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# The Ability of Sos1 to Oligomerize the Adaptor Protein LAT Is Separable from its Guanine Nucleotide Exchange Activity In Vivo

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## Abstract

The activation of the small guanosine triphosphatase Ras by the guanine nucleotide exchange factor (GEF) Sos1 (Son of Sevenless 1) is a central feature of many receptor-stimulated signaling pathways. In developing T cells (thymocytes), Sos1-dependent activation of extracellular signal–regulated kinase (ERK) is required to stimulate cellular proliferation and differentiation. Here, we showed that in addition to its GEF activity, Sos1 acted as a scaffold to nucleate oligomerization of the T cell adaptor protein LAT (linker for activation of T cells) in vivo. The scaffold function of Sos1 depended on its ability to binding to the adaptor protein Grb2. Furthermore, the GEF activity of Sos1 and the Sos1-dependent oligomerization of LAT were separable functions in vivo. Whereas the GEF activity of Sos1 was required for optimal ERK phosphorylation in response to T cell receptor (TCR) stimulation, the Sos1-dependent oligomerization of LAT was required for maximal TCR-dependent phosphorylation and activation of phospholipase C γ1 and $Ca^{2+}$ signaling. Finally, both of these Sos1 functions were required for early thymocyte proliferation. Whereas transgenic restoration of either the GEF activity or LAT-oligomerization functions of Sos1 alone failed to rescue thymocyte development in *Sos1*-deficient mice, simultaneous reconstitution of these two signals in the same cell restored normal T cell development. This ability of Sos1 to act both as a RasGEF and as a scaffold to nucleate Grb2-dependent adaptor oligomerization may also occur in other Grb2-dependent pathways, such as those activated by growth factor receptors.

## Introduction

Receptor-stimulated activation of the small guanosine triphosphatase (GTPase) Ras is central to many signal transduction pathways that control developmental, physiologic, and

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pathologic processes in the immune system and throughout biology. Depending upon the cellular context, Ras is activated by one or more Ras guanine nucleotide exchange factors (RasGEFs), which catalyze the exchange of guanosine triphosphate (GTP) for guanosine diphosphate (GDP) on the surface of Ras. Activated Ras then signals through multiple effector pathways to regulate varied biological outcomes that depend on the specific cellular context. In T cells, receptor-dependent activation of Ras by the RasGEFs Sos1 and RasGRP1 (Ras guanine nucleotide–releasing protein 1) is required for the normal differentiation of thymocytes in the thymus and for the mature effector functions of mature T cells (1).

Unlike receptor tyrosine kinases (RTKs), the T cell antigen receptor (TCR) lacks both the intrinsic kinase activity and the adaptor functions required to signal to Ras. Instead, engagement of the TCR by peptide-bound major histocompatibility complex (pMHC) stimulates the recruitment of the Src family kinases Lck and Fyn and the Syk family kinase ZAP-70 (ζ chain-associated protein kinase of 70 kD) to the TCR complex, which then act in concert to phosphorylate the membrane-bound adaptor protein LAT (linker for activation of T cells). Phosphorylated LAT (pLAT) can associate with critical proteins to form two complexes that regulate Ras activation. The first consists of PLC-γ1 (phospholipase C γ1), the adaptor proteins GADS and SLP-76 [Src homology 2 (SH2) domain–containing leukocyte phosphoprotein of 76 kD], and the tyrosine kinase Itk [interleukin-2 (IL-2)–inducible T cell kinase], whereas the second complex consists of the adaptor protein Grb2 (growth factor receptor–bound protein 2) and the RasGEF Sos1. On LAT, activated PLC-γ1 cleaves phosphatidylinositol-4, 5-bisphosphate ($PIP_2$) to generate inositol 1,4,5-trisphosphate ($IP_3$), which triggers the release of intracellular stores of $Ca^{2+}$, and diacylglycerol (DAG). DAG activates the RasGEF RasGRP1 both directly by recruiting it to the plasma membrane and indirectly by recruiting and activating protein kinase C θ (PKCθ), which phosphorylates RasGRP1 to increase its RasGEF activity (1).

The RasGEF Sos1 is recruited to LAT through the adaptor Grb2, where it has a very low basal catalytic activity. However, its activity is enhanced by the association of active Ras-GTP to an allosteric pocket on Sos1, which relieves auto-inhibition of the RasGEF domain and increases the catalytic activity of Sos1 up to 80-fold (2). The binding of Ras-GTP to Sos1 to activate it can therefore potentially engage a positive feedback loop between Ras and Sos, primed by either RasGRP1 or basal Sos activity, to be used when enhanced Ras activation is required (3, 4). Active Ras then signals to multiple effector pathways to drive both T cell development and mature T cell function (1).

T cell development is a particularly tractable model system in which to study the biologic consequences of Ras activation because (i) Ras is required at multiple stages of thymocyte development, and (ii) RasGEF abundance is regulated at each of these stages. T cell development can be followed by assessing the presence or absence of the cell-surface markers CD4 and CD8, with the most immature T cells being $CD4^-CD8^-$ (double-negative, DN), which then progress through a $CD4^+CD8^+$ (double-positive, DP) stage before becoming either $CD4^+$ helper T cells or $CD8^+$ effector T cells. Developing thymocytes must transit through at least two distinct developmental checkpoints, governed by signaling from either the pre-TCR or the TCR to Ras, before emerging as functional T lymphocytes. At the

first checkpoint (β-selection), the pre-TCR signals in a ligand-independent manner to stimulate the proliferation and differentiation of thymocytes to the DP stage. At the second developmental checkpoint, the strength and quality of signal transduction through the mature TCR is interrogated. Here, cells that fail to signal through the TCR die by neglect, cells with a TCR that binds to self-antigen–bound MHC complexes with a strong affinity generate strong TCR signals to promote apoptosis (negative selection), whereas cells with a TCR that has weak affinity for self antigen–bound MHC complexes signal weakly and survive (a process known as positive selection) (5).

In the thymus, the relative amounts of the RasGEFs Sos1 and RasGRP1 are developmentally regulated: Sos1 abundance decreases five-fold between the DN and DP stages, with a reciprocal five-fold increase in RasGRP1 abundance (6). During β-selection, whereas Ras is preferentially activated by Sos1 to stimulate proliferation and differentiation to the DP stage, RasGPR4 compensates for the low abundance of RasGRP1 so that Sos1, RasGRP1, and RasGRP4 act in concert to promote development in response to signals from the pre-TCR (7, 8). At the DP stage, signaling through RasGRP1 alone promotes positive selection (9), whereas signaling through either Sos1 or RasGRP1 is sufficient to promote negative selection (7).

A second, RasGEF-independent, role for Sos1 downstream of the TCR has been described. Extensive microscopic analysis of T cells undergoing activation has revealed that the TCR, the critical tyrosine kinases, and most of the proteins involved in the activation of signaling pathways downstream of the TCR, including LAT, Grb2, and Sos1, are found in large (50 to 200 nm in diameter) microclusters at the site of activation (10). Biophysical studies assessing the interactions between LAT, Grb2, and Sos1 have shown that Sos1 has two distinct Grb2-binding sites in its proline-rich region (PRR), and that Sos1 and Grb2 can form a 1:2 complex (11). Similarly, the SH2 domain of Grb2 can bind to one of three phosphorylated tyrosines in the C-terminal tail of LAT, so that LAT and Grb2 can form a 1:3 complex. Based on the multiplicity of Sos1:Grb2 and LAT:Grb2 interactions, it was hypothesized that the Grb2–Sos1–Grb2 complex could nucleate LAT oligomerization. Mutation of the Grb2-binding sites on LAT (in the LAT-3YF mutant) blocks the recruitment of both the Grb2–Sos1 and PLC-γ1–GADS–SLP-76 signaling complexes to LAT (12), as well as the formation of microclusters containing LAT (11), which suggests that protein-protein interactions are necessary for the formation of LAT microclusters. However, despite extensive biochemical and microscopic studies assessing signaling microclusters in vitro and in cell lines, there has been no test of the role of these structures in vivo.

Here, we showed that Sos1 acts both as a RasGEF and as a scaffold to nucleate Sos1-dependent LAT oligomerization to promote normal T cell development. In experiments with mice in which Sos1 was deleted specifically in developing T cells [*Sos1*$(T)^{-/-}$ mice], we showed that Sos1 was not only required for maximal TCR-induced phosphorylation (and activation) of extracellular signal–regulated kinase (ERK), but was also critical for TCR-stimulated phosphorylation of LAT and for LAT microcluster formation. Inhibition of the Sos1-dependent oligomerization of LAT led to disrupted phosphorylation of PLC-γ1 and $Ca^{2+}$ signaling, events generally thought to be independent of Sos1 function. In experiments with Sos1 transgenic mice that independently restored either the RasGEF activity of Sos1 or

its ability to oligomerize LAT, we showed that these functions of Sos1 controlled different aspects of TCR-dependent signaling. The RasGEF activity of Sos1 was required for maximal TCR-stimulated ERK phosphorylation, whereas the ability of Sos1 to cause LAT oligomerization promoted optimal PLC-γ1 phosphorylation and $Ca^{2+}$ flux. Finally, we showed that both of these Sos1-dependent signals was required for normal T cell development, and that simultaneous restoration of these two signals in trans restored normal intrathymic T cell development. These data highlight the multifunctional nature of Sos1 in TCR-induced signal transduction, and provide a new paradigm for Sos1-dependent signaling in receptor-stimulated signal transduction.

## Results

### Sos1 is required for TCR-dependent LAT signaling

We previously showed that the RasGEFs Sos1 and RasGRP1 act in concert to promote maximal TCR-dependent activation of ERK in developing T cells (7). While characterizing the role of Sos1 in TCR-stimulated signaling in cells isolated from *Sos1*$(T)^{-/-}$ mice (that is, in cells stimulated with antibodies against CD3ε and CD4), we noticed not only a reduction in the extent of ERK1/2 activation (Fig. 1A) (7), but we also unexpectedly saw a similar 40 to 50% reduction in TCR-stimulated $Ca^{2+}$ flux (Fig. 1B). These data suggested that, in addition to its role as a RasGEF, Sos1 played an unexpected role in controlling PLC-γ1–dependent signaling. Western blotting analysis for phosphorylated tyrosine (pTyr) residues to assess the overall activation of signaling components upstream of PLC-γ1 in response to TCR stimulation revealed a reduction in the extent of phosphorylation of several proteins, including a prominent reduction in the abundance of phosphorylated LAT (pLAT, Fig. 1C). Using phospho-specific antibodies, we found that whereas activation of the upstream kinase ZAP-70 was unaltered in the absence of Sos1, the phosphorylation of LAT and downstream PLC-γ1 was reduced (Fig. 1, C and D).

TCR signaling pathways are modulated by a number of ERK-dependent positive and negative feedback phosphorylation events (13, 14), and we hypothesized that perhaps the reduced ERK signaling that we observed in the absence of Sos1 led to a reduction in LAT phosphorylation and the recruitment of downstream signaling molecules. If this were true, LAT phosphorylation would be dependent not only on Sos1, but also on RasGRP1, because ERK phosphorylation is almost completely RasGRP1-dependent in developing thymocytes (fig. S1) (7, 9). However, phosphorylation of ZAP-70, LAT, and PLC-γ1 was unaffected by the loss of RasGRP1 (fig. S1), which suggested that an alternative mechanism was responsible for the reduced TCR-dependent $Ca^{2+}$ signaling in the absence of Sos1.

Sos1 is recruited to the membrane-bound adaptor LAT through its association with Grb2. The existence of multivalent interactions between LAT:Grb2 (1:3) and Sos1:Grb2 (1:2) has led to the hypothesis that Grb2–Sos1–Grb2 complexes may nucleate LAT oligomerization (11). We therefore directly assessed whether Sos1 was required for LAT microcluster formation. Thymocytes isolated from *Sos1*$^{+/+}$ and *Sos1*$(T)^{-/-}$ mice were dropped onto stimulatory coverslips, fixed after 3 min, and incubated with antibodies specific for pLAT and pTyr. The size and number of pTyr clusters were not altered by loss of *Sos1* (Fig. 1E, and fig. S2, A and B), confirming that global tyrosine kinase signaling was unchanged in the

absence of Sos1. In contrast, we observed a substantial defect in the formation of microclusters containing pLAT (hereafter referred to as pLAT microclusters) in the absence of Sos1. In *Sos1*(T)$^{-/-}$ thymocytes, individual pLAT microclusters were smaller and showed less intense staining compared to those in wild-type thymocytes (Fig. 1E and fig. S2A). In addition to having smaller pLAT clusters, *Sos1*(T)$^{-/-}$ thymocytes contained 40% fewer pLAT microclusters per cell (Fig. 1F). Together, these data suggest that the overall size and intensity of pLAT found in microclusters in individual cells was substantially decreased in the absence of Sos1 (Fig. 1F).

To determine the role of Sos1-dependent LAT oligomerization in vivo, we generated a series of Sos1 transgenic mice designed to separate the RasGEF activity of Sos1 from its ability to promote Grb2-dependent oligomerization of LAT (Fig. 2A). To restore Sos1–Grb2-dependent LAT oligomerization in the absence of Sos1 RasGEF activity, we generated transgenic mice expressing Sos1 that contains a point mutation that impairs the catalytic activity of Sos1 (F929A) (15). Because this catalytically inactive Sos1 mutant contains multiple Grb2-binding sites in its PRR, we predicted that it would still be capable of nucleating LAT oligomerization in a Grb2-dependent manner. To restore Sos1 RasGEF activity independently of its ability to promote LAT oligomerization, we generated a chimeric Sos1 (referred to as Sos-SH2) in which the PRR of Sos1 (which contains two Grb2-binding sites) was replaced by the SH2 domain of Grb2 (16, 17). The Sos-SH2 chimeric protein was predicted to directly associate with pLAT through the SH2 domain of Grb2, and this direct recruitment to LAT would enable the appropriate localization of Sos1 RasGEF activity; however, the inability of this chimeric protein to associate with multiple LAT molecules simultaneously would precludes its ability to promote Grb2-dependent LAT oligomerization (see model in Fig. 2B). We generated wild-type Sos1 transgenic mice as a positive control, and we also generated a combined Sos1 F929A/SH2 transgenic mouse both to act as a negative control and to restore any other potential Sos1-dependent signals to LAT complexes. Transgene expression was driven by a human *CD2* promoter to enable stable Sos1 transgene production throughout T cell development (18).

Sos1 transgenic founder mice were individually crossed onto a *Sos1*(T)$^{-/-}$ genetic background, and we performed Western blotting analysis to assess the abundances of the various Sos1 constructs in multiple founder lines for each transgenic construct (fig. S3, A to D). Sos1 transgenic mice (wild-type, F929A, Sos-SH2, and F929A/SH2) showing protein abundance similar to that of endogenous Sos1 were chosen for further study (Fig. 2C). Expression of wild-type Sos1 on a *Sos1*(T)$^{-/-}$ background restored TCR-dependent ERK phosphorylation, LAT phosphorylation, and $Ca^{2+}$ flux, whereas cells expressing the F929A/SH2 mutant Sos1 showed signaling similar to that of cells from *Sos1*(T)$^{-/-}$ mice (Fig. 3, A to F and Fig. 4, A to D), suggesting that the combination of the RasGEF activity of Sos1 and its ability to oligomerize LAT fully account for the role of Sos1 in TCR signaling shown earlier (Fig. 1).

## The RasGEF activity of Sos1 is required for TCR-dependent ERK activation

Deletion of *Sos1* affected TCR-dependent LAT phosphorylation, ERK activation, and $Ca^{2+}$ flux to a similar extent (resulting in a 40 to 50% reduction, see Fig. 1). Because PLC-γ1

signaling controls both the release of intracellular $Ca^{2+}$ stores and most TCR-dependent ERK activation (through RasGRP1), we hypothesized that Sos1-dependent LAT oligomerization and downstream signaling to PLC-γ1 might be responsible for all of the Sos1-dependent signals that we observed. We therefore assessed whether Sos1-dependent LAT oligomerization was responsible for the 40 to 50% reduction in TCR-dependent ERK phosphorylation that we observed in developing T cells. We stimulated purified DP thymocytes with increasing doses of anti-CD3ε antibody (Fig. 3, A and B, 60-s stimulation) or with a constant, intermediate concentration of antibody over different time points (Fig. 3, C and D), and then assessed the extent of ERK phosphorylation by Western blotting analysis.

Whereas expression of wild-type Sos1 restored TCR-stimulated ERK phosphorylation in cells from *Sos1*$(T)^{-/-}$ mice, expression of the catalytically inactive mutant Sos1 (F929A) failed to do so. In contrast, expression of the Sos-SH2 mutant protein rescued TCR-dependent ERK phosphorylation to an extent similar to that in cells expressing wild-type Sos1 (Fig. 3, A to D). Single-cell analysis of pERK abundance by flow cytometry also revealed that expression of either wild-type Sos1 or the Sos-SH2 mutant restored full, digital ERK phosphorylation, whereas ERK phosphorylation was partially defective in cells from mice expressing either the F929A or F929A/SH2 transgenes (Fig. 3, E and F and fig. S4, A and B). These data indicate that the RasGEF activity of Sos1, and not its ability to promote LAT oligomerization, is primarily responsible for the role of Sos1 in promoting TCR-dependent ERK activation.

## Sos1-dependent clustering of LAT is required for TCR-stimulated PLC-γ1 signaling

When we assessed the role of Sos1 in TCR-dependent LAT phosphorylation, we observed a prominent defect immediately after TCR stimulation, whereas LAT phosphorylation remained relatively intact at later times (Fig. 1C). We therefore assessed the initial activation of proximal TCR signaling by stimulating DP thymocytes with increasing concentrations of anti-CD3ε antibody in the presence of anti-CD4 antibody for 30 s (Fig. 4, A and B). TCR-dependent ZAP-70 phosphorylation was unaffected by either the absence of Sos1 or by the expression of any of the Sos1 constructs (Fig. 4, A and B), confirming that Sos1-dependent signaling was not involved in feedback control of the most receptor-proximal TCR signaling events. In contrast, TCR-dependent phosphorylation of LAT and PLC-γ1 were diminished in the absence of Sos1 at all antibody concentrations tested, but were rescued by re-introduction of wild-type Sos1. Furthermore, whereas Sos1-dependent phosphorylation of ERK required its RasGEF activity, the Sos1-dependent phosphorylation of LAT and PLC-γ1 were independent of its RasGEF activity. Expression of the catalytically inactive mutant Sos1 (F929A) completely rescued TCR-dependent LAT and PLC-γ1 phosphorylation in *Sos1*$(T)^{-/-}$ cells. In contrast, expression of the Sos-SH2 construct in cells failed to restore the TCR-dependent phosphorylation of LAT and PLC-γ1 (Fig. 4, A and B), indicating that maximal phosphorylation of LAT and PLC-γ1 required Sos1-dependent clustering of LAT.

Once activated, PLC-γ1 cleaves $PIP_2$ to generate $IP_3$ and DAG, with the former triggering the release of intracellular $Ca^{2+}$ stores and the latter stimulating the RasGRP1-dependent activation of ERK. To examine PLC-γ1–dependent increases in $Ca^{2+}$ signaling, we assessed

cytosolic $Ca^{2+}$ flux upon TCR stimulation in DP thymocytes. The catalytically inactive Sos1 mutant (F929A) completely rescued the defective $Ca^{2+}$ signaling observed in *Sos1*$(T)^{-/-}$ cells, whereas the Sos1 mutant defective in its ability to cluster LAT (Sos-SH2) failed to rescue TCR-induced $Ca^{2+}$ signaling in *Sos1*$(T)^{-/-}$ mice (Fig. 4, C and D). Therefore, similar to PLC-γ1 phosphorylation, maximal TCR-dependent $Ca^{2+}$ signaling required Sos1-dependent clustering of LAT. These data contrast sharply with the role played by Sos1 in TCR-dependent ERK activation. Whereas a Sos1 mutant (Sos-SH2) defective in its ability to cluster LAT resulted in a marked reduction in PLC-γ1 phosphorylation and downstream $Ca^{2+}$ flux compared to that in cells expressing wild-type Sos1 (Fig. 4), ERK phosphorylation was normal in these cells (Fig. 3), which suggests that DAG-dependent activation of RasGRP1 in these cells was normal. Therefore, the markedly reduced amounts of pLAT observed in cells expressing the Sos-SH2 mutant were fully capable of supporting TCR-dependent ERK phosphorylation, but not $Ca^{2+}$ flux. Together, these data (Figs. 3 and 4) suggest that PLC-γ1–dependent ERK phosphorylation and $Ca^{2+}$ signaling may be differentially sensitive to the amount of pLAT in developing T cells.

## The negative selection of thymocytes is Ras-dependent

The data thus far (Figs. 1 to 4) suggest that there are at least two Sos1-dependent signals that promote optimal TCR signaling in developing T cells. We next wanted to examine which of these Sos1-dependent signals was important for the effects of Sos1 on T cell development. Signal transduction from the pre-TCR and the TCR through the RasGEFs Sos1 and RasGRP1 to the small GTPase Ras is required at two distinct developmental checkpoints during T cell maturation. We and others have shown that at the first (pre-TCR) checkpoint, Sos1, RasGRP1, and RasGRP4 act in concert to promote pre-TCR–dependent proliferation and differentiation of thymocytes, and that deletion of *Sos1* results in the most marked effect of any of the single knockout models (6–8). In contrast, at the second (TCR) checkpoint, deletion of *Rasgrp1* blocks positive selection (9), whereas combined deletion of *Sos1* and *Rasgrp1* is required to efficiently block negative selection (7). Because of the redundancy between Sos1 and RasGRP1 in promoting negative selection of thymocytes, we rescued *Sos1* expression with the various Sos1 transgenes in a *Sos1*$(T)^{-/-}$*Rasgrp1*$^{-/-}$ background. We examined the effects of Sos1-dependent signaling during negative selection with $HY^+$ TCR-transgenic mice. HY mice express a high-affinity TCR early during T cell development that recognizes a male-specific peptide in the context of MHC class I, which causes strong signaling and negative selection early in DP thymocytes in male, but not female, $HY^+$ mice (fig. S5A). We chose this classic model of negative selection because it induces negative selection and loss of DP thymocytes; a strong model of negative selection is essential when assessing mice that also have a severe block in positive selection, such as *Rasgrp1*$^{-/-}$ mice.

Although both Sos1 and RasGRP1 are required for negative selection in $HY^+$ male mice, we previously reported a graded effect of deleting these two RasGEFs (7). Here, we found that negative selection was intact in *Sos1*$(T)^{-/-}$ $HY^+$ male mice, because these mice showed an almost complete loss of $CD4^+CD8^+$ (DP) thymocytes (Fig. 5A and fig. S5, B and C). Negative selection also occurred in *Rasgrp1*$^{-/-}$ $HY^+$ male mice, because these mice had fewer thymocytes compared to those of non-transgenic control mice (Fig. 5A). However,

unlike *Sos1*(T)$^{-/-}$ mice, *Rasgrp1*$^{-/-}$HY$^{+}$ male mice exhibited moderate survival of DP thymocytes (fig. S5, B and C) (7), indicating that negative selection is incomplete in *Rasgrp1*$^{-/-}$ mice. In contrast, *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$HY$^{+}$ mice showed a complete defect in negative selection, because both the numbers (fig. S5B) and percentages (fig. S5C) of surviving DP thymocytes were similar to those of non-transgenic *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$ control mice (Fig. 5A). Furthermore, the increase in the cell-surface abundance of CD69, a marker of Ras- and ERK-dependent cellular activation during thymocyte selection, was reduced in *Rasgrp1*$^{-/-}$HY$^{+}$ mice compared to that in wild-type controls, and was absent in *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$HY$^{+}$ male mice (Fig. 5B and fig. S5D).

To examine the roles of Sos1-dependent LAT oligomerization and Sos1 RasGEF activity during thymocyte negative selection, we crossed the various Sos1 transgenic mice into a *Sos1*(T)$^{-/-}$ *Rasgrp1*$^{-/-}$ HY$^{+}$ background, and then we analyzed HY-dependent negative selection. Transgenic expression of either wild-type Sos1 or the Sos-SH2 mutant, but not the F929A or F929A/SH2 mutants, was sufficient to completely rescue both negative selection (Fig. 5A and fig. S5, B and C) and the increase in CD69 abundance (Fig. 5B and fig. S5D) in *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$HY$^{+}$ male mice. These data indicate that, in the absence of RasGRP1, the RasGEF activity of Sos1 was required for the negative selection of thymocytes, whereas Sos1-dependent LAT oligomerization was dispensable. Furthermore, these data show that Ras-dependent signaling was absolutely required for thymocyte negative selection.

### Thymocyte proliferation requires multiple independent Sos1-driven signals

Sos1 is required for pre-TCR–mediated proliferation and differentiation of thymocytes (6). Deletion of *Sos1* led to a 50% reduction in the number of developing T cells (Fig. 6A), as well as a partial block in the progression of thymocytes beyond the DN3 stage of thymocyte development (fig. S6A). This partial block at the DN3 stage was exhibited as an increase in the ratio of DN:DP cells (fig. S6, B and C), and more specifically in the ratio of DN3:DN4 thymocytes (fig. S6, D and E). The Sos1-dependent proliferative defect was rescued by introduction of wild-type Sos1, such that both the numbers of thymocytes (Fig. 6A) and the appropriate ratios of the different thymocyte subsets (fig. S6, B to E) were similar to those of *Sos1*$^{+/+}$ mice. In contrast, expression of the F929A, Sos-SH2, or F929A/SH2 constructs in *Sos1*(T)$^{-/-}$ mice failed to rescue the partial block in pre-TCR–dependent development that occurred because of deletion of *Sos1*. These data suggest that both the RasGEF activity of Sos1 and its ability to oligomerize LAT are required for normal pre-TCR–dependent developmental signaling.

Although Sos1 is the single most important RasGEF at the pre-TCR checkpoint, we previously showed that combined deletion of *Sos1* and *Rasgrp1* led to a marked reduction in thymocyte cellularity [(7) and compare Fig. 6, A and B], underlining the importance of RasGEF signaling at this early checkpoint. We hypothesized, therefore, that in the absence of both Sos1 and RasGRP1, we might observe an increased importance of the RasGEF activity of Sos1 relative to its ability to promote LAT oligomerization. However, when we assessed thymocyte development in *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$ mice expressing the various Sos1 constructs, we found that only wild-type Sos1, but none of the signaling-defective Sos1

mutants, rescued thymocyte numbers in *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$ mice (Fig. 6B). These data underline the importance of Sos1-dependent LAT oligomerization in pre-TCR–dependent developmental signaling.

To further assess the role that various Sos1-dependent signals had on thymocyte proliferation during T cell development, we crossed *Sos1*(T)$^{-/-}$Sos1-TG$^{+}$ mice to *Rag2*$^{-/-}$ mice. Western blotting analysis confirmed the expression of the various Sos1 constructs in *Sos1*(T)$^{-/-}$*Rag2*$^{-/-}$Sos1-TG$^{+}$ mice, confirming that the hCD2 promoter drove *Sos1* transgene expression in *Rag2*$^{++}$ mice (fig. S7A). *Rag2*$^{-/-}$ mice lack the ability to induce TCR gene rearrangement, and thymocyte development is arrested at the pre-TCR (DN3) checkpoint in these mice. However, proliferation and differentiation of cells to the DP stage can be induced in these mice by injecting a TCR-stimulatory (anti-CD3ε) antibody (19), presumably by stimulating signaling through preformed CD3δε, CD3γε, and TCRζζ dimers (20, 21). We previously showed that this proliferation is Sos1-dependent, but RasGRP1-independent (6). Similar to our observation assessing pre-TCR–dependent thymocyte development in *Sos1*(T)$^{-/-}$Sos1-TG$^{+}$ mice, we found that wild-type Sos1, but none of the signaling defective Sos1 mutants, fully restored anti-CD3ε–stimulated proliferation in *Sos1*(T)$^{-/-}$ *Rag2*$^{-/-}$ mice (Fig. 6C). In contrast, anti-CD3ε–stimulated differentiation of thymocytes to the DP stage was restored both by wild-type Sos1 and the Sos-SH2 mutant, indicating that this developmental signal was primarily RasGEF-dependent (fig. S7B).

These data suggested that the RasGEF activity of Sos1 and its ability to cluster LAT were independently required for pre-TCR–dependent proliferation during T cell development. If this were true, we hypothesized that we could restore normal T cell development in *Sos1*(T)$^{-/-}$ mice by simultaneous and independent expression of both the F929A and Sos-SH2 mutants, thereby simultaneously providing each of these two Sos1-dependent signals. To test whether Sos1 RasGEF activity and Sos1-dependent LAT oligomerization could be independently restored in trans, we generated *Sos1*(T)$^{-/-}$ mice simultaneously expressing the F929A and Sos-SH2 transgenes (Intercross F929A×SH2, Fig. 7, A to E). Simultaneous expression of the Sos1 F929A and Sos-SH2 constructs restored normal TCR-dependent LAT and ERK phosphorylation in *Sos1*(T)$^{-/-}$ DP thymocytes such that signaling was indistinguishable from that seen in *Sos1*$^{+/+}$ cells, confirming that both Sos1-dependent signals could be restored in trans (Fig. 7, A and B). Imaging of the formation of pLAT microclusters in response to anti-CD3 and anti-CD4 antibodies showed that expression of the F929A mutant (which contains two Grb2-binding sites), but not the Sos-SH2 construct (which lacks the ability to simultaneously bind to two Grb2 molecules), rescued the number (Fig. 7, C and D), size (fig. S8A), and intensity (fig. S8B) of pLAT microclusters in *Sos1*(T)$^{-/-}$ thymocytes. Simultaneous expression of the F929A and Sos-SH2 constructs resulted in a similar restoration of both the numbers (Fig. 7D) and size (fig. S8A) of pLAT microclusters; however, these cells showed an intermediate intensity of pLAT staining that was not statistically different from that of either *Sos1*$^{+/+}$ or *Sos1*(T)$^{-/-}$ cells (fig. S8B). This intermediate extent of pLAT abundance might suggest that there is competition between the F929A and Sos-SH2 constructs for binding sites on LAT that could limit the maximal extent of LAT microcluster formation. The restoration of signaling that we observed in mice simultaneously expressing the F929A and Sos-SH2 constructs was sufficient to restore

normal T cell development, because thymocyte numbers (Fig. 7E) and the appropriate ratios of thymocyte subsets (fig. S9, A to D) were restored by independent re-introduction of Sos1 RasGEF activity and Sos1-dependent LAT oligomerization to *Sos1*(T)$^{-/-}$ mice.

## Discussion

TCR-induced signal transduction to Ras is essential for both normal T cell development and for the activation of mature T cells during a productive immune response. We previously showed the importance of Ras activation by Sos1 in receptor-mediated signaling during thymocyte differentiation and proliferation. Here, we demonstrated that Sos1 has distinct and structurally separable functions in vivo: its well-known RasGEF activity and a recently recognized role in mediating the oligomerization of other crucial signaling molecules. Our work highlights the importance of higher order structures of signaling molecules (microclusters), well-studied in artificial systems, in vivo. In experiments with *Sos1*(T)$^{-/-}$ mice, we showed that Sos1 plays a role in LAT phosphorylation and the formation of TCR-dependent LAT microclusters. Next, with Sos1 transgenic mice designed to specifically ablate either Sos1-dependent LAT oligomerization (Sos-SH2) or Sos1 RasGEF activity (F929A), we showed that these mutant Sos1 constructs could independently restore optimal Sos1-dependent ERK phosphorylation or LAT–PLC-γ1–Ca $^{+}$ signaling in *Sos1*(T)$^{-/-}$ mice, respectively. Finally, we provided evidence that each of these Sos1-dependent signals were (i) required for optimal T cell development and (ii) were independent signaling events, because simultaneous introduction of both the Sos-SH2 and F929A constructs in trans rescued TCR signaling and T cell development in *Sos1*(T)$^{-/-}$ mice. Finally, given the cooperative nature of proximal signaling in multiple systems, these data suggest a new paradigm whereby Grb2–Sos1–Grb2 complexes can provide a framework to build the larger signaling machines that are required to drive many biological processes.

### Why might early T cell development be particularly sensitive to a partial loss in LAT oligomerization?

Whereas the RasGEF activity of Sos1 was required at both of the major T cell developmental checkpoints, Sos1-dependent LAT oligomerization was required for pre-TCR–dependent proliferation at the β-selection checkpoint, but not for TCR-stimulated negative selection (Fig. 7F). Although many of the downstream signaling components are similar at each of these checkpoints, the mechanisms of receptor activation at these two checkpoints are quite different (1). At the DP stage, mature TCR signaling (activated by peptide-bound MHC) is interrogated during positive and negative selection. In contrast, at the pre-TCR (β-selection) checkpoint, pre-TCR signaling is ligand-independent and is initiated upon TCRβ rearrangement (22). Furthermore, in DN thymocytes, surface pre-TCR abundance is low relative to the abundance of the TCR in DP thymocytes. The combination of low receptor abundance and ligand-independent signaling might make cells much more sensitive to perturbations in downstream signaling pathways, and promote an environment in which LAT oligomerization is absolutely required to generate sufficiently increased localized signaling to stimulate proliferation.

We previously showed that the relative abundances of the RasGEFs Sos1 and RasGRP1 are developmentally regulated (6). Sos1 protein abundance is increased in DN thymocytes, but is reduced precipitously at the DP stage, whereas RasGRP1 abundance is reciprocally low in DN thymocytes, but is increased in DP thymocytes. We hypothesized that these regulated changes in Sos1 and RasGRP1 abundance might help to determine the relative usage of these RasGEFs during T cell development (7). However, we observed that both the RasGEF activity of Sos1 and its ability to cluster LAT were required for optimal pre-TCR–dependent proliferation of DN thymocytes (Figs. 6 and 7). Therefore, increased Sos1 protein abundance might not only determine RasGEF usage, but may also be required to promote sufficient LAT oligomerization to stimulate proliferative responses at this stage. In addition to Sos1, c-Cbl (11) and Gab1 (23) can form 1:2 complexes with Grb2 and therefore have the potential to promote Grb2-dependent LAT oligomerization. It will be interesting to examine whether, similar to what is seen for the RasGEF activities of Sos1 versus RasGRP1, different scaffolds are differentially regulated to promote LAT oligomerization at different stages of T cell development.

## What advantages might be afforded by LAT oligomerization?

After TCR stimulation, pLAT recruits numerous signaling proteins, through both direct and indirect interactions, into multi-protein signaling complexes at the cell surface (10, 24). Whereas association with a single LAT molecule would limit the total number of signaling components in an individual complex, LAT oligomerization enables these individual complexes to be brought into close proximity with each other. This increased proximity between molecules in different complexes might enhance signal propagation between different complexes, potentially driving kinase and enzyme activation en masse (for example, for PLC-γ1 and PI3K) and thereby promote signal amplification. Similarly, the close proximity of different signaling complexes might enable signaling crosstalk by enhancing, modulating, or inhibiting signaling in adjacent complexes. Furthermore, given the intrinsically low affinity of interactions between some signaling molecules, the enhanced associations afforded by stimulus-dependent LAT oligomerization might be a way to threshold signaling, enabling immune cell activation when receptor is sufficiently stimulated, but reducing biologic noise when receptor stimulation is low. Finally, oligomerization of LAT, and occupancy of its pTyr residues by multiple signaling complexes in the center of large oligomers might protect LAT from dephosphorylation by phosphatases, thereby increasing the time that different complexes remain associated with each other to further enhance productive signaling events (25).

## Complexes versus clusters: How might interactions of LAT with different protein complexes regulate oligomerization?

Although LAT associates with both PLC-γ1 and Grb2 through direct SH2-pTyr interactions, these individual binding events require other cooperative interactions to form stable complexes. The binding of PLC-γ1 to $pTyr^{136}$ of LAT is stabilized by the adaptors GADS and SLP-76 (12, 26, 27), and formation of a LAT-PLC-γ1-GADS-SLP-76 complex seems to be required for the phosphorylation and activation of PLC-γ1 by Itk on LAT (28). Similarly, whereas Grb2 can associate with any of three pTyr residues in the C-terminus of LAT ($pTyr^{175}$ , pTyr , or $pTyr^{235}$ ), the presence of at least two of these sites is required for

efficient LAT-Grb2 binding (27), suggesting cooperativity between Grb2-binding sites on LAT. Furthermore, biophysical (29) and modeling (30) studies showed that multiple, cooperative interactions between LAT, Grb2, and Sos1 regulate the efficiency of higher-order LAT oligomerization. In this modeling work, it is predicted that whereas the binding of LAT to two Grb2 molecules enables linear LAT chains to form, the formation of large LAT clusters requires the association of LAT with three Grb2 molecules.

These modeling studies are particularly interesting in the context of recent PALM (photoactivated localization microscopy) imaging studies assessing the nanoscale organization of LAT (31). At the molecular level, LAT exists in preformed nanoclusters (containing 2 to 3 molecules) before TCR stimulation, which then aggregate into larger structures upon TCR stimulation. None of these structures are observed in cells expressing a phosphorylation-defective mutant of LAT (LAT-4YF), underlining the importance of protein-protein interactions in LAT aggregation. Furthermore, two-color PALM studies assessing interactions between labeled LAT and either Grb2 or SLP-76 revealed an interesting architecture of larger, TCR-stimulated nanoclusters: whereas Grb2 was found throughout LAT microclusters, SLP-76 tended to localize to the periphery of larger LAT clusters. Because the GADS-SLP–76 complex would occupy either $pTyr^{175}$ or $pTyr^{195}$ of LAT, this binding would tend to limit the number of Grb2 molecules bound to any individual LAT molecule. These data suggest an interesting hypothesis whereby multivalent, cooperative interactions between LAT, Grb2, and Sos1 promote LAT oligomerization, whereas recruitment of PLC-γ1–GADS-SLP-76 complexes block Grb2-LAT associations, and therefore limit the overall size of LAT oligomers.

## Is Sos1-Grb2–dependent oligomerization a generalized mechanism in signal transduction?

Studies have shown that Grb2 and Sos1 play complex negative and positive regulatory roles in growth factor receptor signaling. Before stimulation of fibroblast growth factor receptor 2 (FGFR2) by FGF, Grb2 homodimers hold dimeric FGFR2 in an inactive state that limits receptor trans-autophosphorylation and inhibits basal signaling. After receptor stimulation, Grb2 is tyrosine-phosphorylated and released from the receptor, which enables full activation of FGFR2 (32). In contrast, multiple cooperative Grb2-Sos1 protein-protein and protein-lipid interactions are required to both activate and fine-tune Ras- and ERK-dependent developmental signals downstream of FGFR2 (16). Grb2–Sos1 complexes are recruited to FGFR2 by the membrane-bound adaptor protein FRS-2 (fibroblast growth factor receptor substrate 2), which, similar to LAT, contains multiple Grb2-binding (SH2) sites (33). It is intriguing to hypothesize that similar to LAT, the association of multiple Grb2 complexes to FRS-2 might add additional Grb2-mediated regulation to this receptor signaling system.

The Grb2-dependent recruitment of Sos1 is a ubiquitous mechanism for receptor-induced Ras activation, and many growth factor receptors and adaptor molecules contain multiple Grb2-Sos1 docking sites that facilitate Ras activation (34, 35). The epidermal growth factor receptor (EGFR) contains at least two Grb2- and two Shc-binding sites, and Grb2 colocalizes with clustered EGFR. Furthermore, EGFR oligomerization is both tyrosine kinase- and phosphorylation-dependent (36), suggesting a potential role for Grb2-Sos1–

Grb2-nucleated complexes in EGFR oligomerization and signaling. It will be interesting to examine whether, similar to the regulation of LAT oligomerization, Grb2-Sos1 complexes play a regulatory role by promoting the oligimerization of signaling proteins in different RTK signaling systems.

Here, we provided evidence that, in addition to its RasGEF activity, Sos1 also regulates TCR signaling in a RasGEF-independent manner. By providing a framework for LAT oligomerization, Sos1 regulates the formation of focal regions of enhanced signaling that are essential to optimally activate downstream signaling in vivo. These data provide a new paradigm for understanding Sos1-dependent signaling in receptor-induced signal transduction, and suggest that, independent of targeting the Sos1-Ras interface (37–39), targeting Grb2-Sos1 interactions might have additional therapeutic potential.

## Materials and Methods

### Mice

*Rasgrp1*$^{-/-}$ mice (9) were a gift from J. Stone (University of Alberta, Canada). HY mice (40) and Lck-Cre (41) mice were purchased from Taconic. *Rag2*$^{-/-}$ mice (42) were purchased from the Jackson Laboratory. T cell–specific deletion of *Sos1* [denoted *Sos1*(T)$^{-/-}$] was achieved by crossing *Sos1*$^{f/f}$ mice to mice expressing Lck-Cre (6). Genotyping of HY, *Rasgrp1*$^{-/-}$, *Sos1*(T)$^{-/-}$, *Rag2*$^{-/-}$, and Lck-Cre mice was performed as detailed in the original publications. All mice were housed at the NIH according to guidelines set forth by the NCI-Bethesda Animal Care and Use Committee.

### Production of *Sos1* transgenic mice

Plasmid encoding myc-tagged Sos1 was a generous gift from E. Santos (Universidad de Salamanca, Salamanca, Spain) (43). After insertion of a silent mutation to remove an internal *Eco* R1 site, a complementary DNA (cDNA) encoding the Sos1-SH2 fusion protein containing amino acid residues 1 to 1091 of Sos1 fused to the SH2 domain of murine Grb2 (residues 53 to 164) was performed with standard techniques. The F929A mutation in both wild-type Sos1 and the Sos1-SH2 fusion was performed by site-directed mutagenesis. The hCD2-WT, F929A, SH2, and F929A/SH2 Sos1 constructs were generated by replacing TCRζ with the appropriate Sos1 transgene in hCD2-TCRζ (theζ-CT108 vector, a generous gift from P. Love, NICHD). Transgenic founder mice were generated with standard methods by the National Cancer Institute Transgenic Mouse Core. Germline transmission was verified by Southern blotting and polymerase chain reaction (PCR) analysis. Sos1 transgene protein amounts for each construct were determined by immunoprecipitations and Western blotting analysis, and transgenic lines were chosen that showed protein amounts similar to that of endogenous Sos1 in *Sos1*$^{+/+}$ mice. Genotyping of *Sos1* transgenic mice was performed at an annealing temperature of 60°C in a three-primer reaction with the following primers: Sos1TG-F: CAGTCCCCACCCATTAGTAGGAAG; Sos1genomic-R: CAAAGTTTCAGAGTGACACCGAGAC; Sos1TG-R: CATCTGCTGGCTGTAAAACCGAC. To distinguish F929A transgenic mice from Sos-SH2 transgenic mice in Fig. 7, PCR was performed at an annealing temperature of 60°C in a three-primer reaction with the following primers: Sos1TGmix-F:

CGGATTCCTGAAAGTGAGACGG; Sos1SH2-R: GCTGCTGTGGCATCTGTTCTATG; Sos1WT-R: GGAGGGAGTTTGGGGATAAGTTG.

### In vivo 2C11 stimulations

Mice were injected intraperitoneally (i.p.) with 100 μg of anti-CD3ε antibody (2C11 NA/LE, BD Biosciences). At the post-injection times indicated in the figure legends, total thymocytes were isolated and subjected to flow cytometric analysis.

### Flow cytometry

Single-cell suspensions from thymus were stained with the fluorochrome-conjugated monoclonal antibodies described in the text (all from BD Biosciences). To profile DN1 to DN4 thymocyte populations and to analyze pERK abundance, staining was performed as previously described (7). Flow cytometry was performed with a FACSCalibur flow cytometer and CELLQuest software (BD Biosciences), and data were analyzed with FlowJo software (Tree Star, Inc).

### Cell purification, stimulation, and Western blotting analysis

Pre-selection DP thymocytes were isolated and stimulated as previously described (6). Samples were loaded at $0.5 \times 10^6$ cells per lane ($1.5 \times 10^6$ cells for the Western blotting analysis of Sos1) and were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Blots were incubated overnight at 4°C with the following primary antibodies directed against: pZAP-70 (Cell Signaling Technology #2704 and #2717), ZAP-70 (44), pLAT ( Cell Signaling Technology #3584), LAT (45), pPLC-γ1 (Cell Signaling Technology #2821), PLC-γ1 (Santa Cruz #sc-7290), pERK (Cell Signaling Technology #4370), ERK1/2 (Cell Signaling Technology #4695), p-Tyr (4G10, Invitrogen #05–321), β-actin (Sigma-Aldrich #AC-40), Sos1 (Santa Cruz #sc-256), myc epitope (9E10, Santa Cruz #sc-40). Blots were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Millipore) for 1 hour at room temperature. ECL (enhanced chemiluminescence, Super Signal West Pico and Super Signal West Femto, Pierce) was used to visualize protein bands, which were quantified with ImageJ software (NIH).

### Measurement of $Ca^{2+}$ flux

Total thymocytes were incubated in Hank's balanced salt solution (HBSS) containing 5 mM Indo-1-AM (Molecular Probes) and 0.5 mM probenicid (Sigma) for 45 min at 30°C. Cells were then washed with HBSS and incubated with fluorochrome-conjugated anti-CD4 and anti-CD8 antibodies for 15 min. Cells were again washed with HBSS, resuspended in HBSS, and incubated at 37°C for 5 min before measurements were made. Antibodies against CD3ε (3 μg/ml) and CD4 (5 μg/ml) were then added, and a baseline reading was taken for 30 s before antibody crosslinking was stimulated with streptavidin (20 μg/ml). Samples were analyzed with an LSR II flow cytometer (BD Biosciences), and data were analyzed with FlowJo software.

### Fixed-cell imaging, image processing, and quantitation of image data

The cell spreading assay has been described previously (46). Chambered coverslips (LabTek) were coated with anti-CD3ε (3 μg/ml) and anti-CD4 (10 μg/ml) antibodies in phosphate-buffered saline (PBS) overnight at 4°C. Cells were plated onto coverslips containing imaging buffer (RPMI 1640 without phenol red, 10% fetal calf serum, 20 mM Hepes) and were fixed after 3 min with 2.4% paraformaldehyde. Images from fixed cells were collected with a Zeiss 510 LSCM (laser scanning confocal microscope), with a 63X, 1.4 NA objective (Carl Zeiss Inc.). Imaris 7.4.0 (Bitplane, Andor) software was used to process the images of fixed cells. Single z slices containing the microclusters were analyzed. The 488 channel (used for imaging of anti-pLAT signals) was used to generate surfaces for the analysis of all punctae, including the numbers of microclusters, and the calculation of microcluster area and channel intensity.

### Statistical analysis

Quantified Western blotting data are presented as means ± SD, whereas animal and imaging data are presented as means ± SEM. For Western blotting and pERK timecourse data, statistical significance was determined by 2-way ANOVA with GraphPad Prism software. For the imaging data in Fig. 1, the statistical significance of the difference between the means was determined by two-tailed Student's *t* test. For data from mouse experiments, peak $Ca^{2+}$ flux, peak pERK staining, and for the imaging data presented in Fig. 7, the statistical significance of the difference between means of groups of mice was determined by one-way ANOVA, and a Tukey correction was applied to enable multiple comparisons. Statistically significant data are denoted as follows: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ when compared to $Sos1^{+/+}$ (or to $Rasgrp1^{-/-}$ in Fig. 5B); $\#P \leq 0.05$, $\#\#P \leq 0.01$, $\#\#\#P \leq 0.001$ when compared to $Sos1(T)^{-/-}$ [or to $Sos1(T)^{-/-}Rasgrp1^{-/-}$ in Fig. 5B]. $P \leq 0.05$ was considered statistically significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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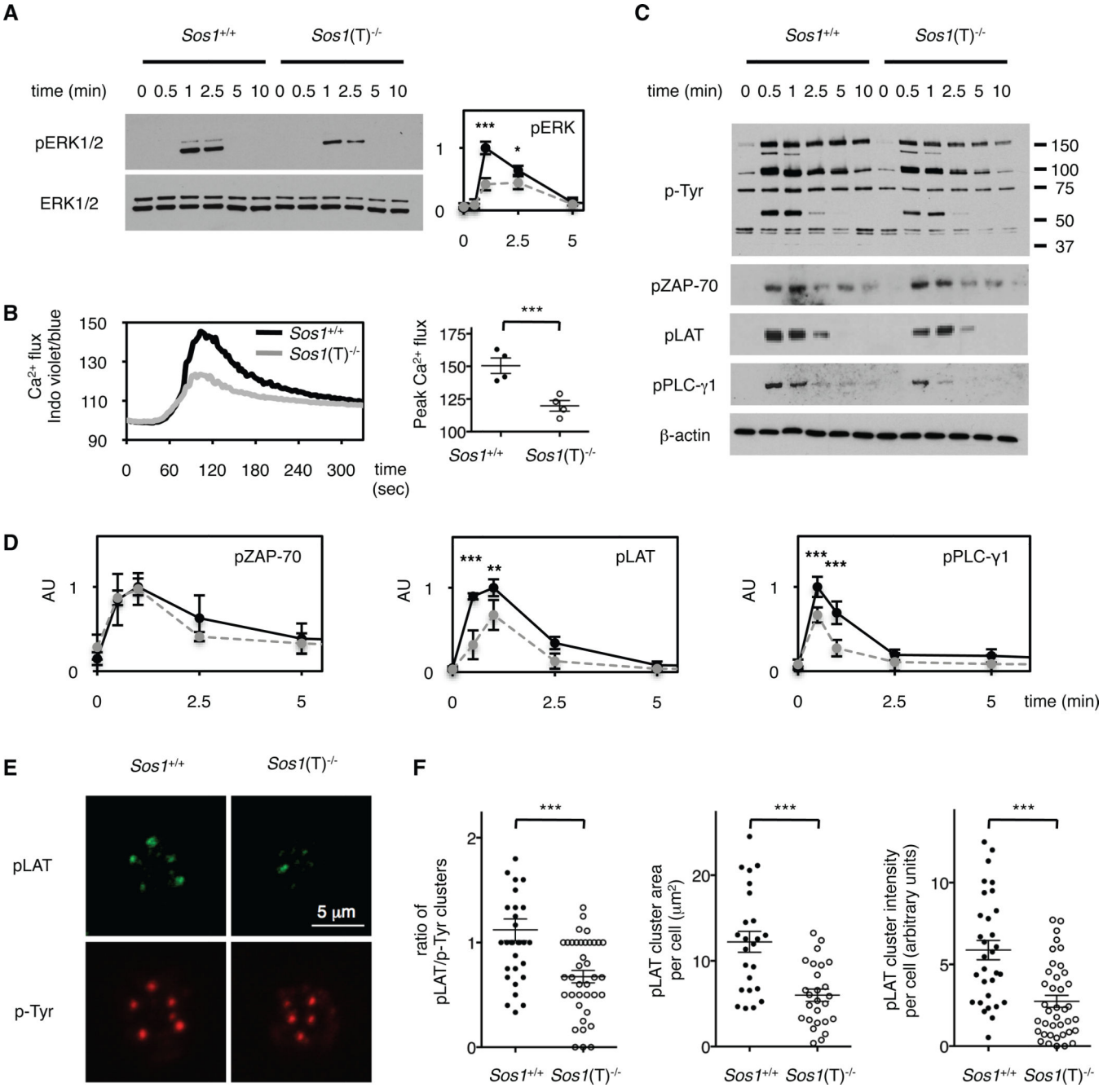


**Fig. 1. Sos1 is required for optimal TCR-dependent LAT phosphorylation**

(**A**) Left: DP thymocytes purified from *Sos1*$^{+/+}$ or *Sos1*(T)$^{-/-}$ mice were stimulated with anti-CD3ε antibody (α-CD3ε, 3 μg/ml) and anti-CD4 antibody (α-CD4, 5 μg/ml) for the indicated times, and then whole-cell lysates (WCLs) were analyzed by Western blotting with antibodies specific for pERK1/2 and total ERK1/2. Blots are representative of three independent experiments. Right: Quantitation of the ratio of pERK to total ERK at the indicated times. Data are means ± SD of three independent experiments, and statistical significance was determined by 2-way ANOVA. *$P \leq 0.05$, ***$P \leq 0.001$. (**B**) Left: Representative $Ca^{2+}$ flux experiments (showing the ratio of indo-blue to indo-violet) with

gated DP thymocytes stimulated with α-CD3ε (3 μg/ml) and α-CD4 (5 μg/ml) antibodies for the indicated times. Plots are representative of four independent experiments. Right: Quantitation of peak $Ca^{2+}$ flux measurements (indo-blue to indo-violet ratio). Data are means ± SEM from four independent experiments, and statistical significance was determined by 2-tailed t-test. ***$P \leq 0.001$. (**C**) WCLs from the cells shown in (A) were analyzed by Western blotting with antibodies specific for total pTyr (pTyr, 4G10), pZAP-70 ($Tyr^{319}$), pLAT ($Tyr^{191}$), pPLC-γ1 ($Tyr^{783}$), and β-actin. Blots are representative of three independent experiments. (**D**) Quantitation of the abundances of pZAP-70, pLAT, and pPLC-γ1 proteins relative to those of the corresponding total proteins from the experiments represented in (C). Data are means ± SD from three independent experiments, and statistical significance was determined by 2-way ANOVA. **$P \leq 0.01$, ***$P \leq 0.001$; AU= arbitrary units. For each plot in (A), (B). and (D), *Sos1*$^{+/+}$ mice are shown in black and *Sos1*(T)$^{-/-}$ mice are shown as a gray dashed line. (**E**) Purified DP thymocytes from *Sos1*$^{+/+}$ or *Sos1*(T)$^{-/-}$ mice were dropped onto stimulatory coverslips and were fixed after three min. After fixation, cells were permeabilized and immunostained for pLAT ($Tyr^{191}$, green) and pTyr (4G10, red). Images are representative of three experiments. (**F**) Quantitation of the ratio of the number of pLAT clusters relative to the number of pTyr clusters, the surface area of pLAT clusters, and the intensity of pLAT clusters per cell. Data are pooled from n ≥ 28 cells for each genotype. Each symbol denotes an individual cell, and the bars denote the mean ± SEM for the group. Statistical significance was determined by 2-tailed t-test. ***$P \leq 0.001$.

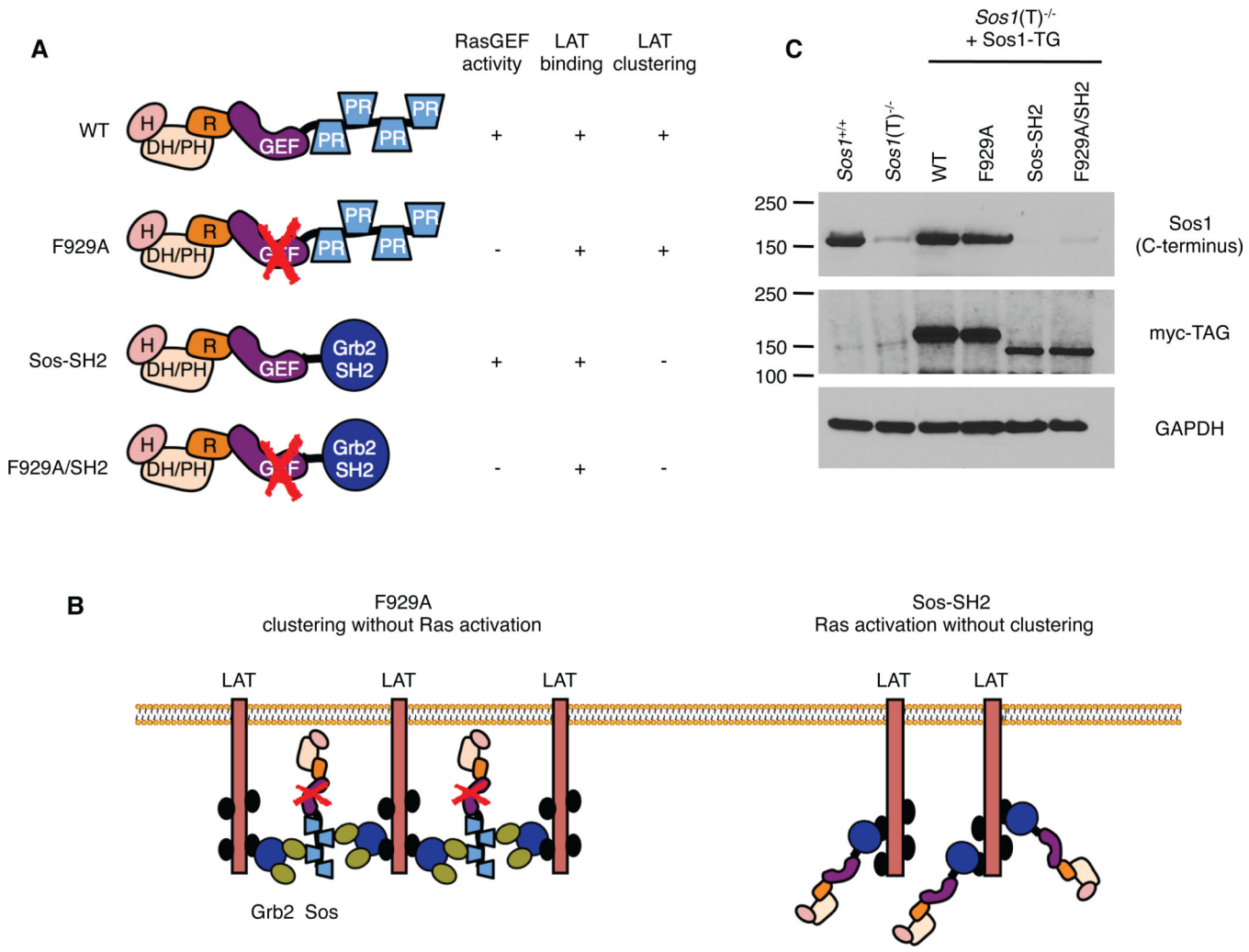


**Fig. 2. Sos1 mutant constructs that separate its RasGEF activity from its ability to promote LAT clustering**

(**A**) Diagram of Sos1 constructs. The F929A construct contains a point mutation in Sos1 that ablates its RasGEF activity, whereas Sos-SH2 is a chimeric molecule in which the PRR of Sos1 is replaced with the SH2 domain of Grb2. The F929A/SH2 construct is a combined construct of the F929A and Sos-SH2 mutations. Each transgenic construct contains a C-terminal myc tag. The ability of each construct to act as a RasGEF, to physically associate with LAT (either directly or through Grb2), and to promote LAT clustering are indicated to the right. (**B**) Model of LAT-Grb2-Sos1 interactions in cells expressing either the F929A or Sos-SH2 mutant Sos1 constructs. The F929A construct has no intrinsic RasGEF activity, but can still interact with two Grb2 molecules through its PRR, and thus it can promote LAT oligomerization. The Sos-SH2 construct does not interact with Grb2, but can instead directly interact with one of three Grb2-binding sites on LAT. Because of the lack of multiple Grb2 proteins attached to Sos-SH2, this construct cannot promote LAT oligomerization. (**C**) WCLs of total thymocytes from *Sos1*$^{+/+}$*Sos1*(T)$^{-/-}$, and *Sos1*(T)$^{-/-}$ mice crossed to the indicated Sos1-transgenic lines shown in (A) were analyzed by Western blotting with antibodies specific for Sos1, the myc tag, and GAPDH (loading control). The anti-Sos1

antibody recognizes the PRR of Sos1, and thus does not recognize either the Sos-SH2 or F929A/SH2 constructs. Blots are representative of three experiments. Numbers to the left of the blots indicate molecular mass markers (kD).

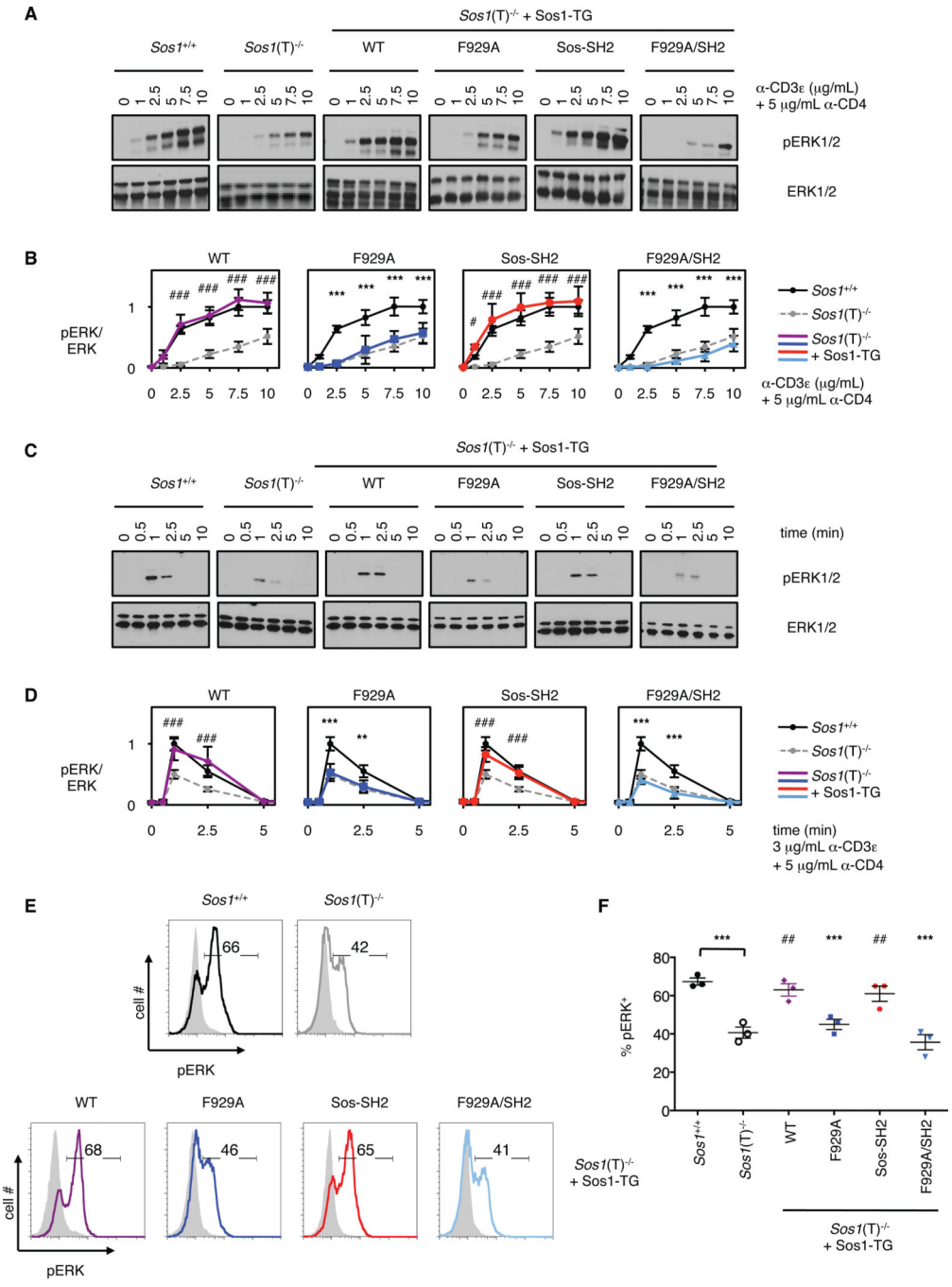


**Fig. 3. The RasGEF activity of Sos1 is required for optimal TCR-dependent phosphorylation of ERK1/2**

(**A** and **B**) DP thymocytes purified from the indicated mice were stimulated with the indicated concentrations of α-CD3ε antibody together with α-CD4 antibody (5 μg/ml) for 60 s, and then WCLs were analyzed by Western blotting with antibodies specific for pERK1/2 and total ERK1/2. (A) Western blots are representative of three independent experiments. (B) Quantitation of the ratios of pERK to total ERK. (**C** and **D**) DP thymocytes purified from the indicated (8-week-old) mice were stimulated with α-CD3ε (3 μg/ml) and α-CD4 (5

μg/ml) for the indicated times, and then WCLs were analyzed by Western blotting with antibodies specific for pERK1/2 and total ERK1/2. (C) Western blots are representative of three independent experiments. (D) Quantitation of the ratios of pERK to total ERK. Data in (B) and (D) are means ± SD from three independent experiments, and statistical significance was determined by 2-way ANOVA. For each Sos1 transgenic strain: $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ when compared to $Sos1^{+/+}$; ###P ≤ 0.001 when compared to $Sos1^{-/-}$ For each plot in (B) and (D), $Sos1^{+/+}$ mice are shown in black, $Sos1(T)^{-/-}$ mice are shown as a gray dashed line, and $Sos1(T)^{-/-}$ mice expressing the indicated Sos1 transgene are shown in the indicated colors, one for each construct. (**E**) Representative histograms from the flow cytometric analysis of pERK staining in gated DP thymocytes from the indicated mice that were either left unstimulated (gray, shaded) or were stimulated with α-CD3ε (3 μg/ml) and α-CD4 (5 μg/ml) for 2 min (unshaded, bold line). (**F**) Quantitation of the percentages of $pERK^+$ cells from three independent experiments. Each symbol denotes an individual experiment, and the bars denote means ± SEM for the group. Statistical significance was determined by ANOVA. Statistically significant differences are shown for each for each Sos1 transgenic mouse: $^{***}\leq P$ 0.001 when compared to $Sos1^{+/+}$; $^{\#\#}P \leq 0.01$, $^{\#\#\#}P \leq 0.001$ when compared to $Sos1(T)^{-/-}$.

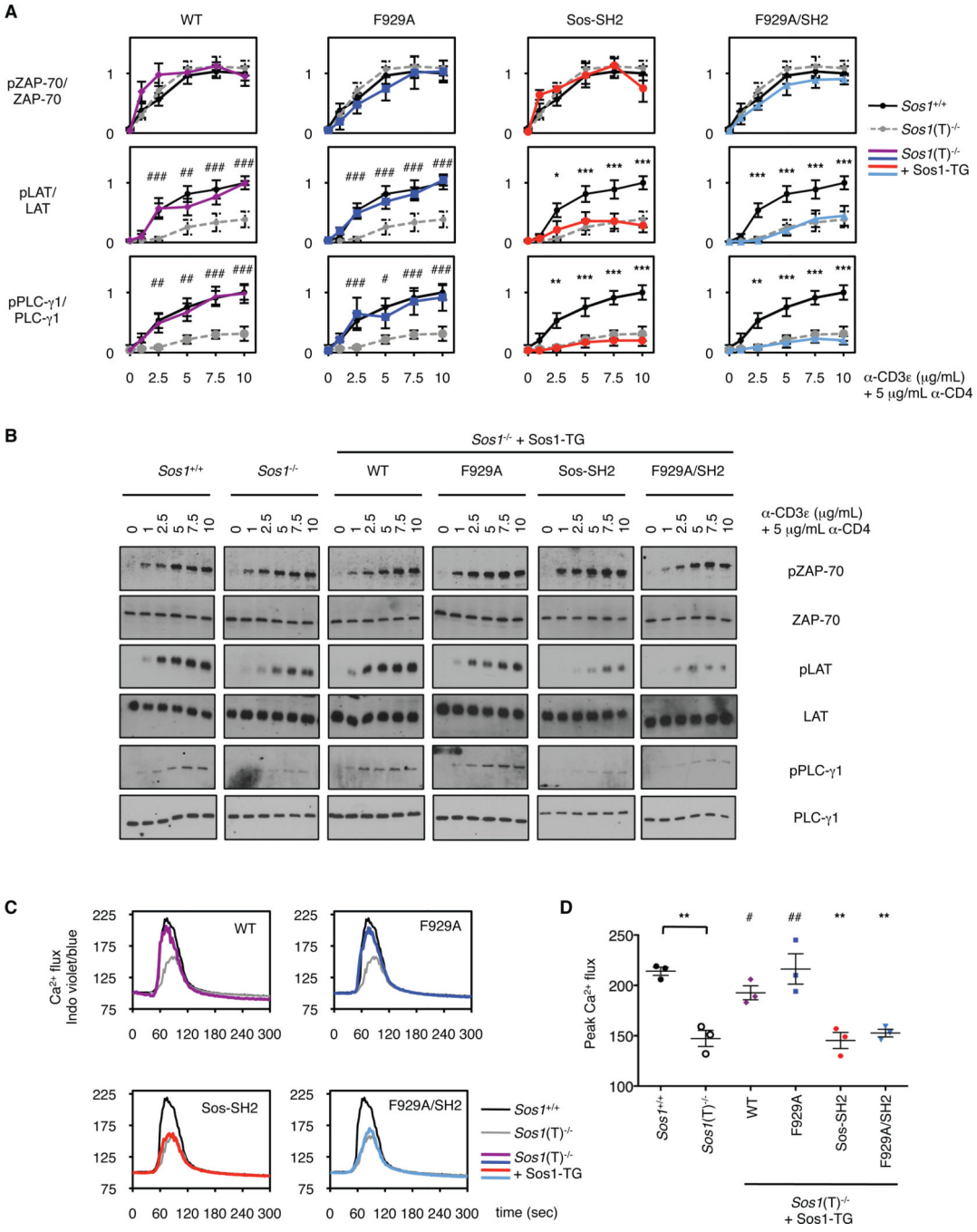


**Fig. 4. The ability of Sos1 to promote LAT oligomerization is required for optimal TCR-dependent phosphorylation of LAT and PLC-γ1 and for $Ca^{2+}$ flux**

(**A** and **B**) DP thymocytes purified from the indicated mice were stimulated with increasing concentrations of α-CD3ε together with a fixed concentration of α-CD4 (5 μg/ml) for 30 s, and then WCLs were subjected to Western blotting analysis with antibodies specific for pZAP70 ($Tyr^{319}$), total ZAP70, pLAT ($Tyr^{191}$), total LAT, pPLC-γ1 ($Tyr^{783}$), and total PLC-γ1. (A) Data are means ± SD from three independent experiments and show quantitation of the abundances of pZAP-70, pLAT, and pPLC-γ1 proteins relative to those

of the corresponding total proteins. Statistical significance was determined by 2-way ANOVA. For each Sos1 transgenic strain: $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ when compared to *Sos1*$^{+/+}$; $\#P \leq 0.05$, $\#\#P \leq 0.01$, $\#\#\#P \leq 0.001$ when compared *Sos1*(T)$^{-/-}$ (B) Western blots are representative of three independent experiments. (**C**) Representative $Ca^{2+}$ flux experiments (showing the ratio of indo-blue to indo-violet) in gated DP thymocytes (isolated from the indicated mice) that were stimulated with α-CD3ε (3 μg/ml) and α-CD4 (5 μg/ml) for the indicated times. Plots are representative of three independent experiments. (**D**) Quantitation of peak $Ca^{2+}$ flux measurements (indo-blue to indo-violet ratio) from three independent experiments. Each symbol denotes an individual experiment, and the bars denote means ± SEM for the group. Statistical significance was determined by ANOVA. $**P \leq 0.01$ when compared to *Sos1*$^{+/+}$; $\#P \leq 0.05$, $\#\#P \leq 0.01$ when compared to *Sos1*(T)$^{-/-}$. For each plot in (A) and (C), *Sos1*$^{+/+}$ mice are shown in black, *Sos1*(T)$^{-/-}$ mice are shown as a gray dashed line, and *Sos1*(T)$^{-/-}$ mice expressing the indicated Sos1 transgenes are shown in the indicated colors.

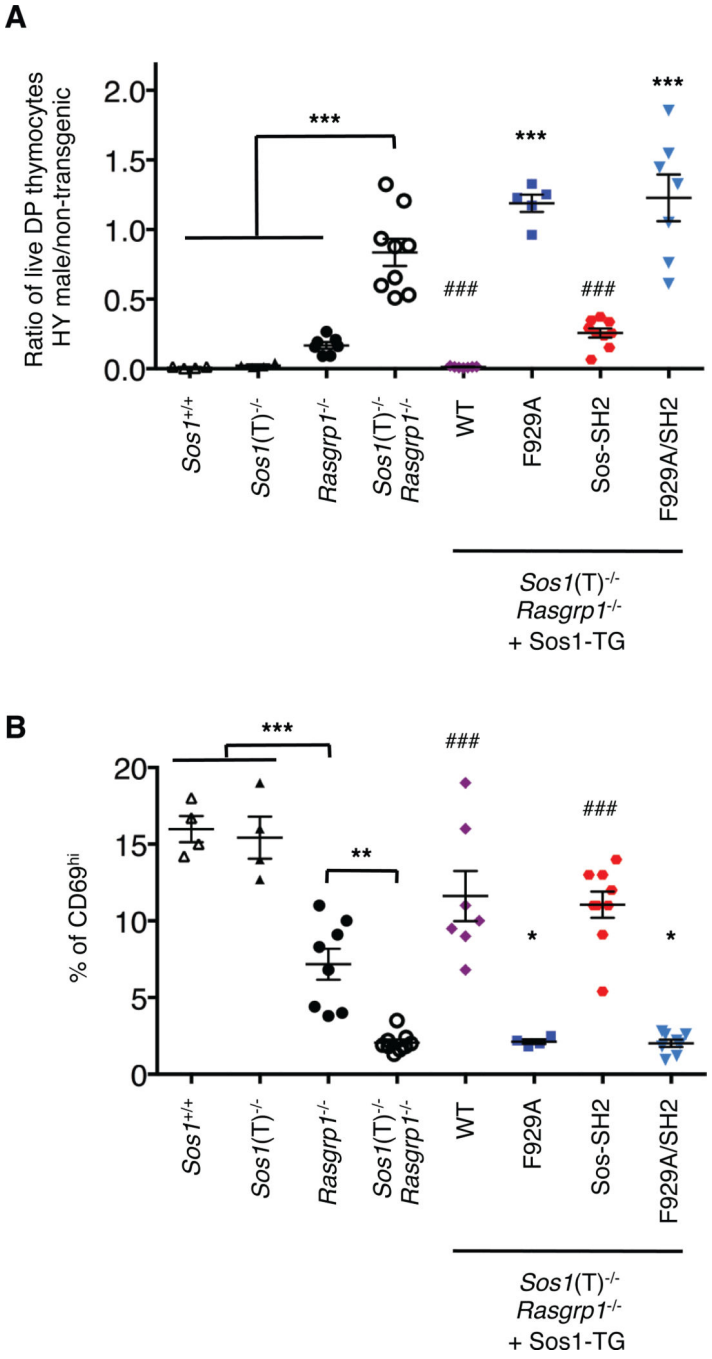


**Fig. 5. The RasGEF activity of Sos1 is required for the negative selection of thymocytes in *Rasgrp1*$^{-}$ $^{-}$ mice**

(**A**) Quantification of the ratio of DP HY$^{+}$ thymocytes to non-transgenic control thymocytes for 6- to 8-week old male mice for each of the indicated genotypes. (**B**) Analysis of the percentages of CD69$^{hi}$ thymocytes from the HY TCR-transgenic male mice shown in (A). Data are from n ≥ 5 mice for each genotype. For (A), each symbol denotes the ratio from a matched pair of mice including an HY-transgenic mouse and a non-transgenic littermate control mouse of the same *Sos1* and *Rasgrp1* genotype. For (B) each symbol denotes an

individual mouse. For (A) and (B) the bars denote means ± SEM for the group. Statistical significance was determined by ANOVA. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ when compared to *Rasgrp1*$^{-/-}$; ##$P \leq 0.01$, ###$P \leq 0.001$ when compared to *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$.

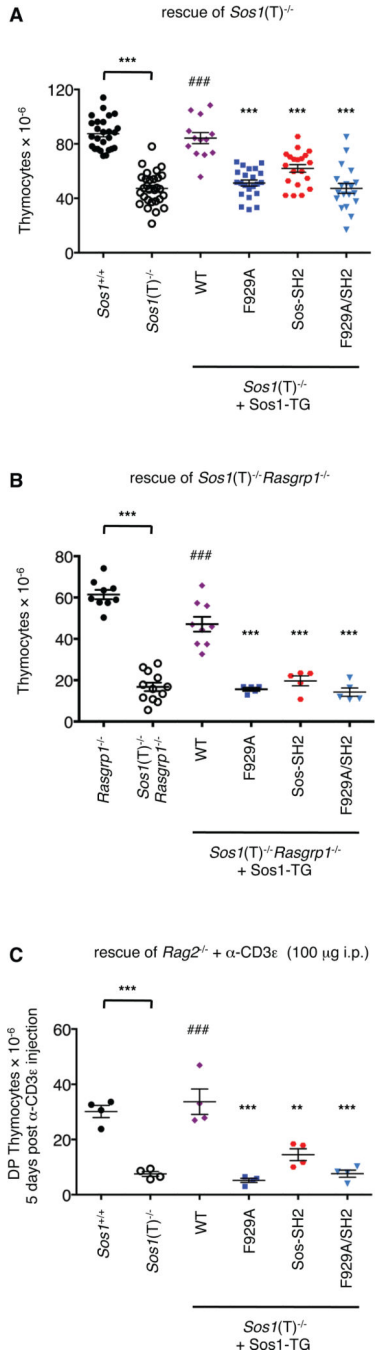


**Fig. 6. Multiple Sos1 functions are required for optimal pre-TCR-dependent development of thymocytes**

(**A**) Total numbers of thymocytes isolated from 8-week-old *Sos1*$^{+/+}$ mice, *Sos1*(T)$^{-/-}$ mice, and *Sos1*(T)$^{-/-}$ mice expressing the indicated Sos1 transgenes. Data are from n ≥ 12 mice for each genotype. (**B**) Total numbers of thymocytes isolated from 8-week-old *Rasgrp1*$^{-/-}$ mice, *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$ mice, and *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$ mice expressing the indicated Sos1 transgenes. Data are from n ≥ 5 mice for each genotype. (**C**) Numbers of DP thymocytes isolated five days after intraperitoneal injection of mice of the indicated

genotypes with 100 μg of α-CD3ε antibody (2C11). Data are from four mice for each genotype. In all panels, each symbol denotes an individual mouse, and the bars denote means ± SEM for the group. Statistical significance was determined by ANOVA. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ when compared to *Sos1*$^{+/+}$ mice [or to *Rasgrp1*$^{-/-}$ mice in (B)]; $^{\#\#\#}P \leq 0.001$ when compared to *Sos1*(T)$^{-/-}$ mice [or to *Sos1*(T)$^{-/-}$*Rasgrp1*$^{-/-}$ mice in (B)].

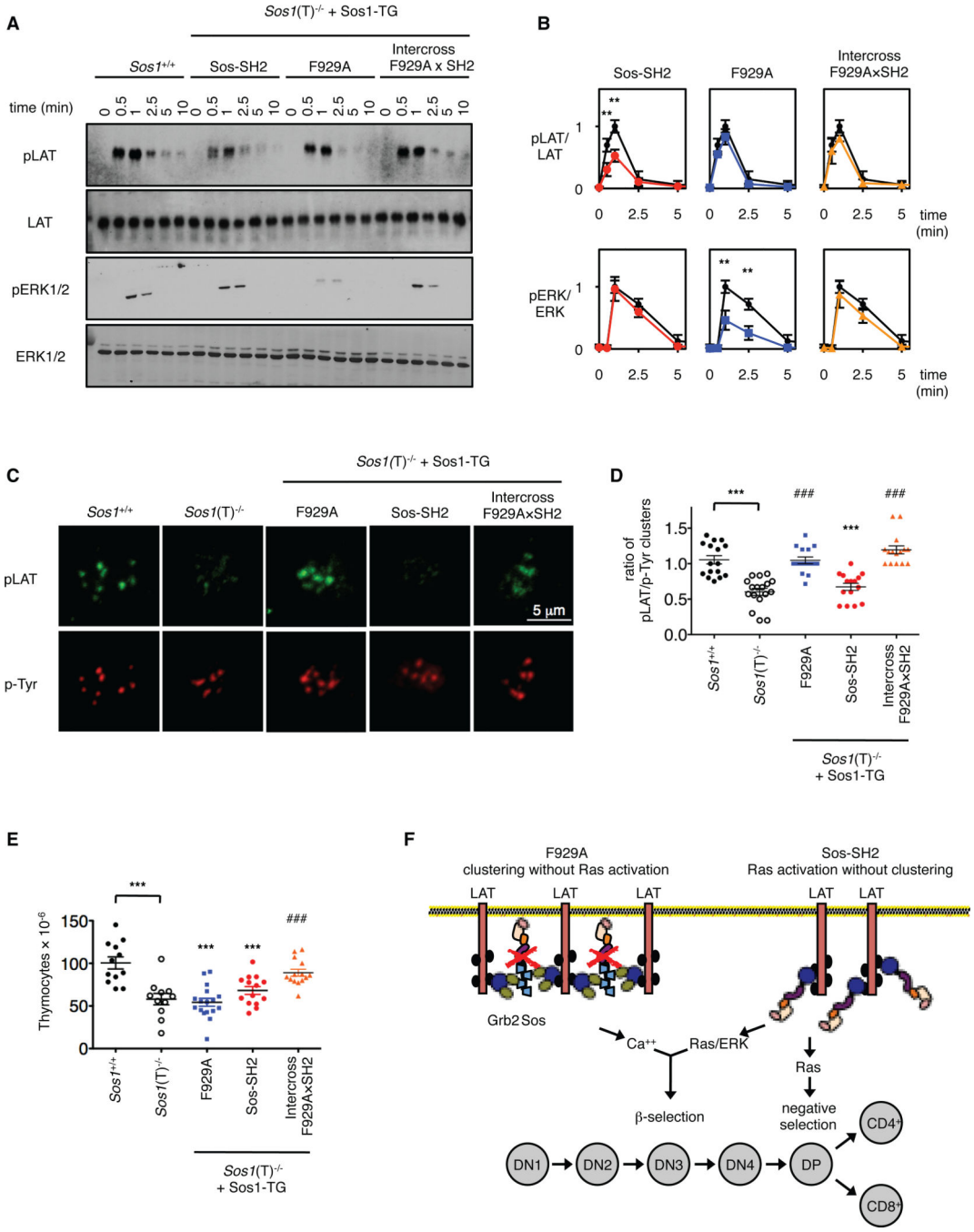


**Fig. 7. Combined expression of the Sos1 F929A and Sos-SH2 proteins rescues thymic cellularity in *Sos1*(T)$^{-/-}$ mice**

(**A** and **B**) DP thymocytes purified from the indicated mice were stimulated with α-CD3ε (3 μg/ml) and α-CD4 (5 μg/ml) antibodies for the indicated times, and then WCLs were analyzed by Western blotting with antibodies specific for pLAT (Tyr$^{191}$ ), total LAT, pERK1/2 and total ERK1/2. The intercross F929A×SH2 denotes *Sos1*(T)$^{-/-}$ mice expressing both the F929A and Sos-SH2 transgenes. (A) Western blots are representative of three independent experiments. (B) Quantitation of the ratios of pLAT and pERK1/2 abundances

relative to those of their respective total proteins. Data are means ± SD, and statistical significance was determined by 2-way ANOVA. For each Sos1 transgenic strain: $^{**}P \leq 0.01$ when compared to $Sos1^{+/+}$. (**C**) Purified DP thymocytes from the mice shown in (A) were dropped onto stimulatory coverslips and fixed after 3 min. After fixation, cells were permeabilized and immunostained for pLAT ($Tyr^{191}$, green) and pTyr (4G10, red). Images are representative of two experiments. (**D**) Quantitation of the ratio of the numbers of pLAT clusters to the numbers of pTyr clusters for the cells shown in (C). Data are pooled from n ≥ 15 cells for each genotype. Data are means ± SEM for the group. Statistical significance was determined by ANOVA. $^{***}P \leq 0.001$ when compared to $Sos1^{+/+}$; $^{\#\#\#}P \leq 0.001$ when compared to $Sos1(T)^{-/-}$ (**E**) Total numbers of thymocytes isolated from 8-week-old mice of the indicated genotypes. Data are pooled from n ≥ 12 mice for each genotype. Each symbol denotes an individual mouse, and bars denote means ± SEM for the group. Statistical significance was determined by ANOVA. $^{***}P \leq 0.001$ when compared to $Sos1^{+/+}$; $^{\#\#\#}P \leq 0.001$ when compared to $Sos1(T)^{-/-}$. (**F**) Proposed model of Sos1-dependent signaling during T cell development. Sos1-dependent oligomerization of LAT promotes maximal TCR-stimulated $Ca^{2+}$ flux, whereas the RasGEF activity of Sos1 is required for maximal TCR-stimulated phosphorylation of ERK. Both of these Sos1-dependent signals are required for optimal T cell development beyond the pre-TCR (DN3) developmental checkpoint. In contrast, the RasGEF activity of Sos1 alone can restore Sos1 function during the negative selection of thymocytes.