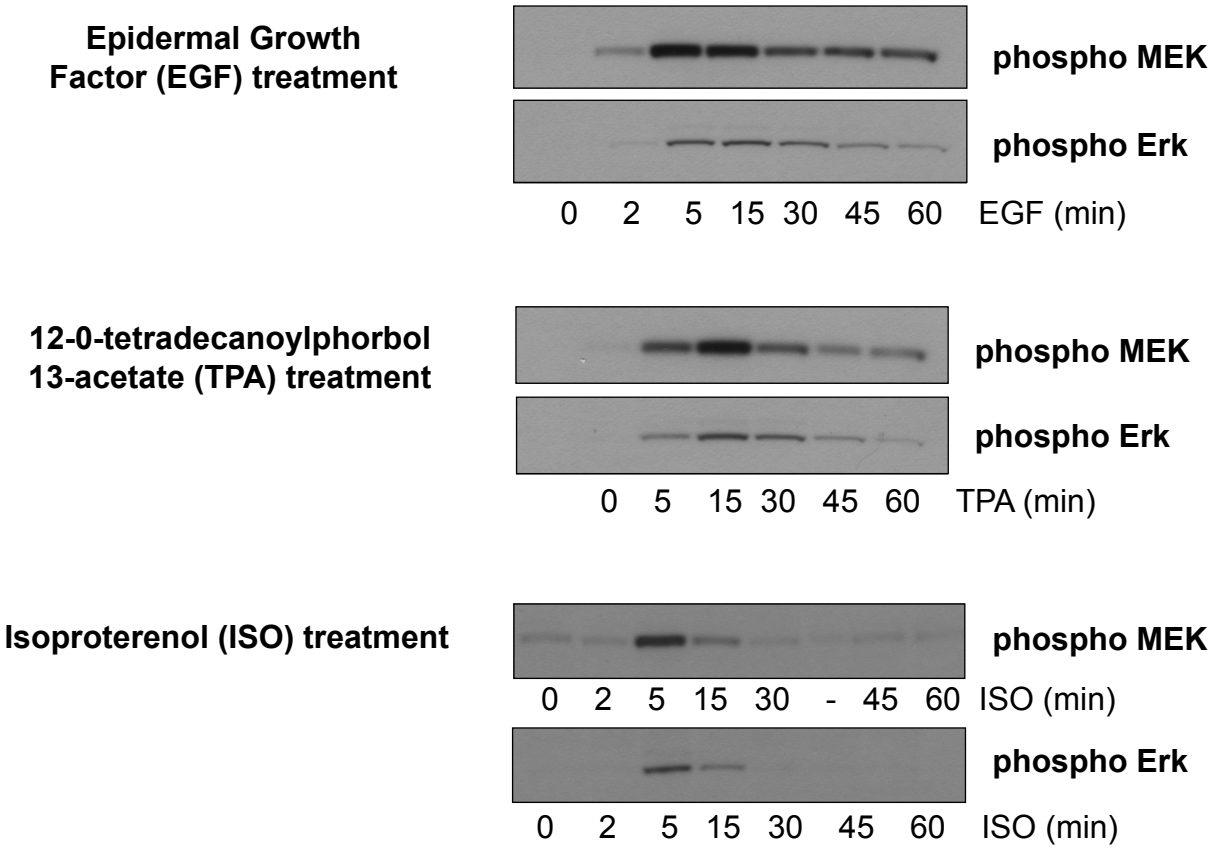
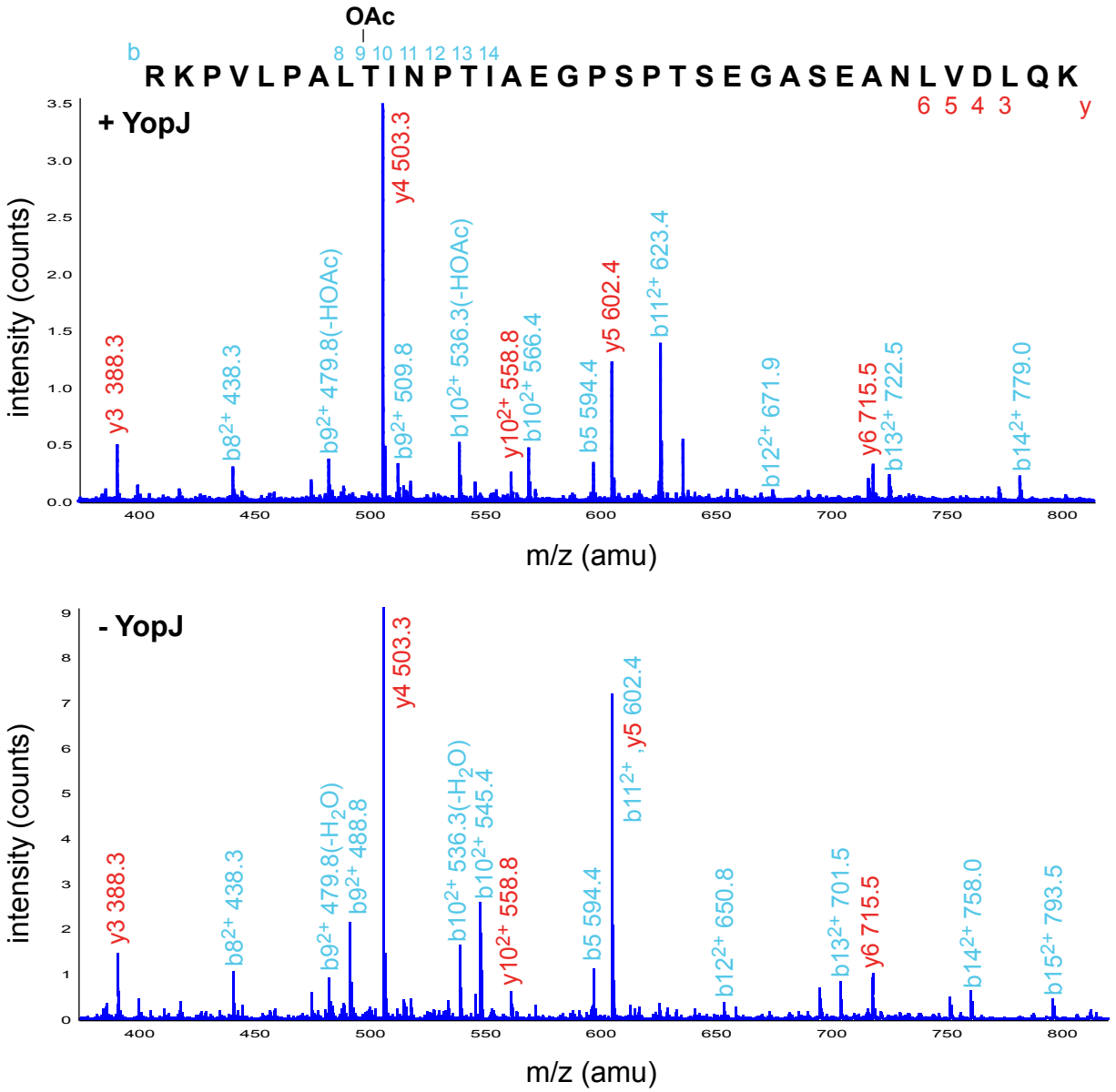
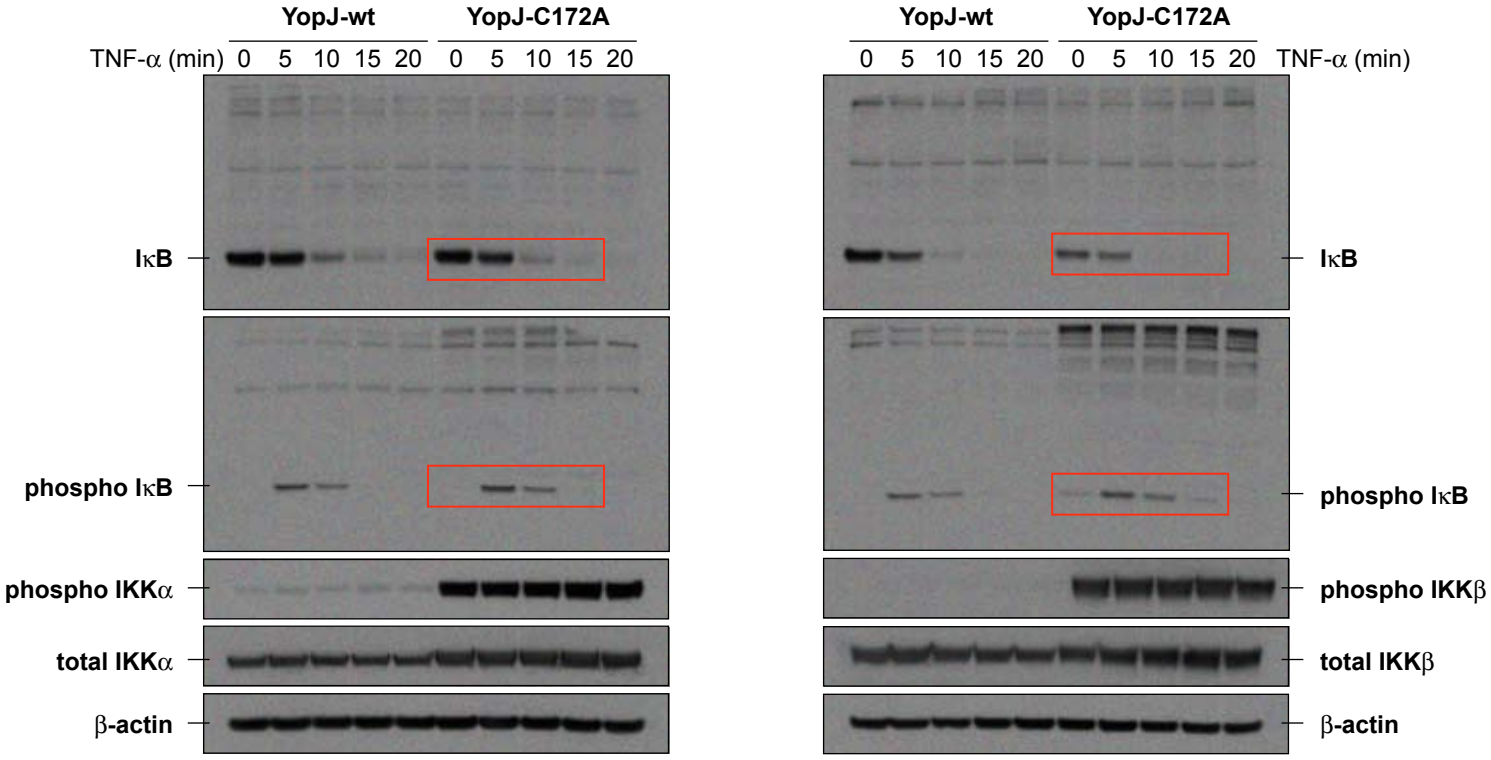


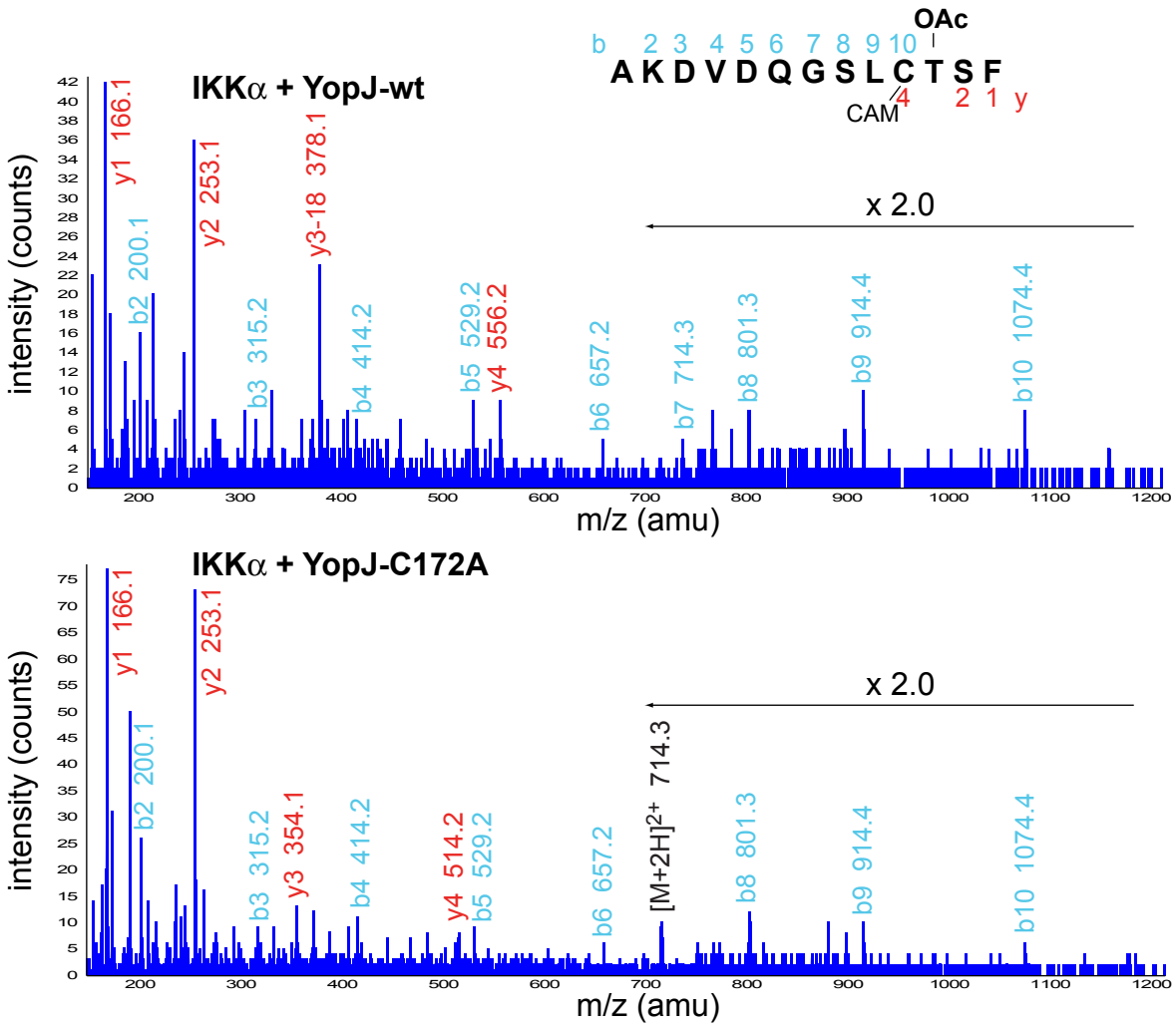
Supporting Figure 4 Stimulation of MEK and Erk phosphorylation by various extracellular stimuli. HeLa cells that had been serum-starved for 16 hours were stimulated with either EGF (20 ng/ml), TPA (200 ng/ml) or isoproterenol (10 μM) at 37ºC and the activation (by phosphorylation) of MEK and Erk was detected by the use of phospho-specific antibodies. Isoproterenol was chosen as the stimulus for the experiments reported in this paper because the rapid activation and decay of the signals allowed us to collect a complete time-course.



Supporting Figure 5 YopJ acetylates MEK2 on Thr13. Tryptic digests of MEK2 purified from YopJ-coexpressing cells (+) and control cells (-) were subjected to LC-MS/MS. A mass difference of 42 amu (corresponding to the mass of one acetyl group) was detected in the peptides corresponding to aa5-aa39. Collision-induced fragmentation of these peptides is depicted here. Both peptides display the same y-ion series (up to y13, although data only up to y6 is shown here) and identical m/z ratios for the b-series ion, b8. The difference between the b9 (and subsequent) ions in the two preparations indicates that the threonine residue at position 13 in MEK2 is O-acetylated by YopJ.



Supporting Figure 6 YopJ protects IκB from degradation in IKKβ-overexpressing cells by modifying IKK. HEK293 cells were cotransfected with IKKα and either YopJ-wt or YopJ-C172A (left panels). Similarly, they were cotransfected with IKKβ and either YopJ-wt or YopJ-C172A (right panels). 24 h after transfection cells were serum-starved for 16 h and then stimulated with 20 ng/ml TNF-α. At the indicated time-points cells were harvested and processed for Western Blotting. The boxed regions in the upper panel of anti-IκB blots show that the resting amount of IκB is much lower in cells overexpressing IKKβ and inactive YopJ compared to those expressing IKKβ and active YopJ. This is owing to the fact that (even in the absence of TNF-α stimulation) overexpressed IKKβ autoactivates and phosphorylates IκB leading to its ubiquitination and degradation by the proteasome. This results in the reduced resting levels of IκB seen in YopJ-C172A coexpressing cells. Co-expressed wild type YopJ, however, modifies IKKβ inhibiting its ability to phosphorylate IκB - thus resulting in higher resting levels of IκB. This interpretation is reinforced by the phospho IκB blot that shows elevated levels of high molecular weight poly-ubiquitinated phospho IκB in the C172A co-expressing cells. Also, the phospho IκB blot shows that upon TNF-α treatment the kinetics of appearance of phospho IκB is reduced in C172A co-expressing cells (red box) reflecting the lowered amount of IκB present in the cells at the time of TNF-α stimulation. TNF-α treatment results in the complete degradation of IκB presumably due to phosphorylation by the small percentage of unmodified IKKβ that would be present in YopJ-wt co-expressing cells. Similar effects are not seen with IKKα overexpressing cells because even though IKKα autoactivates (see phospho IKKα blot) its preferred target is not IκB. The lower three panels show that both IKKa and IKKβ are modified by wild type YopJ such that their activation is inhibited, but total protein amounts are unaffected.



Supporting Figure 7 YopJ acetylates IKKα on Thr179. Flag-IKKα was prepared by immunoprecipitation from cells co-expressing either YopJ-wt or the inactive mutant YopJ-C172A. Peptides resulting from chymotrypsin treatment of the preparations were resolved by nanoscale liquid chromatography and analysed by mass spectrometry. From such an LC-MS/MS experiment, a doubly charged ion at m/z 735.3 corresponding to the amino acid sequence A^{169}KDVDQGSLC*TSF^{181} (C* is carbamidomethylcysteine), with an additional mass of 42 amu (one acetyl group) was detected. This ion was selected and subjected to collision-induced fragmentation. Comparison of the fragmentation data from this modified peptide (upper panel) with that from the unmodified doubly charged ion at m/z 714.3 (lower panel), shows that they have the same b-ion series (b2-10) and y1 and y2 ions. The difference of 42 amu in y4 ions indicates that the acetylation site is on threonine 179 of IKKα.