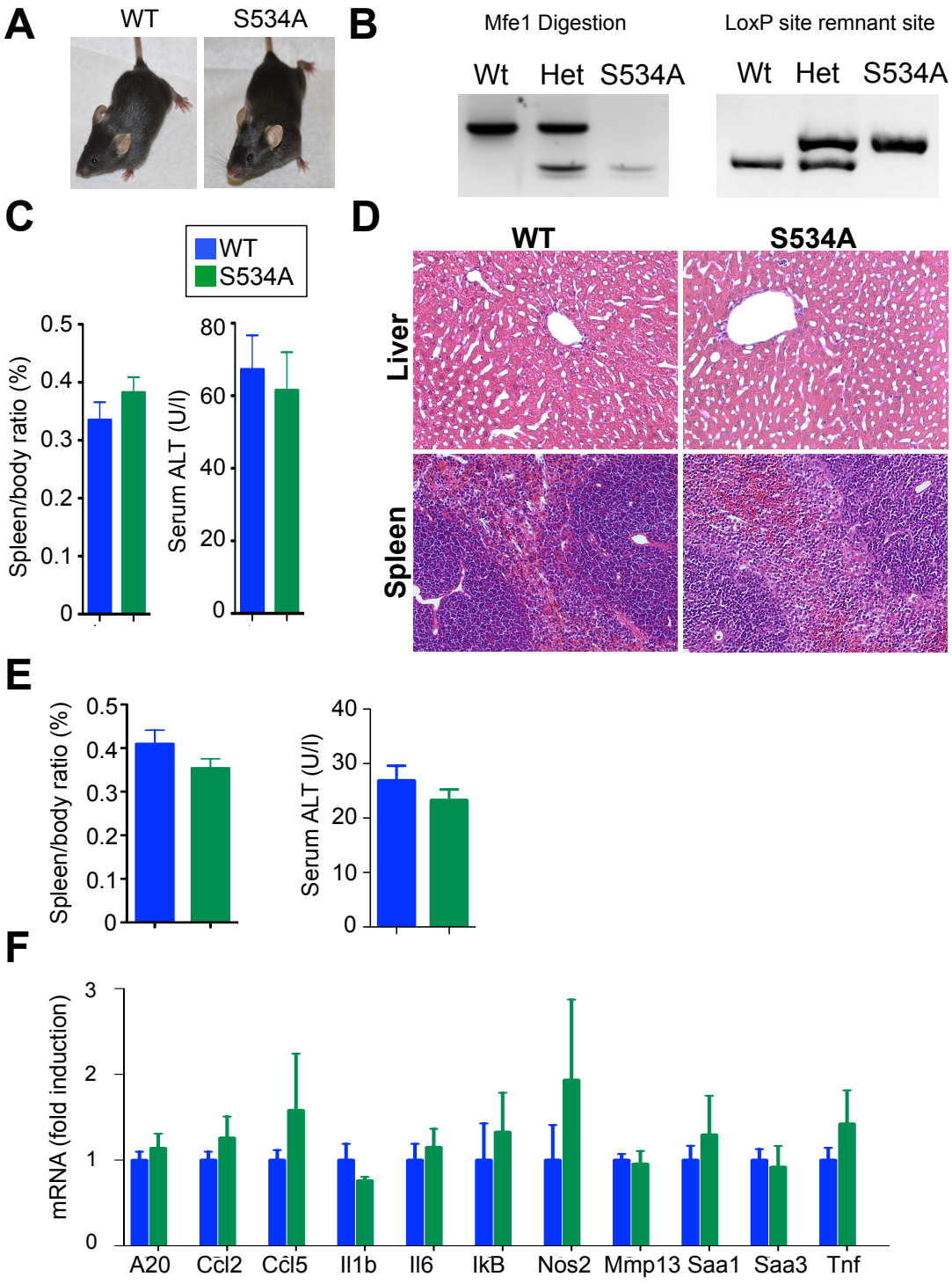

Fig. S1. Phenotypic characterization of S534A knock-in mice. (**A**) Close-up pictures of wild-type (WT) and S534A mice. (**B**) Two strategies for PCR-based genotyping of the mutant allele were used. Left: PCR amplification of a region flanking an Mfe I restriction site that was coupled to the mutation in the targeted allele. Right: Amplification of the remnant loxP site (after excision of the neomycin cassette) that was present on the mutant allele. (**C** and **D**) Spleen-body weight ratio and serum ALT levels were measured and H&E staining of the liver was performed on eight-week-old WT (n = 6) and S534A (n= 6) mice. (**E** and **F**) Spleen-body weight ratio and serum ALT were measured (E) and qPCR analysis of inflammatory gene expression in the liver (F) was performed on one-year-old male S534A (n = 5) and WT (n = 5) mice. Data are means ± SD of 5 mice of each genotype.

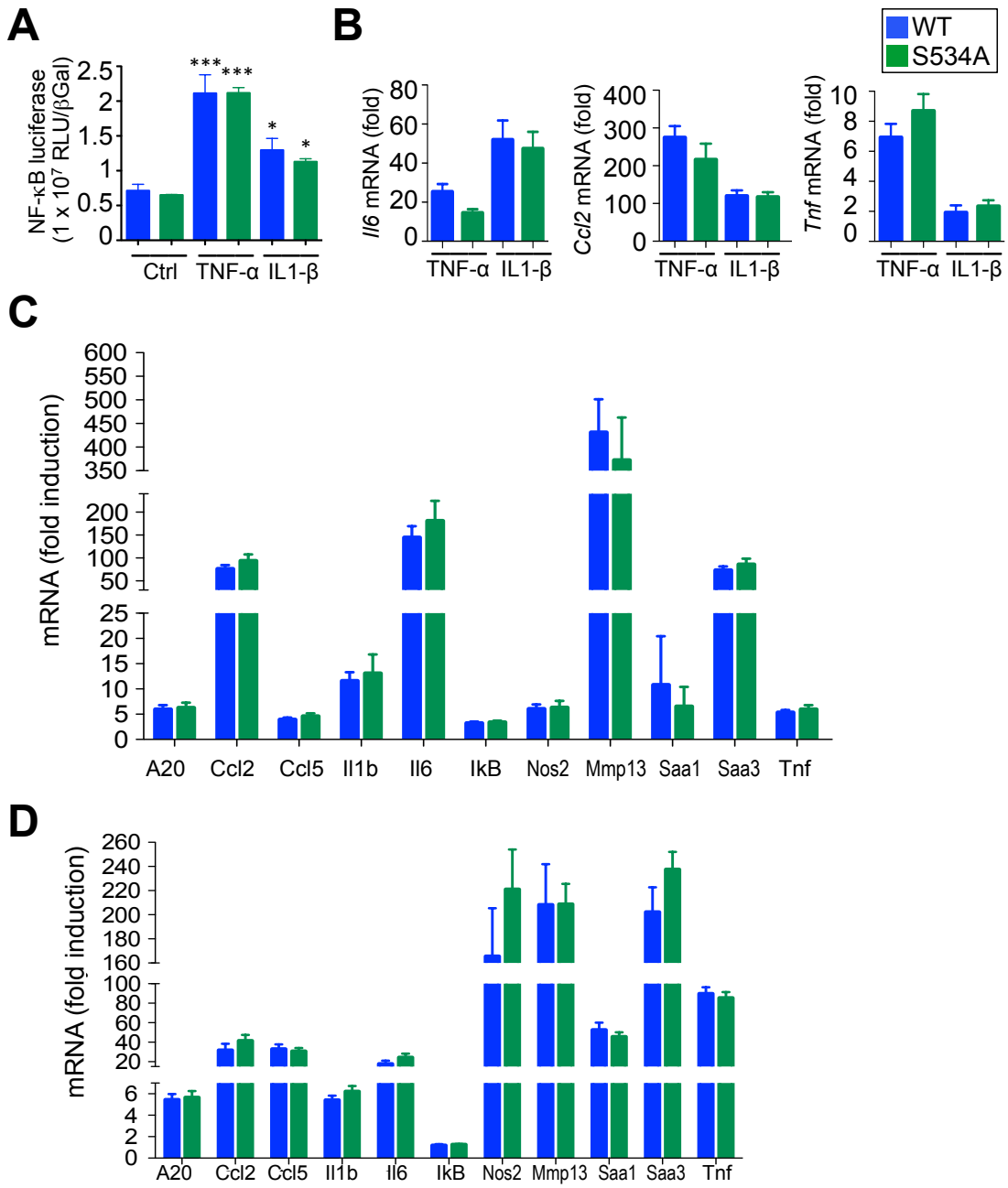

Fig. S2. Determination of NF-κB activation in MEFs, spleens, and livers from WT and S534A mice. (**A**) WT and S534A MEFs were transduced with an adenoviral NF-κB reporter. The cells were left untreated (Ctrl) or were treated for 6 hours with TNF-α (30 ng/ml) or IL-1β (5 ng/ml) before luciferase activity was measured. (**B**) WT and S534A MEFs were treated as described in (A) before being subjected to qPCR analysis of the abundances of the indicated mRNAs. Data in (A) and (B) are means ± SD of three independent experiments, each performed in triplicate. (**C**) WT (n = 9) and S534A (n = 9) mice were injected intravenously with LPS (1 mg/kg) and sacrificed 4 hours later. Splenocytes were analyzed by qPCR to determine the relative abundances of the indicated mRNAs. Data are means ± SD of 9 mice of each genotype. (**D**) WT (n = 8) and S534A (n = 10) mice were injected intravenously with TNF-α (5 μg/kg) and the mice were sacrificed 4 hours later. mRNA was extracted from the liver and analyzed by qPCR to determine the relative abundances of the indicated mRNAs. Data are means ± SD of 8 WT and 10 S534A mice. **P* < 0.05, ****P* < 0.001 vs. Ctrl.

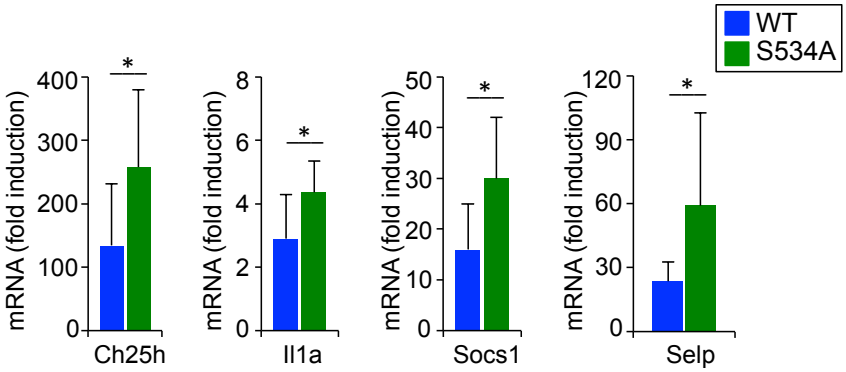

Fig. S3. Analysis of gene expression in the livers of WT and S534A mice injected with low-dose LPS. WT (n=7) and S534A (n=7) mice were injected intravenously with LPS (1 μg/kg) and sacrificed 4 hours later in one single experiment. Expression of *Ch25h*, *Il1a*, *Socs1*, and *Selp* mRNAs was determined by qPCR analysis and is expressed as fold induction in comparison to liver samples from untreated mice. Data are means ±SD of 7 mice per genotype .*$P < 0.05$ vs. WT.

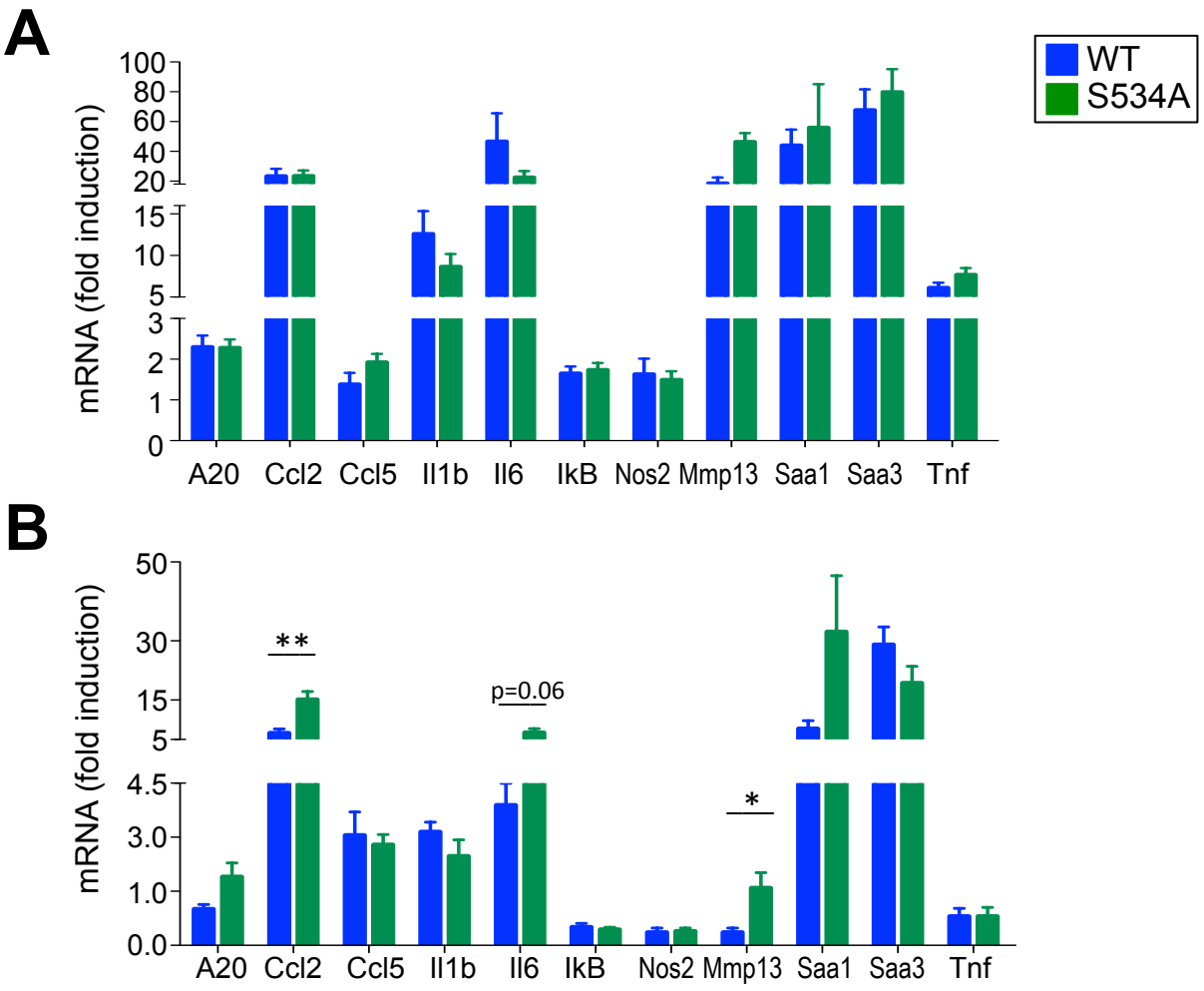

Fig. S4. Expression of NF-κB–dependent genes in the spleens of WT and S534A mice at early and late time points after injection with low-dose LPS. (A and **B)** WT and S534A mice were injected intravenously with low-dose LPS (1 μg/kg) and spleens were collected 4 (A) and 8 (B) hours later. Expression of the indicated mRNAs was determined by qPCR analysis and is expressed as fold induction in comparison to liver samples from untreated mice. Data are means ± SD of 9 mice of each genotype (A) or of 6 WT and 10 S534A mice (B). $*P < 0.05$, $**P < 0.01$.

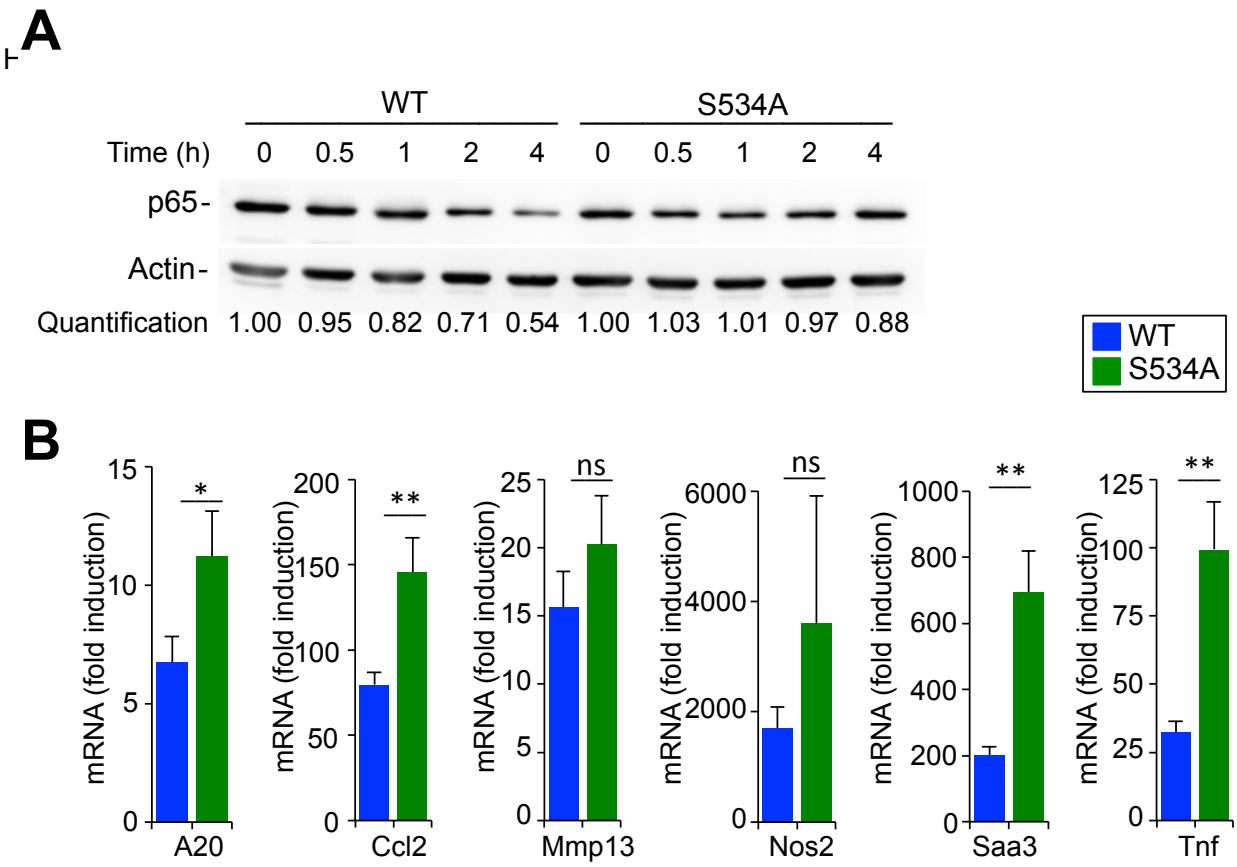

Fig. S5. Analysis of p65 protein abundance and NF-κB target gene expression in macrophages from WT and S534A mice. (**A**) Bone marrow-derived macrophages (BMDM) from WT and S534A mice were left untreated or were treated with LPS (100 ng/ml) for the indicated times. Cells were analyzed by Western blotting with antibodies against the indicated proteins. (**B**) BMDM from WT (blue) and S534A (green) mice were treated with LPS for 6 hours. Expression of the indicated mRNAs was determined by qPCR analysis and is expressed as fold induction in comparison to untreated control macrophages. Data are means ± SD of four experiments. * $P<0.05$; ** $P<0.01$; ns, non-significant

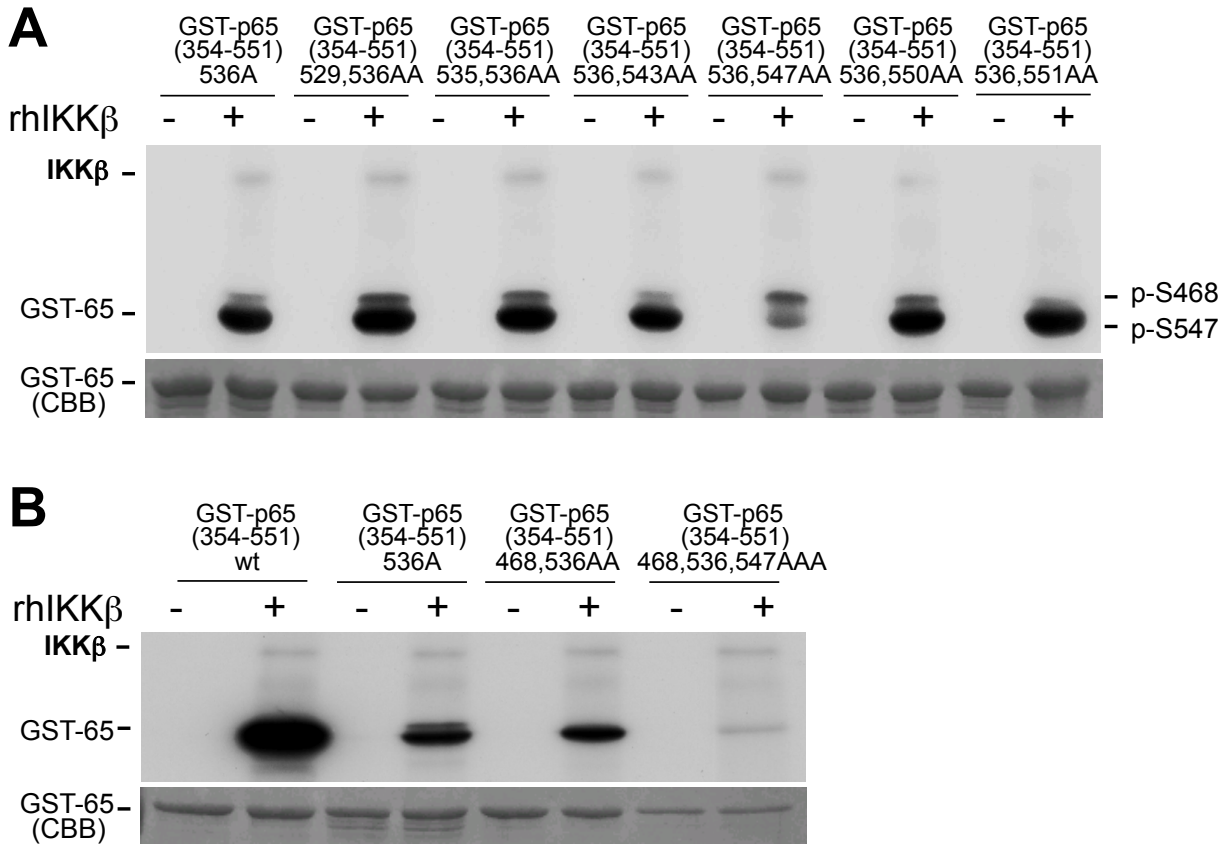

Fig. S6. IKK phosphorylates p65 at multiple sites. (**A** and **B**) p65 phosphorylation sites were analyzed by in vitro kinase assay in which recombinant IKKβ protein was incubated with WT or mutant GST-p65 proteins (containing amino acid residues 354 to 551). The presence of equal amounts of GST-p65 substrate was confirmed by coomassie blue ("CBB") staining. The presence of IKKβ was confirmed by detection of an autophosphorylation band of the appropriate size. (**C**) IKK phosphorylation sites in addition to the known Ser^{468} (S468) and Ser^{536} (S536) phosphorylation sites were investigated with different S536A constructs that had additional serine residues mutated. (**D**) Analysis of the in vitro phosphorylation of triple S468, S536A, and S547A mutants.