Supplemental Information

TNF-stimulated MAP kinase activation mediated by a Rho family GTPase signaling pathway.

Shashi Kant, Wojciech Swat, Sheng Zhang, Zhong-Yin Zhang, Benjamin G. Neel, Richard A. Flavell, and Roger J. Davis

Supplemental Figures S1 – S4
Supplementary Figure S1. Expression of MLK isoforms.

Total RNA isolated from wild-type MEF was employed to measure the expression of *Mlk1, Mlk2, Mlk3*, and *Mlk4* mRNA by quantitative Taqman® RT-PCR analysis. The data are normalized for the amount of *Gapdh* mRNA detected in each sample (mean ± SD; n = 4).
Supplementary Figure S2. Construction of MLK2-deficient mice.

(A) Strategy for the creation of $\text{Mlk2}^{-/-}$ mice. The structure of the $\text{Mlk2}$ genomic locus and the targeted locus are illustrated. The kinase domain is encoded by exon I and the CRIB domain is encoded by exons V and VI. Homologous recombination was employed to replace $\text{Mlk2}$ exons I - VI with a $\text{Neo}^R$ cassette. The deletion starts within exon I (260 bp 5' of the translation initiation site) and extends to intron VI.

(B) Genomic DNA isolated from mouse tails was examined by PCR to detect wild-type and disrupted alleles of $\text{Mlk2}$ and $\text{Mlk3}$.

(C) Extracts prepared from WT and $\text{Mlk2}^{-/-} \text{Mlk3}^{-/-}$ MEF were examined by immunoblot analysis using antibodies to MLK2, MLK3, and $\alpha$-Tubulin.

Quantitative RT-PCR assays of WT and $\text{Mlk2}^{-/-} \text{Mlk3}^{-/-}$ MEF demonstrated no significant differences in the expression of $\text{Tak1}$, $\text{Tnfr1}$, and $\text{Tnfr2}$ mRNA (data not shown).
Supplementary Figure S3. Effect of MLK2 or MLK3-deficiency on the response to TNF.

WT, Mlk2\(^{-/-}\), and Mlk3\(^{-/-}\) MEF were treated without or with 10 ng/ml TNF\(\alpha\). Protein extracts were examined by immunoblot analysis by probing with antibodies to MAP kinases, phospho-MAP kinases, and I\(\kappa\)B\(\alpha\).

MLK2 or MLK3-deficiency did not alter the activation of the NF-\(\kappa\)B pathway, as monitored by measurement of I\(\kappa\)B\(\alpha\) degradation. Similarly, MLK2 and MLK3-deficiency did not cause major defects in MAP kinase activation.


**Supplementary Figure S4. Effect of MLK-deficiency on Rac-stimulated JNK activation.**

(A) WT and *Mlk2<sup>−/−</sup> Mlk3<sup>−/−</sup> MEF were transduced with a retroviral vector that expresses activated Rac (pBABE-Rac<sup>Q61L</sup>-Puro) or with a control vector (pBABE-Puro). Transduced MEF were selected by incubation with medium supplemented with 2 µg/ml puromycin (72 hrs) at 48 hrs post-infection. Cell lysates prepared from the MEF were examined by immunoblot using antibodies to JNK and phospho-JNK. The immunoblots were quantitated using an ImageQuant LAS4000 machine and ImageQuant TL software (General Electric).

(B) Rac-stimulated JNK activation was compared using cultures of WT and *Mlk2<sup>−/−</sup> Mlk3<sup>ΔCRIB/ΔCRIB</sup> MEF.