Receptor tyrosine kinases participate in several signaling pathways through small G proteins such as Ras (rat sarcoma). An important component in the activation of these G proteins is Son of sevenless (SOS), which catalyzes the nucleotide exchange on Ras. For optimal activity, a second Ras molecule acts as an allosteric activator by binding to a second Ras-binding site within SOS. This allosteric Ras-binding site is blocked by autoinhibitory domains of SOS. We have reported recently that Ras activation also requires the actin-binding proteins ezrin, radixin, and moesin. Here we report the mechanism by which ezrin modulates SOS activity and thereby Ras activation. Active ezrin enhances Ras/MAPK signaling and interacts with both SOS and Ras in vivo and in vitro. Moreover, in vitro kinetic assays with recombinant proteins show that ezrin also is important for the activity of SOS itself. Ezrin interacts with GDP-Ras and with the Dbl homology (DH)/pleckstrin homology (PH) domains of SOS, bringing GDP-Ras to the proximity of the allosteric site of SOS. These actions of ezrin are antagonized by the neurofibromatosis type 2 tumor-suppressor protein Merlin. We propose an additional essential step in SOS/Ras control that is relevant for human cancer as well as all physiological processes involving Ras.

ERM proteins | autoinhibition | GEF regulation

The small GTPase Ras (rat sarcoma) regulates essential cellular processes such as proliferation, motility, and differentiation. Activation of Ras by receptor tyrosine kinases (RTKs) is mediated by the guanine nucleotide-exchange factor (GEF) Son of sevenless (SOS). SOS is recruited by activated RTKs and subsequently engages Ras. In recent years, however, it has been recognized that this simple activation process is subject to a complex regulation. A number of regulatory motifs on SOS have been identified: the C-terminal catalytic Ras-binding domain for nucleotide exchange (1), the N-terminal half that carries histone-like sequences rich in positively charged amino acids, a Dbl homology (DH) domain, and a pleckstrin homology (PH) domain (1, 2). The DH/PH domains decrease the catalytic activity of SOS by folding back on the catalytic domain, thereby restricting accessibility to a second Ras-binding site that is distinct from the catalytic site (2). This allosteric Ras-binding site is important for the activation of SOS. Thus, Ras itself is an essential determinant of SOS regulation (2). Finally, lipid interaction contributes to the activation of SOS: The positively charged histone-like sequences interact with the negatively charged plasma membrane (3, 4). Moreover, binding of both phosphoinositides to the PH domain (5) and phosphatidic acid (PA) to the histone-like domain enhances SOS activity by relieving autoinhibition and exposing the allosteric Ras-binding site (6–8).

Our interest in small GTPases was triggered originally by the observation that members of a family of actin-binding proteins—ezrin, radixin, and moesin (ERM)—appear to enhance Ras activity (9). We showed that in response to growth factors ERM proteins form a multiribosomal complex at the plasma membrane that comprises Ras, SOS, filamentous actin, and coreceptors such as β1-integrin. Coreceptors focus these complexes to relevant sites of RTK activity at the plasma membrane/F-actin interface. We defined binding sites on ezrin for both Ras and SOS, mutations of which destroy the interactions and inhibit the activation of Ras. Our data revealed that ERM proteins are essential intermediates in the control of Ras activity that fine-tune growth factor signals. In the present study we dissect the level of ERM action in a purified system: In addition to the direct assembly of Ras and SOS, ezrin (the ERM protein prototype) participates in the control of SOS activity by facilitating the encounter of Ras and SOS. We conclude that ezrin mediates the spatiotemporal control of Ras activity by acting as a regulatory scaffold for Ras and SOS.

Results
We demonstrated that SOS precipitated from cells exerts significant nucleotide exchange activity only when bound to ezrin (9). This finding suggests that ezrin not only acts as a scaffold assembling Ras and SOS but also participates in SOS activity control (9).

In this paper we explore the regulatory condition under which ezrin participates in Ras activation. We then characterize the ERM-dependent complex in vitro by examining SOS activity using purified recombinant proteins. This method excludes the possibility that additional components present in the cell-derived ezrin complexes participated in SOS activation. Specifically, we focused

**Significance**

**Activity of the small GTPase Ras (rat sarcoma) needs to be tightly regulated because aberrant activity has a potent onco-genic effect, causing several forms of cancer as well as developmental disorders known as RASopathies.** We have identified the ezrin, radixin, and moesin protein family as a previously unknown component in the control of Ras activity. Ezrin interactions in the assembly of a protein complex that facilitates the encounter of Ras with its activating enzyme, Son of sevenless. Thus ezrin mediates a spatiotemporal control of Ras activity that helps coordinate and safeguard Ras signaling output.


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on whether ezrin is subject to regulation itself and whether ezrin exerts control over its partners, possibly contributing to the release of SOS from autoinhibition and enabling the presentation of Ras to the allosteric regulatory site of SOS.

**Active Ezrin Interacts with both SOS and Ras.** In vivo, ezrin can be activated by threonine phosphorylation (10). Threonine phosphorylation of the ERM proteins (ezrinT567, radixinT564, and moesinT558) releases intramolecular and/or intermolecular head-to-tail associations. An open active conformation allows ezrin to complex with SOS and Ras before growth factor stimulation (Fig. 1A). We observed that the addition of PDGF to NIH 3T3 cells caused ezrin (ezrinWT), formed a complex with SOS and Ras independent of PDGF stimulation (Fig. 1B and Fig. S1A, quantitation of exogenous ezrin expression). These data indicate that an active ezrin can interact with both SOS and Ras.

According to our previous data, this interaction with ezrinT567D should increase the GTP loading of Ras. Although the pseudoactive ezrin can complex with SOS and Ras before growth factor stimulation, ezrinT567D expression in NIH 3T3 fibroblasts showed enhanced and prolonged activation of Ras (determined as GTP-Ras) and MAPK/Erk activation (Fig. 1A). We tested whether this active ezrin can interact with Ras and SOS from cellular lysates in vitro and whether this active ezrin is sufficient for or contributes to Ras activation. GST- growth factor receptor-bound protein 2 (Grb2) pull-down experiments from cell lysates showed that pseudoactive ezrin, unlike wild-type ezrin (ezrinWT), formed a complex with SOS and Ras independent of PDGF stimulation (Fig. 1B and Fig. S1A, quantitation of exogenous ezrin expression). These data indicate that an active ezrin can interact with both SOS and Ras.

**Active Ezrin Stimulates SOS Activity in Vitro.** The DH/PH domains of the phosphatidylinositol (3′)-4,5′-bisphosphate (PI(3,4,5)P3) binding site of ezrin interacted with SOS, the GTPase activator. Recombinant SOS with a point mutation R40L (9) was introduced in the T567D background and resulted in increased activity vis-à-vis full-length SOS. SOS mutants containing the D38A mutation in the switch 1 region (13) could interact with ezrinT567D, the phospho-mimicking double mutant ezrinR40L/T567D, which lacked the ability to bind to Ras and phosho-Erk over total Ras illustrates an enhanced and prolonged signaling activation (graph in Fig. S1B). Apparently ezrin action on Ras is specific, because the pseudoactive ezrin had no significant effect on other small GTPases (e.g., Rac and Rho) (Fig. S1C). As a consequence of an increase in Ras activity, the proliferation rate of ezrinT567D-expressing cells was increased as compared with cells expressing ezrinWT (Fig. 1D).

**Active Ezrin Forms a Complex with SOS and Ras in Vitro.** To ensure that it is the active ezrinT567D that binds its partner proteins and that no other components were required, we analyzed the association of recombinant ezrinT567D with SOS and Ras in vitro. Recombinant SOS containing the histone, DH, PH, and catalytic Rem-Cdc25 domains (SOS HDPcat) (see Table S1 for construct overview; see Fig. S3 for SOS domain organization) bound to ezrinT567D, but not to ezrinWT (shown by His pull-down in Fig. 2A), suggesting that activation of ezrin is a prerequisite for ezrin–SOS interaction. EzrinT567D bound directly most strongly to the PH domain of SOS, indicating that the PH domain is at least one docking site for ezrin (GST pull-downs in Fig. S2A). In the ezrin–Ras interaction, ezrinT567D, like ezrinWT (9), interacted directly with GDP-Ras in vitro (Fig. 2B), as demonstrated by the concentration-dependent increase of fluorescence when ezrinT567D was added to fluorescent 2′,3′-O-(4′-Methylanthraniloyl)GDP (mantGDP)-Ras (Fig. 2B Datasheet and Methods). Size-exclusion chromatography further supported the finding that Ras interacts directly with ezrinT567D (Fig. S2B; ezrin–Ras interaction in fraction D1).

The critical test is whether ezrinT567D can form a complex with both Ras and SOS in vitro. To avoid nucleotide exchange that would disturb the fluorescence measurements, we used the fluorescent mantGDP loaded onto the Ras mutant Y64A that preferentially interacts with the allosteric Ras-binding site of SOS (11, 12). We could show clearly that SOS interacts with RasY64A and that ezrinT567D interacts with RasY64A, indicating an increase in GTP loading when added to fluorescent mantGDP-labeled Ras (Fig. 2C). Mixing all three proteins together further enhanced the fluorescence (Fig. 2C), suggesting that ezrinT567D, Ras, and SOS form a complex in vitro.

Finally we showed that, unlike wild-type Ras (RasWT), Ras containing the D38A mutation in the switch 1 region (13) could not interact with ezrin (Fig. 2D), indicating that GDP-Ras interacts with ezrin through the Ras switch 1 region. We conclude that active ezrin can recruit both SOS and Ras and thus serves as a scaffold, which is required for Ras activation in vivo.

**Active Ezrin Stimulates SOS Activity in Vitro.** The DH/PH domains impede SOS activation by shielding the allosteric Ras-binding site (2). Our identification of the PH domain as a direct interaction site for ezrin suggests that ezrin might facilitate SOS activation either by removing this DH/PH shield and/or bringing GDP-Ras to the allosteric site on SOS, that is essential for SOS activity (2). Therefore we investigated the action of ezrin on SOS activity in vitro by monitoring nucleotide exchange activity via the release of fluorescently labeled GDP (i.e., mantGDP) from preloaded Ras (made visible as a time-dependent reduction of fluorescence intensity; Fig. 3B and Fig. S3). Recombinant SOS (autoinhibited SOS HDPcat) exhibited low activity in vitro compared with an N-terminally truncated form of SOS (SOScat) that is not autoinhibited because it lacks the DH/PH and histone-like domains but still carries the allosteric site (a comparison of the nucleotide-exchange activity of different SOS constructs in the in vitro GEF assay is shown in Fig. S3). Interestingly, the addition of ezrinT567D caused a 41% increase in SOS HDPCat activity (Fig. 3B, indicating that ezrin alone is a modulator of SOS activity. Because Ras activation in a cellular system requires ezrin’s direct interaction with Ras (9), the Ras-binding site mutation in ezrin (R40L) (9) was introduced in the T567D background and tested for relevance in the purified system. Compared with the stimulating effect of ezrinT567D, the phospho-mimicking double mutant ezrinR40L/T567D, which lacked the ability to bind to Ras...
by its N terminus, could not increase SOS HDPcat activity (Fig. 3C); this result clearly indicates that the Ras-binding to ezrin is essential for ezrin’s effect on SOS activation. Taken together, these data show that in a purified system the nucleotide exchange activity of SOS is increased significantly when bound to active ezrin.

The lack of stimulating SOS activity could result from the failure of the ezrinR40L/T567D mutant to act as a scaffold bringing SOS and Ras into the complex and releasing autoinhibition. We also considered the possibility that ezrin might bring GDP-Ras closer to the allosteric site of SOS. This allosteric site drives nucleotide exchange and is subject to positive feedback control by nucleotide-bound Ras (2). The rate of nucleotide exchange by SOS is dependent on the concentration and on the nucleotide species bound to allosteric Ras (2); GTP-Ras is more potent than GDP-Ras. Generally, high concentrations of both GDP-Ras and GTP-Ras also will enhance the occupancy of the allosteric site and thereby increase the catalytic activity of SOS. To see whether ezrin indeed promotes the association of Ras with the allosteric site of SOS, we first exploited the unique property of the RasY64A mutant as a potent allosteric stimulator in a purified system with the self-masking and low-activity SOS HDPcat. The addition of the RasY64A stimulator at a high concentration increased nucleotide exchange significantly (46%) compared with a low concentration (19%) (Fig. 3D). Interestingly, the addition of the low concentration of RasY64A stimulator in combination with ezrinT567D increased SOS activity by 52%, mimicking the effect of the high concentration of stimulator alone (Fig. 3D). However, disruption of ezrinT567D’s GDP-Ras binding (R40L/T567D) abolished its SOS-enhancing ability (Fig. 3D). These results suggest that part of ezrin’s action might be to bring Ras to the proximity of the allosteric site on SOS.

Interaction of the Ezrin–Ras Complex with the Allosteric Site of SOS Enhances SOS Activity. The notion that phosphorylated, Ras-bound ezrin stimulates SOS activity was analyzed further in a second experimental approach. SOScat composed of the Ras exchange motif (Rem) and the Cdc25 domains interacted with the ezrinT567D mutant when isolated together with ezrin from cell lysates (coimmunoprecipitation in Fig. 4A). The interaction with ezrinWT was weaker than that with ezrinT567D (Fig. 4A), perhaps because of insufficient activation of the bulk of transfected ezrin. Disruption of the allosteric site by mutation (SOScat W729E; Table S1) (2) completely inhibited the interaction with ezrin (Fig. 4A). These data indicate that the phospho-mimicking ezrin can interact with SOScat. EzrinT567D and SOScat might interact directly when stabilized by other cellular components, or ezrinT567D may bind SOScat indirectly through Ras at the allosteric Ras-binding site.

We also tested whether ezrin could enhance the activity of SOScat and performed fluorescent GEF activity measurements analogous to the experiments with SOS HDPcat shown in Fig. 3. Interestingly, biexponential fitting of the kinetics data revealed two
components describing the exchange of mantGDP-Ras by SOScat: a faster and a slower rate acting at timescales of seconds and minutes, respectively (Fig. S3). We propose that the two kinetics components might reflect different conformational states of SOScat acting at different exchange rates. Addition of the allosteric stimulator RasY64A further enhanced both rate constants in a concentration-dependent manner (Fig. S4A and Table S2), increasing concentrations of GDP- and GTP-RasY64A support the faster exchange, with GTP-RasY64A being more potent, demonstrating the moderate binding affinity of GDP-RasY64A (Fig. S4C). In addition, GTP-RasY64A shifted the relative contributions of the two rates (Table S2). In the presence of GDP, 30% of the nucleotide exchange is related to fast-acting SOScat, whereas in the presence of increasing concentrations of GTP-RasY64A the contribution of fast-acting SOScat increased progressively up to 80% (Table S2). Mutation of the allosteric site (SOScat W729E) completely eliminated the fast-kinetics component (Fig. S4 B and D), demonstrating the importance of allosteric regulation of SOS.

These data set the stage for testing the effect of ezrin on the allosteric site of SOScat. A mixture of ezrinT567D, SOScat, and fluorescent mantGDP-Ras (substrate as well as stimulator) (raw data are shown Fig. 4B) simultaneously both the fast-kinetics component (52% increase of the nucleotide dissociation rate) (Fig. 4C, Left) and the slow-kinetics component (36% increase) (Fig. 4C, Right) compared with mantGDP-Ras and SOScat in the absence of ezrin. Disruption of ezrinT567D’s GDP-Ras binding (R40L/T567D) abolished this stimulation of SOScat activity of both kinetics components (Fig. 4D), indicating that ezrin–Ras interaction is required to elevate SOScat activity. Disruption of the allosteric site in SOScat (SOScat W729E) also abolished the stimulation by ezrin–Ras (Fig. 4E). We also studied ezrin action at different SOScat concentrations (Fig. S5A). Remarkably, ezrin does not potentiate nucleotide exchange at low and high concentrations of SOScat (Fig. S3 B, C, and E) as compared with moderate SOScat concentrations (0.5 μM) (Fig. S2D). We investigated this behavior in more detail by analyzing the relative contribution of the fast and slow components of the exchange reaction (Fig. S5F). At low SOScat concentrations, the slow rate dominates the reaction; at high SOScat concentrations, the fast rate takes over. We reason that ezrin acts predominantly on the mechanism that is responsible for the fast rate of nucleotide exchange, thus explaining why ezrin is less effective at low SOScat concentrations with little contribution of the fast rate. At very high concentrations of SOScat, the exchange reaction saturates, and ezrin again is less effective. In conclusion, ezrin-dependent stimulation of SOS requires an intact allosteric site and suggests that ezrin could bring GDP-Ras to the allosteric site on SOS more efficiently than free GDP-Ras.

We also show that ezrin is a regulatory scaffold for SOS activity control in vivo. We exploited another SOS mutant, the Noonan mutant M269R. Noonan syndrome is caused by a mutation that eliminates SOS autoinhibition (14, 15). Therefore this mutant behaves similarly to SOScat, permitting Ras access to the allosteric site. Cells expressing the Noonan mutation displayed elevated levels of activated Ras and MAPK/ERK (Fig. 4F, lanes 1 and 2). Down-regulation of ERM proteins inhibited Ras activation, demonstrating the dependence of SOS M269R on ERM proteins (Fig. 4F, lanes 2 and 4). However, a mutated Ras with high affinity to the allosteric site (D38E/A59G) (16) stimulated SOS activity even in the absence of the ERM proteins (Fig. 4F, Right), indicating that the need for the ERM proteins can be overcome by stronger Ras binding to the allosteric site. In conclusion, ezrin brings GDP-Ras to the proximity of the allosteric site on SOS both in vitro and in vivo.

**The Lipid Environment Contributes to Full SOS and Ras Activation.** Ras activation in vivo occurs at the cytoplasmic side of the plasma membrane. In vitro a highly structured artificial surface mimicking a membrane enhances SOS activity (5, 6). Moreover, binding of phosphoinositides to the PH domain and the binding of PA to the histone-like domain also modulate SOS autoinhibition (5–8). Therefore we considered whether other cellular components might be missing in vitro that contribute to full SOS activation in vivo. The addition of PA to SOS HDPcat in the assay with recombinant proteins indeed led to an increased activity (17%) (Fig. 5A). A combination of ezrinT567D and PA further enhanced SOS activity (63%) (Fig. 5B). Although the recombinant proteins do not fully recapitulate the in vivo conditions, they do demonstrate that ezrin has a decisive role in the regulation of SOS activity and that ezrin and PA cooperate.

If membrane targeting and lipid association in vivo are critical components for this unique signaling complex, we speculated that
artificially tethering ezrin to the plasma membrane would lead to activation of Ras. Interestingly, tethering of ezrin to the membrane by the Src membrane-association domain was sufficient to induce Ras activation spontaneously (Fig. 5B, left; without FCS stimulation) and cell proliferation (Fig. 5B; without FCS stimulation). The membrane-anchored ezrin was more heavily phosphorylated than overexpressed WT ezrin (Fig. 5C) and induced MAPK/ERK activity (Fig. 5C) and cellular transformation (determined as soft-agar colony formation) (Fig. 5D). Despite increased phosphorylation, the membrane-anchored ezrin mutated in the Ras-binding site (R40L) (9) could not induce cellular transformation and carried a reduced MAPK/Erk activity (Fig. 5C and D). Taken together, these results suggest that in vivo targeting of ezrin to the plasma membrane and its direct interaction with Ras and SOS are essential for full activation of SOS.

### Physiological Regulation of Ezrin Activity

Merlin, the neurofibromatosis type 2 tumor-suppressor protein, can counteract ezrin by competing for the same binding sites on coreceptors (17). Merlin activation at high cell density antagonizes the formation of the SOS–ezrin complex (18). Neither activated merlin from high cell density cells (18) nor an active merlin mutant (S518A) interacted with SOS (Fig. S7A). However, we did observe a complex between merlin and Ras (Fig. 5B and C). Purified Ras loaded with GDP pulled down merlin from cellular lysates (Fig. S7B). Also incubation with fluorescent mantGDP-Ras indicated that merlin interacted directly with GDP-Ras (Fig. S7C), likely through the N-terminal 4.1 protein/ezrin/radixin/moesin (FERM) domain also found in ezrin. Because merlin cannot interact with SOS, we reasoned that, unlike ezrin, merlin is unable to bring GDP-Ras to the allosteric site. Indeed, a mixture of recombinant merlin and SOS HDPCat (Fig. S7D) showed no increase in SOS activity. Moreover, purified merlin added to SOSCat significantly inhibited nucleotide exchange activity.

### Discussion

Ras is an essential signaling protein that cycles between inactive GDP-bound and active GTP-bound states. Triggering nucleotide exchange and thereby activating Ras through RTKs is a crucial step in mediating multiple cellular functions. Downstream of RTKs, the GEF SOS is a necessary intermediate in Ras control. This step of SOS-mediated Ras stimulation includes several regulatory actions to coordinate and safeguard biological output. SOS, a large molecule with several domains, contains two binding sites for Ras, the catalytic site and an allosteric site. GDP-Ras or GTP-Ras binding to the allosteric site drives the catalytic function of SOS. The DH/PH domains block the access to the allosteric site (2) and therefore must dissociate from the allosteric site, a process promoted by the interaction with lipids of the plasma membrane (2, 5, 7).

Our results add to the complexity of the SOS-Ras regulation. Phosphorylated ezrin (the ERM protein prototype) directly binds Ras and SOS. In addition, we show that active ezrin elevates the nucleotide exchange activity of SOS (model in Fig. 6), probably by facilitating the encounter of Ras with the allosteric regulatory site of SOS. Our conclusions are based on several experiments, which included the disruption of ezrinT567D binding GDP-Ras, thereby inhibiting ezrin’s ability to enhance SOS. Because the affinity of GDP-Ras for the allosteric site is weaker than that of GTP-Ras (12), ezrin appears to stimulate predominantly the initial allosteric regulation through its preferential interaction with GDP-Ras. To exert these functions, ezrin must be activated at least by phosphorylation of threonine 567, a conclusion supported by the experiments with the phospho-mimicking ezrin mutant (T567D).

We have demonstrated recently and in this study that the FERM domain of ezrin interacts with Ras (9). The recent structure determination of the FERM domain of Krev interaction trapped 1 (KRIT1) with Ras-related protein 1 (Rap1) revealed the mode of FERM-KRIT1 binding (Fig. S8). Structural superposition of the structures of ezrin FERM and GDP-Ras onto KRIT1 and Rap1 of the complex structure, respectively, illustrates the probable interaction of ezrin with Ras (Fig. S8). The lack of ezrinT567D/R40L binding to GDP-Ras and of GDP-Ras/D38A binding to ezrinT567D is consistent with ezrin R40 and Ras D38 being part of the resulting interface. In addition, a Ras switch region is involved in binding, in agreement with the observed nucleotide-specific binding of ezrin to GDP-Ras. Interestingly, according to the superposition, GDP-Ras binds to ezrin with a surface area resembling those that are used by Ras to bind to the allosteric site of SOS (2), suggesting that ezrin might not simply transfer GDP-Ras directly to the SOS allosteric target site. Therefore, a possible mechanism of SOS activation could be that SOS binding to ezrin might evoke subtle conformational changes in ezrin leading to the release of GDP-Ras and re-binding of Ras to the allosteric regulatory site of SOS. Alternatively, the interaction of GDP-Ras with phosphorylated ezrin might be necessary for full opening of the C-terminal and middle domain of ezrin (Fig. S8A) (19), thereby enabling the productive interaction of ezrin with SOS and contributing to the release of SOS autoinhibition (Fig. 6). Obtaining high-quality structures and interactions of SOS–ezrin and Ras–ezrin would be the ultimate goal in achieving a complete mechanistic understanding of how ezrin contributes to the control of SOS activity.

The assembly of the ezrin–SOS–Ras complex using recombinant and purified components indeed proves the regulatory interactions. Although the stimulation is modest in vitro, it still is
significant. In our assay ezrin by itself is not an SOS allosteric stimulator but rather assists its direct partner Ras to localize efficiently to this allosteric Ras-binding site in SOS. Nevertheless, ERM-dependent Ras activation in vivo is obviously more efficient than the stimulation by active ezrin in our purified system (9). The assembly at the inner leaflet of the lipid membrane is one obvious component. A modest increase in SOS activity was observed in vitro by the addition of lipids. A combination of PA and ezrinT567D produced an additive stimulatory effect on SOS activation, suggesting that indeed the in vivo targeting of active ezrin to the plasma membrane and its direct interaction with SOS are both essential features for the full activation of SOS.

Scaffold proteins play a critical role in regulating diverse signaling events by organizing biochemical reactions at the right time and place. Because Ras activity must be tightly controlled in all cells, misregulation of such scaffolds (e.g., ERM proteins) also could contribute to human pathology. Here two disease-relevant results are worth stressing: The neurofibromatosis type 2 tumor-suppressor protein merlin interferes with SOS activation both in vivo and in the complex with recombiant proteins in vitro. Ezrin fused to a membrane anchor and thus constitutively bound to the inner phase of the plasma membrane exerts tumorigenic properties. ERM proteins often are overexpressed in cancer. Our findings suggest that elevated expression of ezrin (20, 21), radixin (22), and moesin (23) may contribute to cancer progression and metastasis by increasing Ras activity. We propose an additional level of SOS/Ras control involving a family of actin-binding proteins as a molecular step that is relevant in Ras-dependent physiological processes.

Materials and Methods

Detailed information about cell lines, plasmids, antibodies, siRNA sequences, purification of recombinant proteins, preparation of ezrin- and merlin-expressing cell lines, and pull-down methods can be found in SI Materials and Methods.

Proliferation Assay. Proliferation assays were performed using the PrestoBlue Cell viability reagent (Invitrogen) following the manufacturer’s instructions. Details are given in SI Materials and Methods.

Soft Agar Assay. Stably transduced NIH 3T3 cells or doxycycline-inducible RT4ethif2 cells were used based on the principles described (21). Details are given in SI Materials and Methods.

Fluorescent GEF Activity Measurements in Vitro. Nucleotide loading and nucleotide exchange measurements for Ras were performed as described previously (12).

Protein Interaction Studies. Protein interaction was analyzed in vitro based on the 2′,7′-dichlorofluorescin diacetate (DCFDA) uptake assay. Upon binding to a protein, the fluorescence quantum yield of the mant fluorophore increases, thus leading to an increase in fluorescence (24). Details are given in SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Growth Factors, Antibodies, and Reagents. Recombinant human PDGF BB was obtained from Biomol; Igepal CA-630, Triton X-100, GDP, GTP/S, PEG 6000, Polybrene, and docycycline (dox) from Sigma; 2'-3'-O-(N-Methylanthranilyl)GDP (mantGDP), manTGTP and GST protein from Jena Bioscience, Lubrol 17A17 from Uniqyma; GST-growth factor receptor-bound protein 2 (Grb2) glutathione agarose (GST-Grb2), RAF proto-oncogene serine/threonine-protein kinase (c-Raf/Raf1)–Ras-binding domain glutathione agarose (GST-Raf1-RBD), and GST-Ras- and GST-agarose from Upstate; glutathione agarose from Santa Cruz. GST-B (1-14), His (H-3), Erk1 (K23), ezrin (C-19), moesin (C-15), radixin (C-15), merlin (A-19), Son of sevenless homolog 1 (SOS1) (C-25), Myc (9E10), and tubulin (TU-02) antibodies were obtained from Santa Cruz; Ras (rat sarcoma) antibody (RAS10) was obtained from Upstate; VSVG, HA antibody was obtained from Roche; Flag (F1804) was obtained from Sigma; ezrin antibody (3C12) was obtained from NeoMarkers. Antibodies against the phosphorylated proteins Erk (Thr202/Tyr204) and ezrin (Thr567)/radixin (Thr564)/moesin (Thr558) were obtained from Cell Signaling.

Cell Culture. NIH 3T3 cells (immortalized Swiss mouse embryo fibroblasts; European Collection of Animal Cell Cultures) were grown in DMEM (4.5 g/L glucose, with 1-glutamine) (PAALaboratories GmbH) supplemented with 10% (vol/vol) donor FCS (PAA Laboratories GmbH). For RT4 cells, high cell density (the equivalent of logarithmic or exponential growth) was determined. Measurements were performed in triplicate.

Definition of Growth Condition in Culture Dishes. For NIH 3T3 cells, low cell density (logarithmic or exponential growth) was defined as the density recorded 24 h after seeding of 500 cells per square centimeter. For RT4 cells, high cell density (the equivalent of confluent growth condition) was defined as the density recorded 24 h after seeding of 5,000 cells per square centimeter.

Generation of Stable Clones. To generate stable NIH 3T3 clones (NIH 3T3 ezrinWT, NIH 3T3 ezrinT567D, NIH 3T3 ezrinR40L, NIH 3T3 Src-EzrinWT, and NIH 3T3 Src-EzrinR40L), cells were transfected with lentivirus coding for GFP and wild-type ezrin (ezrinWT), ezrinT567D, ezrinR40L, Src-EzrinWT, or Src-EzrinR40L. Positive cells were selected using FACS. Generation of RT4 cells carrying dox-inducible merlinWT (designated RT4tetNf2) or merlinS518A (designated “RT4tetNf2/S518A”) has been described earlier (1).

Transient Plasmid and siRNA Transfection of Cells. Transfections were performed using Lipofectamint 2000 (Invitrogen) in accordance with the manufacturer’s instructions. If growth factor stimulation was required, cells were serum starved overnight before PDGF stimulation. Knock-down efficiency was checked after 2–3 d. siRNA sequences (Dharmacon, Thermo Scientific) were as follows: ezrin, SMARTpool (mouse): GGU ACA GGA GCC UCC GAA AUU, CCG CAC AGG AGG UCC GAA AUU, GCG CAA GGA GGA CGA GGU AUU, UAAUACG ACG CUG CGG CAA AUU; radixin, SMARTpool (mouse): CCA ACA CAU CAC AGC GAU AUU, CCA AUC AUA GUA AGA GUA AUU, GCC CAA UAC AAG GCA AUU, GAU CGU UAU UGU AGC AUA UUU; moesin, SMARTpool (mouse): CGG AUU AAC AAG CGG AUC UUU, UUU CAG UAU UAG CGA CUU AUU, AGU UGG AAA UGG CUC GAA AUU, GAG AAG AUU GAG CGG GAG GAA AUU.

Plasmids. Myc-H-RasWT and HA-H-RasWT in pcDNA3.1+ were purchased from the University of Missouri cDNA Resource Center, University of Missouri-Rolla, Rolla, MO. For protein purification, ezrinWT was cloned in a pET15T7E expression vector (C.B.), and merlin was cloned in pET30a+ (BamHI/NotI). SOScat (amino acids 564–1,094) in pPreX HTB expression vector was obtained from A. Wittinghofer, Max Planck Institute for Molecular Physiology, Dortmund, Germany. SOS DHPhCat (amino acids 198–1,049), SOS HDPeat (amino acids 1–1,049), and H-Ras (amino acids 1–166) were cloned in pET15T7E expression vector (BamHI). All point mutations were generated using the QuickChange site-directed mutagenesis kit (Stratagene) in accordance with the manufacturer’s instructions. Lentiviral ezrin constructs were cloned in the SparQ Dualpromoter vector (QMS11B-1; System Biosciences).

Proliferation Assay. Proliferation assays were performed using the PrestoBlue Cell viability reagent (Invitrogen) following the manufacturer’s instructions. Briefly, cells were seeded and grown in normal medium. At the indicated time, medium was replaced by fresh medium containing PrestoBlue. After 1.5 h the metabolic activity was detected by means of fluorescence (excitation 530 nm, emission 600 nm). Measurements were made in triplicate.

Soft Agar Assay. Stably transduced NIH 3T3 cells or dox-inducible RT4tetNf2 cells were detached from culture plates with 0.25% trypsin, resuspended in complete medium, and counted. Cells (1 x 10⁴) were diluted in 3.6 mL medium containing 10% (vol/vol) FCS (PAALaboratories GmbH). In all cell-culture experiments 1 μg/mL dox was used. Stimulation with PDGF was performed at a final concentration of 10 ng/mL.

Affinity Precipitation and Immunoprecipitation. GTP-Ras was pulled down from cell lysate with GST-Raf1-RBD. Cells were lysed in 25 mM Hepes (pH 7.5), 150 mM NaCl, 10 mM MgCl₂, 1% Igepal CA-630, 10% (vol/vol) glycerol, 1 mM EDTA, 1 mM sodium vanadate, 1 mM PMSF, 10 mg/mL aprotinin, and 10 mg/mL leupeptin. Immunoblotting quantifications were performed using QuantiiOne software (Bio–Rad Laboratories) or ImageJ. Immunoprecipitation was performed as described previously (1). In brief, cells were lysed in 25 mM Hepes (pH 7.5), 20 mM NaCl, 0.5% Lubrol 17A17, 1 mM sodium vanadate, 1 mM PMSF, 10 mg/mL aprotinin, and 10 mg/mL leupeptin. Supernatants were incubated with 2 μg of antibody or 5 μg of GST-Grb2 fusion protein rotating at 4°C for 1 h.

Rac and Rho G-Lisa. Activation of Rac (Ras-related C3 botulinum toxin substrate) and Rho (Ras homolog gene family) were measured using the Rac1,2,3 or RhoA G-Lisa kit (BK124 or BK125; Cytoskeleton) according to the manufacturer’s instructions. Measurements were performed in triplicate.

Fluorescent Guanine Nucleotide-Exchange Factor Activity Measurements in Vitro. Nucleotide loading and nucleotide exchange measurements for Ras were performed. Dissociation rates were measured with a stop-flow apparatus (SFM-400; Bio–Logic) equipped with fluorescence.
Plate Reader Exchange Assays. Exchange reaction buffer [40 mM Hepes (pH 7.5), 10 mM MgCl₂, 1 mM DTT] was completed with 2 mM mantGDP-Ras, 400 μM GDP, SOScat (2–0.125 μM), and ezrinT567D protein (5–0.5 μM) in a black plate with 384 round-bottomed wells (Corning). Samples were analyzed immediately with a Mithras LB 940 Multimode Microplate Reader (Berthold Technologies) at 20 °C. Fluorescence emission was recorded at 460 nm after excitation with lamp energy of 5,000 (5.72 W) at 4 °C overnight using ProTEV Plus protease (Promega), and the His tag was cleaved using TEV Protease (Promega) following the manufacturer’s instructions.

Protein Interaction Studies. Protein interaction was analyzed in vitro based on the mant fluorophore. Upon binding to a protein, the fluorescence quantum yield of the mant fluorophore increases, thus leading to an increase in fluorescence. Fluorescence was monitored with a Shimadzu RF-5301PC spectral fluorophotometer (excitation 370 nm, emission 430 nm). Fluorescent mantGDP/mantGTP-Ras and respective unlabelled proteins were incubated in reaction buffer [40 mM Hepes (pH 7.5), 10 mM MgCl₂, 1 mM DTT]. Measurements were done in triplicate. The relative increase in fluorescence was calculated by subtracting the values for the intrinsic fluorescence of respective proteins.

Pull-Down Assays Using Recombinant Proteins. The respective His-tagged ezrin proteins (1–2 μg) were linked to Ni-Sepharose and were incubated with 50–100 ng SOS in 500 μL of interaction buffer [50 mM Hepes (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, and 0.5% Triton X-100] overnight at 4 °C under constant rotation. The samples were washed [50 mM Hepes (pH 7.5), 80 mM NaCl, 10 mM MgCl₂, and 0.5% Triton X-100] and were subjected to SDS/PAGE and Western blotting.

Production of Lentiviral Particles. Virus particles were produced and concentrated as previously described (3). In brief, HEK293T cells were co-transfected with the SparQ Dualpromoter vector and the packaging plasmids pCMV-dR8.91 and pMD.G. Viral particles were concentrated threefold by polyethylene glycol precipitation, resuspended in medium, and used for transduction of NIH 3T3 cells. Polybrene was added to a final concentration of 8 μg/mL.

Fig. S1. (A) NIH 3T3 clones stably expressing GFP alone (Control), Myc-ezrinWT, or Myc-ezrinT567D were serum starved overnight and subsequently were treated with PDGF (3 min) to induce phosphorylation of ezrin. The phospho-specific antibody used recognizes both endogenous and transfected Myc-ezrinWT as well as the phospho-mimicking Myc-ezrinT567D. The amount of overexpressed Myc-ezrin was evaluated in a Western blot and quantified using ImageJ. (B) Quantification of GTP-Ras (Upper) or P-ERK1 (Lower) at indicated PDGF stimulation periods using QuantityOne. Signals were normalized to total Ras levels, and the increase over no PDGF stimulation was calculated. (C) Empty vector (Control), EzrinWT, or ezrinT567D-expressing stable clones of NIH 3T3 cells were seeded, serum starved overnight, and stimulated with PDGF for the indicated time periods. Collected lysates were used for Rac (Left) or Rho (Right) G-Lisa to determine Rac/Rho activation levels. Signals were normalized to the Rac/Rho activation level of ezrinWT cells with no PDGF stimulation. Data are shown as mean ± SD; n = 3; n.s. not significant using the Student t test.
Fig. S2. (A) Active ezrinT567D interacts directly with the pleckstrin homology (PH) domain of SOS. GST-SOS fragments were expressed in E. coli (for domain organization of SOS, see Fig. 3A), immobilized on glutathione Sepharose, and used to pull-down recombinant purified ezrinT567D. EzrinT567D was detected by immunoblotting. The input of GST fusion proteins was stained with Ponceau S (n = 3). All lanes were assembled from the same gel, and immunoblots were taken at the same exposure time. (B) Size-exclusion chromatography was carried out with ezrinT567D or RasWT alone or after incubation overnight at 4 °C. Collected protein fractions based on absorption at 280 nm were subjected to SDS/PAGE and immunoblotted as indicated.
Fig. S3. SOS HDPcat is autoinhibited, whereas truncated SOScat is released in vitro. (Top) Schematic representations of different SOS constructs. The allosteric site, indicated by a yellow star, is accessible and functional only in the truncated SOScat WT. Fluorescent mantGDP-Ras (1 μM) was incubated with unlabeled GDP (200 μM) in the absence (mantGDP-Ras, black) or presence of SOS HDPcat (brown, 1 μM), SOScat WT (red-brown, 1 μM), or SOScat W729E (red, 1 μM), and the release of labeled nucleotide was measured. Exchange is indicated by a decline in fluorescence over time. Raw data (Middle) were subjected to linear (mantGDP-Ras, SOS HDPcat), single (SOScat W729E), or biexponential (SOScat WT) fitting, and apparent nucleotide dissociation rates were plotted in a column diagram (Bottom). Quantitative results represent mean ± SD. n = 3.
Fig. S4. (A and B) SOScat shows a biexponential decay function for nucleotide exchange activity toward Ras (A), whereas SOScat W729E shows single exponential decay only (B). In vitro guanine nucleotide-exchange factor (GEF) activity was measured as shown in Fig. S3. Raw data were subjected to either biexponential (SOScat WT, red) or single exponential (mantGDP-Ras, black; SOScat W729E, brown) fitting. Apparent nucleotide dissociation rates are shown. Data are shown as mean ± SD; n = 3. (C and D) SOScat exchange activity can be allosterically stimulated, unlike SOScat W729E. (C) The SOScat WT exchange rate can be allosterically stimulated by the weak stimulator GDP-RasY64A or the strong stimulator GTP-RasY64A. In vitro GEF activity was measured as described in Fig. S3. The SOScat WT-catalyzed exchange reaction was measured in the absence or presence of 1 μM and 5 μM GDP-loaded RasY64A or GTP-loaded RasY64A. 1 μM (1x) is the equivalent of the mantGDP-Ras concentration. The mantGDP–Ras curve was fitted to a single exponential, whereas SOScat WT reactions were biexponentially fitted. Apparent nucleotide dissociation rates are shown (mean ± SD; n = 3). (D) SOScat W729E cannot be stimulated by GDP-RasY64A or GTP-RasY64A. The SOScat W729E-catalyzed exchange reaction was measured in the absence or presence of 1 μM and 5 μM GDP-loaded RasY64A or GTP-loaded RasY64A. The mantGDP–Ras curve was fitted to a single exponential, whereas SOScat W729E reactions were biexponentially fitted. Apparent nucleotide dissociation rates are shown (mean ± SD; n = 3).
Fig. S5. Nucleotide exchange at different SOScat concentrations. MantGDP-Ras (2 μM) was incubated with different concentrations of SOScat (0, 0.5, 1, 2, 3.5, and 5 μM) in the absence and presence of ezrinT567D (5 μM). (A) Traces for SOScat alone. The nucleotide exchange reaction speeds up with the concentration of SOScat and saturates above 1 μM SOScat. (B–E) The nucleotide exchange reaction was analyzed by fitting biexponential functions to the data. The resulting functions were normalized \[F = (F - F_{\text{min}})/(F_{\text{max}} - F_{\text{min}})\] and plotted for 0 (black) and 5 μM (red) ezrinT567D with 0.125 μM (B), 0.25 μM (C), 0.5 μM (D), or 1 μM (E) SOScat. (F) Relative contribution of the fast component \[F = (A \tau_f)/(A \tau_f + A \tau_s)\] of the exchange reaction in the absence (black) and presence (red) of ezrinT567D as a function of the SOScat concentration.

NIH 3T3 cells

Fig. S6. The proliferation rate of NIH 3T3 ezrinWT cells is lower than that of cells expressing Src-Ezrin as determined by the Presto Blue proliferation assay. Data are shown as mean ± SD; \(n \geq 3\).
Fig. S7. (Continued)
Fig. S7. Merlin antagonizes ezrin function. (A) RT4 cells carrying dox-inducible Flag-Merlin5518A (RT4tetNf2S518A) were cotransfected with a construct expressing Myc-SOS (Myc IP) or Flag-Merlin (FLAG IP) were immunoblotted. Mouse IgG used as control IP. (B) Purified recombinant His-Ras loaded with either GDP or GTP was incubated with lysates of RT4 cells carrying dox-inducible merlinWT. His pull-down was performed, and immunoblots are shown. (C) Fluorescent mantGDP-Ras (1 μM) was incubated in a 1:1 ratio with ezrinWT (blue) or merlinWT (turquoise) protein, and fluorescence intensity was measured. Data are shown as mean ± SD; n = 3. (D) Merlin has no influence on SOS HDPcat-catalyzed nucleotide exchange. Merlin (turquoise, 1 μM) was added to the SOS HDPcat exchange reaction shown in Fig. 3B and Fig. S3. (Left) Raw data. (Right) Nucleotide dissociation rates are results of linear fitting. Quantitative results are shown as mean ± SD; n = 3. (E) Merlin inhibits SOScat-catalyzed nucleotide exchange. Merlin (turquoise, 1 μM) was added to the SOScat exchange reaction described in Fig. 3B and Fig. S3. Curves (Left) and apparent nucleotide dissociation rates are results of single (SOScat + merlin; Lower Right) or biexponential (SOScat alone, Upper Right) fitting. Quantitative results represent mean ± SD; n = 3. (F) One clone from G expressing either VSVg-ezrinWT or VSVg-ezrinT567D was plated at high density, and merlin expression was induced by dox in the presence or absence of PDGF stimulation. Lysates were immunoblotted as indicated. (G) RT4 tetNf2 cells carrying dox-inducible WT merlin (RT4tetNf2) were cotransfected with constructs encoding VSVg-ezrinWT or VSVg-ezrinT567D and a hygromycin-resistance construct and were plated in soft agar without (-) or with (+) dox. Results shown are mean ± SD of three clones of each type; n ≥ 3; ***P < 0.001 using the Student t test. Lysates were immunoblotted to confirm ezrin expression.
Fig. S8. Structural basis of the GDP-Ras–ezrin interaction. (A) Overall structure of ERM (ezrin-radixin-merlin) proteins [Protein Data Bank (PDB) ID code: 2I1J (1)]. The N-terminal 4.1/ezrin/radixin/moesin (FERM) domain (shown in blue and green) adopts a cloverleaf-like structure composed of the three subdomains F1, F2, and F3. The central coiled coil domain (beige) covers the F1 and F2 lobes, whereas the C-terminal region (CERMAD, red) binds to the F2 and F3 lobes. Phosphorylation of Thr567 (ezrin numbering, shown in yellow) causes dissociation of the CERMAD from the FERM domain, corresponding to an opening of the structure (2). Phosphatidylinositol 4,5-bisphosphate and receptor binding to the FERM domain (3–5) are thought to contribute to the full opening and activation of the ERM proteins (6). (B) Complex structure of the Krev interaction trapped 1 (KRIT1) FERM domain [shown in blue and green; PDB ID code: 4DXA (7)] and GTP–Ras-related protein 1b (Rap1b) (shown in orange and red). Both switch regions (highlighted in light red) of Rap1b are involved in binding. The binding of KRIT1-F1 to Rap1b resembles the Ras–Raf interaction, whereas KRIT1-F2 and Rap1b exhibit a unique interaction. Insets I–III show the putative binding mode of ezrin FERM to GDP-Ras. For a comparative analysis, the F1 and F2 subdomains of ezrin FERM [1NI2 (8)] were superimposed onto those of KRIT1 FERM [rmsd of 2.8 Å with 185/200 superimposed Cα atoms using DaliLite (9)], and GDP-Ras [PDB ID code: 4Q21 (10)] was superimposed onto Rap1b of the KRIT1 complex structure (rmsd of 1.2 Å with 249/249 superimposed Cα atoms using Pymol). For better visibility the Cα trace is depicted as a cartoon; the accurate positions of loop- and helix-Cα atoms are maintained. (Ezrin FERM is shown in green and blue, GDP-Ras in orange and red, and the KRIT1 FERM–Rap1b complex in pale colors). (Inset I) A close-up view of the switch I region supports the importance of ezrin R40 for binding Ras and of Ras D38 for binding ezrin. R40 is located in hydrogen bonding distance of Ras E31 after superposition. D38 occupies a central position in the interface. In addition, Ras D38 of the superimposed GDP-Ras–Raf N71R/A85K complex is shown in orange to illustrate that this residue can adopt a GTP-Ras–like conformation that prevents clashes when binding to Ras-binding domains (11). (Inset II) Different loop conformations of ezrin and KRIT1 near switch region II could contribute to the observed differences in GTPase nucleotide binding. (Inset III) The overall good agreement of the Cα positions of the KRIT1 and ezrin F2 lobe at the distal GTPase-binding site indicates that this region is involved in GTPase binding in ezrin as well. Figures were prepared using Pymol (Schrödinger, LLC).

Table S1. Overview of used SOS, ezrin, Ras, and merlin mutants

<table>
<thead>
<tr>
<th>Construct name</th>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOS WT</td>
<td>Full-length (amino acids 1–1,333) (1)</td>
</tr>
<tr>
<td>SOS M269R</td>
<td>Full-length (amino acids 1–1,333), mutation in DH domain of SOS (2, 3)</td>
</tr>
<tr>
<td>SOS HDPCat</td>
<td>Amino acids 1–1,049, Histone, Db1 (DH), PH, Ras exchange motif (Rem), and Cdc25 domains included (4)</td>
</tr>
<tr>
<td>SOS DHPH</td>
<td>Amino acids 199–546, only DH and PH domain</td>
</tr>
<tr>
<td>SOS PH-</td>
<td>Amino acids 442–566, only PH domain and the PH-Rem linker region</td>
</tr>
<tr>
<td>SOScat</td>
<td>Amino acids 564–1,049, only Rem and Cdc25 domain (5)</td>
</tr>
<tr>
<td>SOScat W729E</td>
<td>Amino acids 564–1,049, only Rem and Cdc25 domain, mutation within allosteric site (6)</td>
</tr>
<tr>
<td>SOS Rem</td>
<td>Amino acids 567–749, only Rem domain</td>
</tr>
<tr>
<td>SOS Cdc25</td>
<td>Amino acids 750–1,050, only Cdc25 domain</td>
</tr>
<tr>
<td>EzrinWT</td>
<td>Full-length (amino acids 1–586)</td>
</tr>
<tr>
<td>EzrinT567D</td>
<td>Full-length (amino acids 1–586), phospho-mimicking mutant</td>
</tr>
<tr>
<td>EzrinR40L/T567D</td>
<td>Full-length (amino acids 1–586), Ras-binding and phospho-mimicking mutant</td>
</tr>
<tr>
<td>RasWT</td>
<td>H-Ras variant 1, for protein purification: amino acids 1–166, for cell-based experiments: amino acids 1–189</td>
</tr>
<tr>
<td>RasY64A</td>
<td>Amino acids 1–166, mutation leads to a specific high-affinity binding to the allosteric but not catalytic site in SOS (7)</td>
</tr>
<tr>
<td>RasD38E/A59G</td>
<td>Amino acids 1–189, “Ras enhancer” (8) (i) has impaired GTP hydrolysis and nucleotide exchange; (ii) specifically interacts with the allosteric site of SOS; and (iii) has disturbed interaction with its Raf1 effector protein</td>
</tr>
<tr>
<td>RasD38A</td>
<td>Switch mutant (9)</td>
</tr>
<tr>
<td>MerlinWT</td>
<td>Amino acids 1–595, isoform 1</td>
</tr>
<tr>
<td>MerlinSS18A</td>
<td>Amino acids 1–595, isoform 1, dephospho-mimicking mutant</td>
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</tbody>
</table>

SOS constructs are always based on the human SOS1 protein sequence. WT, wild-type protein sequence.


Table S2. The biexponential characteristic of SOScat kinetics

<table>
<thead>
<tr>
<th>Kinetics</th>
<th>Amplitude 1 (fast kinetics), %</th>
<th>S.D. amplitude 1, %</th>
<th>Amplitude 2 (slow kinetics), %</th>
<th>S.D. amplitude 2, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOScat</td>
<td>29</td>
<td>6.5</td>
<td>71</td>
<td>6.5</td>
</tr>
<tr>
<td>+GDP-RasY64A (1x)</td>
<td>21</td>
<td>3.6</td>
<td>79</td>
<td>3.6</td>
</tr>
<tr>
<td>+GDP-RasY64A (5x)</td>
<td>29</td>
<td>5.8</td>
<td>71</td>
<td>5.8</td>
</tr>
<tr>
<td>+GTP-RasY64A (1x)</td>
<td>32</td>
<td>3.9</td>
<td>68</td>
<td>3.9</td>
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<tr>
<td>+GTP-RasY64A (5x)</td>
<td>66</td>
<td>8.4</td>
<td>34</td>
<td>8.4</td>
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<tr>
<td>+GTP-RasY64A (10x)</td>
<td>79</td>
<td>6.7</td>
<td>21</td>
<td>6.7</td>
</tr>
<tr>
<td>+GTP-RasY64A (20x)</td>
<td>83</td>
<td>7.8</td>
<td>17</td>
<td>7.8</td>
</tr>
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</table>

The strong stimulator GTP-RasY64A shifted the relative contribution of the two kinetics of SOScat in a concentration-dependent manner. In vitro GEF assays were performed using SOScat (1 μM) and different concentrations (1x = 1 μM, equal to mantGDP-Ras substrate concentration) of the allosteric stimulator RasY64A, loaded with either GDP (weak stimulator) or GTP (strong stimulator). Raw data were biexponentially fitted; only relative amplitudes are shown. Quantitative results represent mean ± SD; n = 3.