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Supplementary Materials for

Complementary Phosphorylation Sites in the Adaptor Protein SLP-76 Promote Synergistic Activation of Natural Killer Cells

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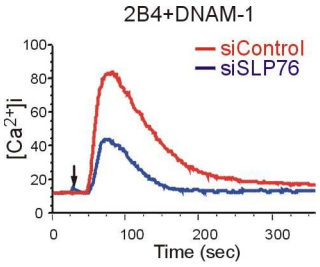

Fig. S1. SLP-76 is required for synergy between 2B4 and DNAM-1. Ca^{2+} mobilization in rested NKL cells transfected with either control siRNA or siRNA specific for SLP-76. NKL cells were stimulated with mAbs against 2B4 and DNAM-1 as described for Fig. 1A. Data are representative of three experiments.

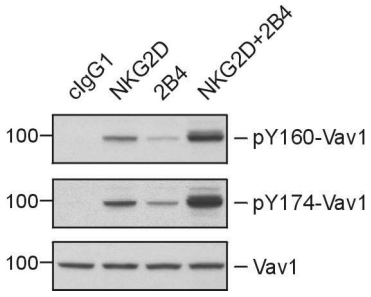

Fig. S2. NKG2D and 2B4 independently induce the phosphorylation of Vav1 at Tyr^{160} and Tyr^{174}. Rested NKL cells were preincubated with isotype control mAb (cIgG1) or mAbs against NKG2D and 2B4 and were stimulated by crosslinking for 2 min as described for Fig. 3A. Cell lysates were analyzed by Western blotting with antibodies against total Vav1 or Vav1 phosphorylated at Tyr^{160} (pY160-Vav1) or Tyr^{174} (pY174-Vav1). Data are representative of three experiments.

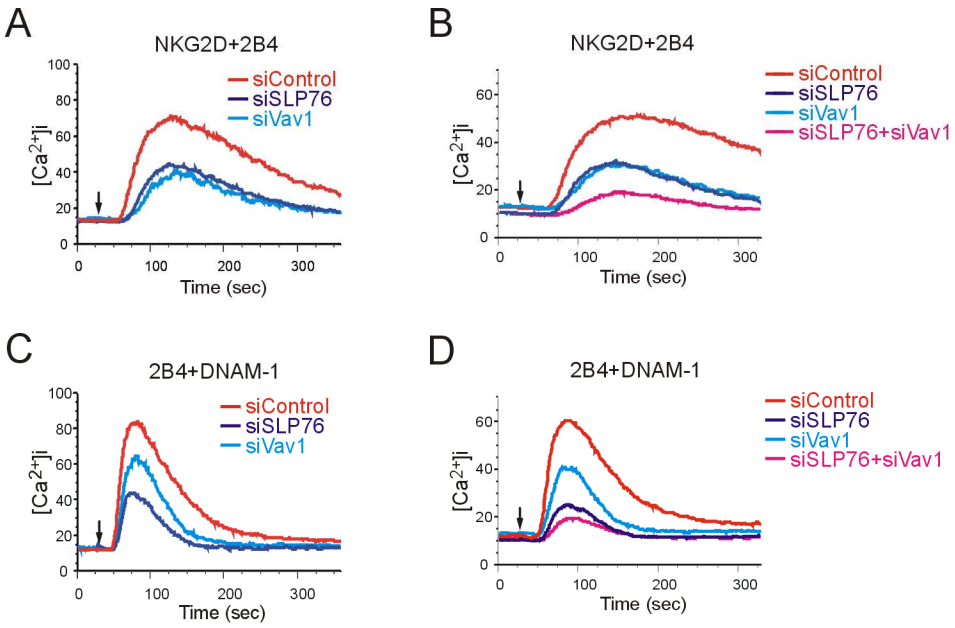

Fig. S3. Synergistic Ca^{2+} mobilization requires both SLP-76 and Vav1. (**A** and **C**) Ca^{2+} mobilization in rested NKL cells transfected with control siRNA or with siRNA specific for SLP-76 or Vav1. Cells were stimulated with mAbs against (A) NKG2D and 2B4 or (C) 2B4 and DNAM-1 as described for Fig. 1A. (**B** and **D**) Ca^{2+} mobilization in rested NKL cells transfected with control siRNA or with siRNAs specific for SLP-76 and Vav1, alone or in combination. Cells were then stimulated with mAbs against (B) NKG2D and 2B4 or (D) 2B4 and DNAM-1 as described for Fig. 1A. Data are representative of three experiments.

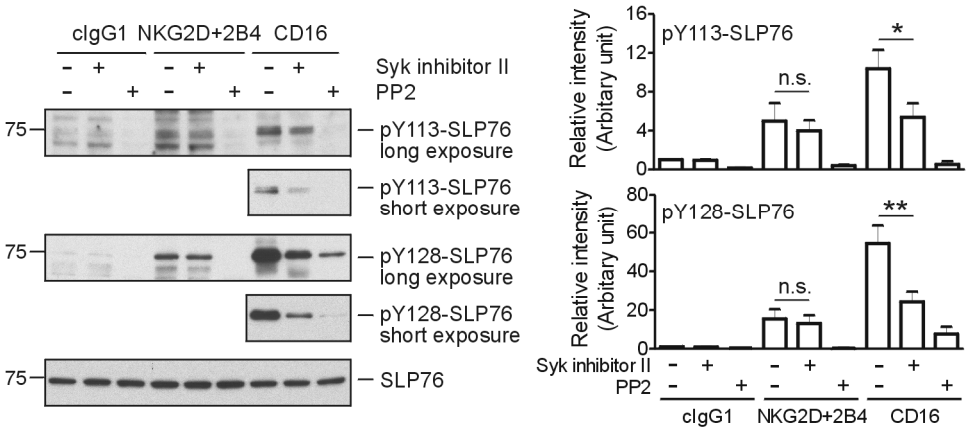

Fig. S4. SLP-76 phosphorylation through combined stimulation of NKG2D and 2B4 is Syk-independent. Primary, resting NK cells were preincubated with isotype control mAb or mAbs specific for the indicated receptors in the presence of 2 μM Syk inhibitor II or 2 μM PP2 for 30 min. After receptor crosslinking for 2 min in the presence of inhibitors, cells were lysed and lysates were analyzed by Western blotting for pY113-SLP-76, pY128-SLP-76, or total SLP-76 (left). Band intensities corresponding to SLP-76 phosphorylated at Tyr^{113} (right, top) or Tyr^{128} (right, bottom) relative to that of total SLP-76 were quantified with ImageJ software. Western blotting data are representative of three experiments, whereas densitometric data are the average of three experiments. n.s., not significant; * $P < 0.05$; ** $P < 0.01$.

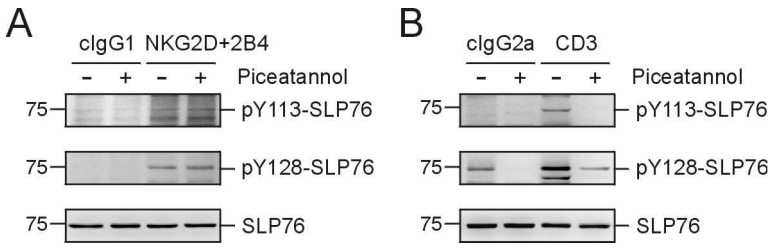

Fig. S5. SLP-76 phosphorylation by combined stimulation of NKG2D and 2B4 is not sensitive to treatment with piceatannol. (**A**) Rested NKL cells were preincubated with isotype control mAb or mAbs against NKG2D and 2B4 in the absence or presence of 10 μM piceatannol for 30 min. After receptor crosslinking for 2 min in the absence or continued presence of the inhibitor, cells were lysed and analyzed by Western blotting for pY113-SLP-76, pY128-SLP-76, and total SLP-76. (**B**) Jurkat cells were preincubated with isotype control mAb or mAb specific for CD3 in the absence or presence of 10 μM piceatannol for 30 min and were stimulated for 2 min as described for Fig. 3A. SLP-76 phosphorylation was analyzed as described for (A). Data are representative of three experiments.

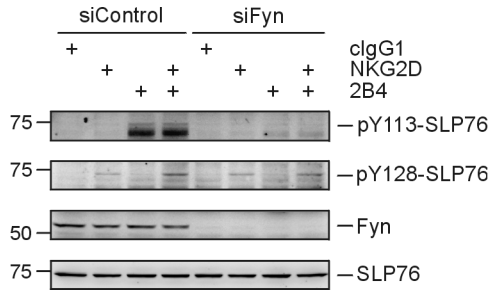

Fig. S6. Fyn is required for the phosphorylation of SLP-76 at Tyr^{113} stimulated by 2B4 but not for the phosphorylation of Tyr^{128} stimulated by NKG2D. Rested NKL cells transfected with control siRNA or Fyn-specific siRNA were stimulated with isotype control mAb (cIgG1) or mAbs against NKG2D, 2B4, or both. After receptor crosslinking for 2 min, cells were lysed and analyzed by Western blotting for pY113-SLP-76, pY128-SLP-76, Fyn, and SLP-76. Data are representative of three independent experiments.

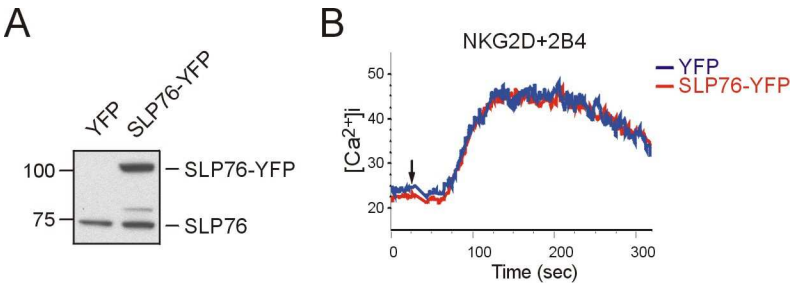

Fig. S7. SLP-76 overexpression does not enhance Ca^{2+} mobilization in response to the combined stimulation of NKG2D and 2B4. NKL cells were transfected with plasmid expressing YFP alone or SLP-76-YFP. (**A**) Rested NKL cells transfected with plasmids encoding the indicated proteins were analyzed by Western blotting for SLP-76. (**B**) Ca^{2+} mobilization in rested YFP^{+} NKL cells transfected with plasmids encoding the indicated proteins. Cells were stimulated with mAbs against NKG2D and 2B4 as described for Fig. 1A. Data are representative of at least three independent experiments.

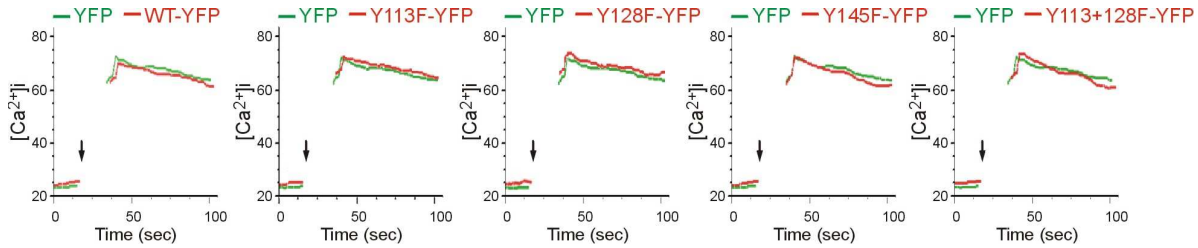

Fig. S8. Treatment of cells with ionomycin results in similar Ca^{2+} mobilization in transfected NKL cells irrespective of the SLP-76 mutant expressed. Ca^{2+} mobilization in rested YFP^{+} NKL cells transfected with siRNA specific for wild-type, endogenous SLP-76 and with plasmids encoding the indicated, siRNA-resistant SLP-76 proteins, as described for Fig. 7. Cells were stimulated with ionomycin. Data are representative of three experiments.

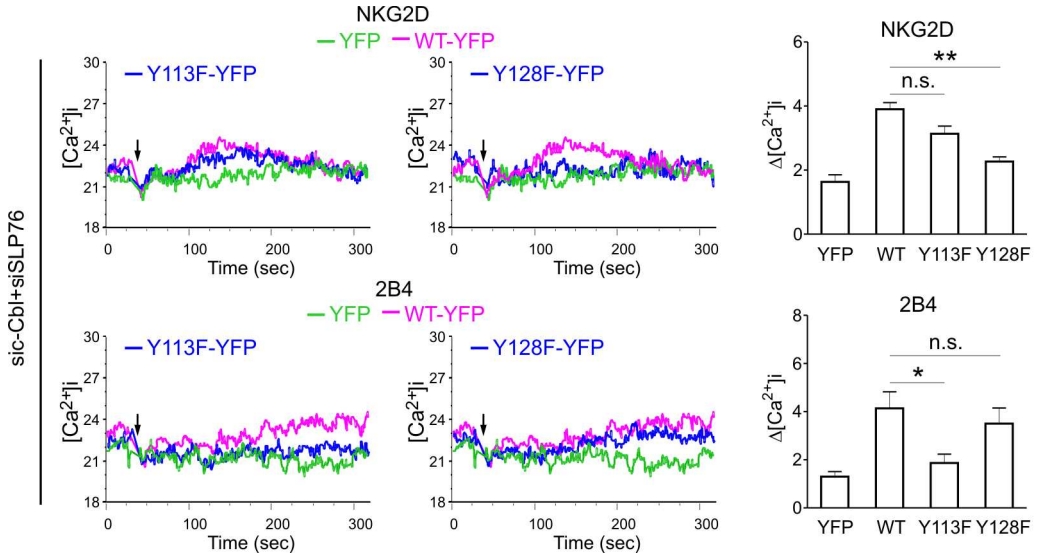

Fig. S9. Different tyrosines in SLP-76 are required for the Ca^{2+} mobilization stimulated by engagement of either NKG2D or 2B4 alone. After knockdown of both c-Cbl and SLP-76 by siRNAs, NKL cells were transfected with plasmids encoding the indicated siRNA-resistant proteins. Ca^{2+} mobilization was measured in rested YFP^+ NKL cells that were stimulated with mAb against NKG2D (top) or 2B4 (bottom) as described for Fig. 1A. The graph on the right shows the difference in peak Ca^{2+} mobilization after stimulation with the indicated mAb relative to the baseline in untreated cells ($\Delta[Ca^{2+}]i$), and is the average of three independent experiments. n.s., not significant; $*P < 0.05$; $**P < 0.01$.