SUPPLEMENTARY MATERIAL FOR:

A high-throughput assay for phosphoprotein-specific phosphatase activity in cellular extracts

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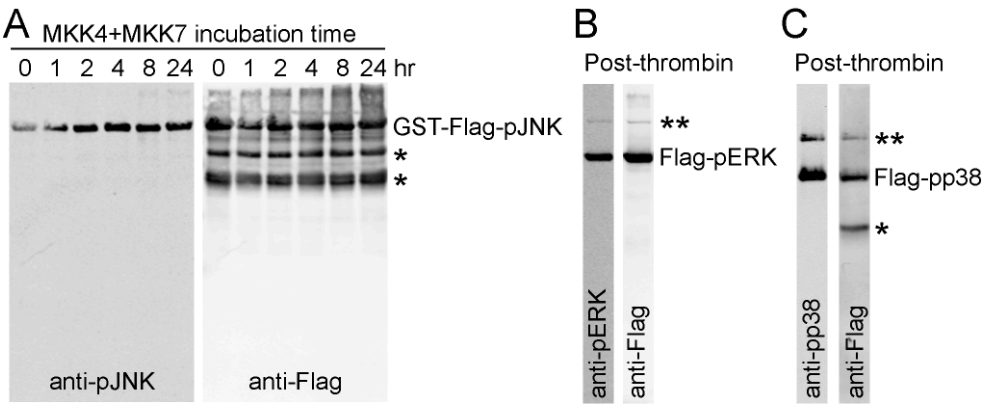
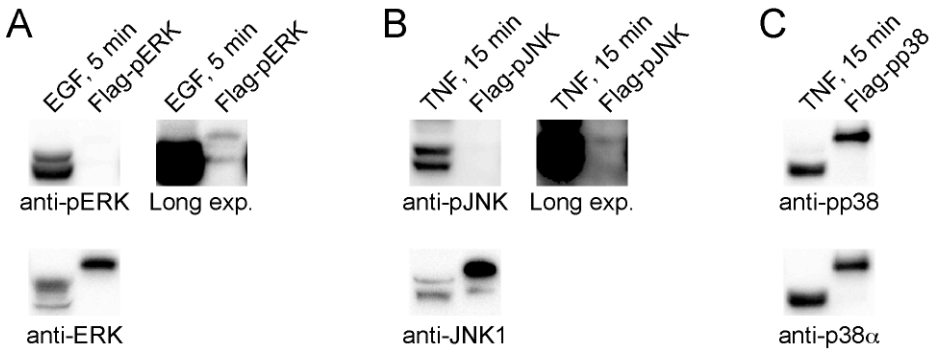
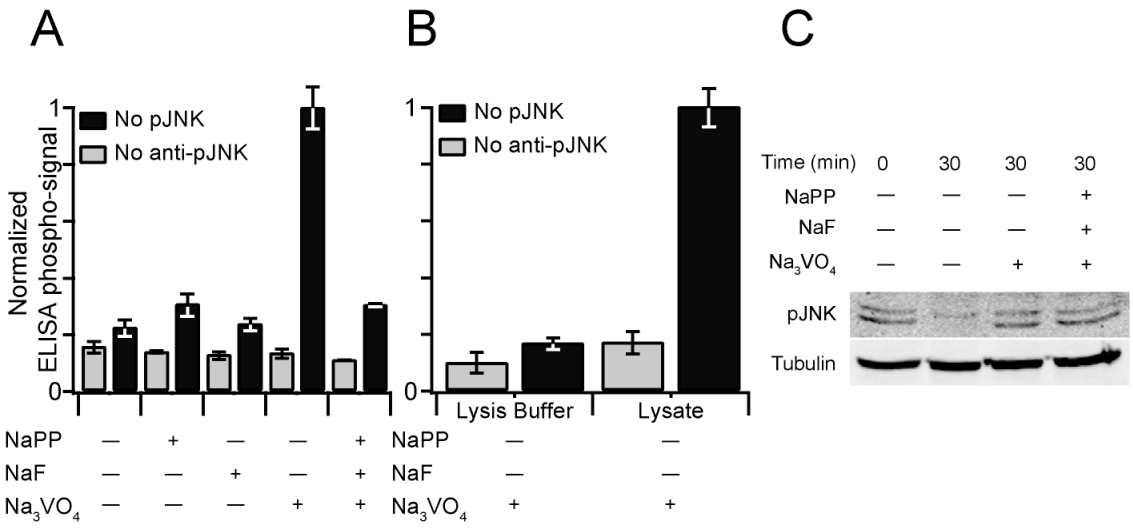


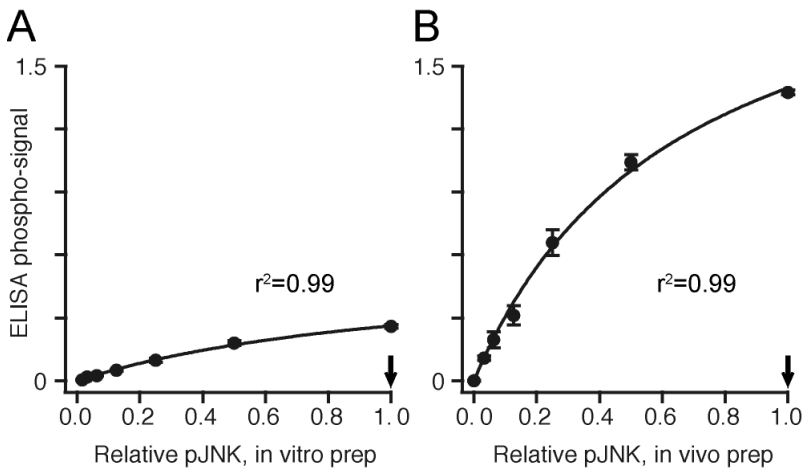
SUPPLEMENTAL FIG. 1. In vitro phosphorylation and thrombin digestion of MAPKs does not give rise phosphorylated cleavage products. *A*, extended phosphorylation of GST-Flag-JNK leads to saturated phosphorylation but no change in JNK cleavage products. Agarose beads carrying recombinant GST-Flag-JNK were incubated with constitutively active MKK4 and MKK7 for the indicated times and analyzed by immunoblotting for pJNK (left) or Flag (right). Single asterisks indicate Flag-containing JNK cleavage products, which are not phosphorylated by MKK4 and MKK7. *B* and *C*, thrombin digestion of GST-Flag-pMAPKs releases Flag-pMAPKs without creating pMAPK cleavage products. Agarose beads carrying *B*, GST-Flag-pERK or *C*, GST-Flag-pp38 were incubated with thrombin, and supernatants were analyzed by immunoblotting for *B*, pERK (left) and Flag (right) or *C*, pp38 (left) and Flag (right). Single asterisk indicates Flag-containing p38 cleavage product that is not phosphorylated, and double asterisks indicate small amounts of GST-Flag-pMAPK that was released but not cleaved during the thrombin digestion. In *A*–*C*, note that cleavage products are not detectably phosphorylated and thus should not interfere with the ELISA measurements.



SUPPLEMENTAL FIG. 2. Recombinant pMAPKs are phosphorylated at or below the stoichiometry observed in vivo for endogenous MAPKs. *A*, Flag-pERK was compared with endogenous pERK from HT-29 cells stimulated with 100 ng/ml EGF for 5 min by immunoblotting for pERK (upper; right shows a longer exposure to visualize Flag-pERK) with total ERK (lower) used as a loading control. *B*, Flag-pJNK was compared with endogenous pJNK from HT-29 cells stimulated with 100 ng/ml TNF for 15 min by immunoblotting for pJNK (upper; right shows a longer exposure to visualize Flag-pJNK) with total JNK1 (lower) used as a loading control. *C*, Flag-pp38 was compared with endogenous pp38 from HT-29 cells stimulated with 100 ng/ml TNF for 15 min by immunoblotting for pp38 (upper) with total p38α used as a loading control.



SUPPLEMENTAL FIG. 3. Na_3VO_4-treatment of cell lysates creates a false-positive pJNK signal in the ELISA format. *A,* the Na_3VO_4-induced signal requires anti-pJNK primary antibody. The pJNK PPase assay was performed with inhibitor-treated lysates in the presence and absence of immobilized pJNK or the anti-pJNK primary antibody. Note the large increase in ELISA signal in the absence of immobilized pJNK. *B*, the Na_3VO_4-induced signal requires PPase lysates. The PPase assay was performed with Na_3VO_4-treated lysate or lysis buffer in the presence or absence of immobilized pJNK or the anti-pJNK primary antibody. *C*, Na_3VO_4 does not cause increased phosphorylation of endogenous JNK. PPase lysates were incubated for the indicated times at 30°C and blotted for pJNK with tubulin as a loading control. Data are shown as the mean ± S.E.M. of three independent assay replicates.



SUPPLEMENTAL FIG. 4. JNK phosphoprotein yield is substantially improved by in vivo phosphorylation with MKK4–MKK7 before bacterial protein purification. *A*, two-fold dilution series (relative level 1.0 = ~400 ng) of pJNK prepared by in vitro phosphorylation of purified GST-JNK with purified constitutively active MKK4 and MKK7. Data are replotted from Fig. 2*B* with a change of scale on the y-axis for comparison. *B*, two-fold dilution series (relative level 1.0 = ~500 ng) of pJNK prepared in vivo by co-induction of GST-JNK, MKK4, and MKK7 in bacteria. MKK4–MKK7 were introduced as a bicistronic plasmid with a different origin of replication and selection marker for coexpression with the GST-JNK plasmid. Note the ~fourfold higher pJNK ELISA signal compared to (*A*). Data are shown as the mean ± S.E.M. of 3–4 independent assay replicates.