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Ras activation assays. Activation of Ras in D10 T cells was analyzed by a RasGTP pull-down assay (with 15 μl of Raf-1 RBD agarose and 10 x 10^6 cells for each time point) according to the manufacturer's instructions (Upstate Biotechnology) as described previously (Roose, J.P., M. Mollenauer, V.A. Gupta, J. Stone, and A. Weiss. 2005. *Mol. Cell. Biol.* 25:4426–4441). Cells were loaded with 1 μg/ml anti-CD3ε and anti-CD28 (1:500) in the presence of DMSO (control) or H89 (10 μM) on ice for 30 min. Stimulations were induced by adding an equal volume of PBS at 37°C with crosslinking antibody (goat anti–mouse and goat anti–hamster [10 μg/ml]).

Measurement of intracellular Ca^{2+} levels. D10 T cells were loaded with the Ca^{2+} indicator Fluo-3-AM and with 1 μg/ml anti-CD3ε and anti-CD28 (1:500) in the presence of DMSO (control) or H89 (10 μM) on ice for 30 min as described previously (Roose, J.P., M. Mollenauer, V.A. Gupta, J. Stone, and A. Weiss. 2005. *Mol. Cell. Biol.* 25:4426–4441). Cells were subsequently washed and warmed to 37 °C for 5 min before stimulation by with crosslinking with goat anti–mouse and goat anti–hamster (10 μg/ml) and analyzed by FACS.

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