One-way membrane trafficking of SOS in receptor-triggered Ras activation

SUPPLEMENTARY NOTE

Supplementary Note 1 - Introductory remarks and condensed summary of main findings

Historically, SOS activation was rationalized in terms of a simple membrane recruitment model based on substrate accessibility (**Fig. 1b**). Grb2 binding to activated receptors recruits SOS-Grb2 complex from the cytosol, thereby positioning SOS in proximity to membrane-anchored Ras and thus promoting nucleotide exchange[1-4]. In favor of this hypothesis, artificial membrane-targeting of SOS by farnesylation was found to trigger the Ras pathway in the absence of stimulatory cues[5]. Grb2 interactions with phospho-tyrosine exhibit relatively fast and reversible kinetics, both *in vitro*[6] and in live cells[7], leading to the assumption that SOS recruitment follows the dynamic equilibrium established by Grb2. Recently, transient second scale interactions of SOS with the plasma membrane has also been reported upon activation of epidermal growth factor receptor in HeLa cells[8]. However, the importance of Grb2-mediated membrane recruitment is challenged by observations that truncated SOS constructs lacking the PR domain still localize to the membrane upon receptor stimulation and are fully signaling competent, or even exhibit increased responsiveness, relative to the full length enzyme[9-14]. Recent work with mouse embryonic stem cells (mESC)[15] demonstrated that, besides Grb2-facilitated membrane recruitment, SOS activity is governed by summation of weak to moderate protein-protein and protein-lipid interactions mediated by the multiple domains of SOS[13,15-17]. These studies imply that the recruitment to membrane integral receptors via Grb2 is an oversimplified model for SOS function.

In this work we quantitatively map out the individual contributions of each domain to the membrane association of SOS through a series of single molecule membrane binding dwell time measurements and bulk kinetic observations using a reconstituted membrane assay system. Observations confirm RasGTP binding by the SOS1 allosteric site as a substantial mode of membrane recruitment, and likely the major way that SOS1 lacking the Grb2 binding domain responds to receptor triggered signals. This enhanced membrane recruitment to RasGTP relative to RasGDP provides an apparent allosteric activation effect in the ensemble, even without actual enhancement of the overall molecular catalytic rate, which has been experimentally demonstrated by single molecule experiments to be independent of nucleotide state[18]. Collectively, Ras- and lipid-binding interactions in the N-terminal domains lead to extremely stable membrane association of SOS1, thus raising the possibility that some additional process may be required to get SOS1 off the membrane in living cells. Following up on these observations in reconstituted systems with cell-based assays, we find the C-terminal Grb2 binding PR domain of SOS1 is required both for achieving timely signal response and timely signal attenuation following receptor stimulation in cells. At early time points following receptor stimulation, the PR domain drives spatial localization of SOS1 to microclusters of activated receptors at the membrane, as would be expected. At later time points, however, the PR domain-containing C terminus exhibits a second functionality and promotes desensitization of SOS1 activity by facilitating its removal from the membrane, ultimately by endocytosis. SOS1 constructs lacking the PR domain become trapped on the membrane surface and lead to sustained ERK activation.

Supplementary Note 2- The catalytic domain of SOS1 in SLB and cellular assays

Single molecule studies of SOS1 activity revealed that allosteric binding of RasGTP to SOS does not increase the average rate of Ras activation per molecule of SOS. Instead, fluctuation dynamics of SOS among several activity states exhibit marked sensitivity to allosteric binding of RasGTP—possibly representing a new form of allosteric regulation[18].

In agreement with the membrane recruitment characteristics of SOS^{Cat} defined by our SLB approaches, we had noted in the past that introduction of SOS^{Cat} into lymphoid cell lines results in spontaneous Ras-ERK signals that induce expression of the activation marker CD69[10,14,19,20]. Since this occurs without the need for receptor stimulation, SOS^{Cat} must be able to somehow spontaneously find its substrate Ras. By contrast, the allosteric pocket mutation, $SOS^{Cat\text{-}W729E}$, which abrogates membrane recruitment in our SLB experiments, did not upregulate CD69 robustly in this cellular assay (**Supplementary Fig. 2a,b**). We also established that SOS^{Cat} spontaneously colocalizes with H-Ras at the plasma membrane of unstimulated fibroblast-like COS1 cells. Significantly, in these experiments a specific variant of H-Ras ($H\text{-}Ras^{A59D\ D38E}$) that only binds to the allosteric site of SOS was employed. Moreover, a SOS mutant impaired in allosteric Ras binding ($SOS^{Cat\text{-}L687E\ R688A}$) did not colocalize with H-Ras at the membrane (**Supplementary Fig. 2c,d** and ref. [21]).

Supplementary Note 3 - SOS^{Cat}-flanking domains have evolved to dampen SOS activity in the basal state but enhance SOS activity upon receptor stimulation

As observed in the comparison of SOS^{Cat} to SOS^{FL} (**Fig. 4**), full-length SOS stands out in two ways; (i) its ability to signal efficiently at physiologically relevant intermediate SOS1 expression levels and (ii) its attenuated signaling at later time points. We uncovered that the Grb2-interacting PR domain plays a critical role in both aspects. First, addition of PR to SOS^{HDPC} (**Supplementary Fig. 5g**) and even to SOS^{Cat} (**Fig. 5f**) enhances the efficiency of SOS signaling to Ras-pERK during the initial phase of BCR stimulation. Note that this is exactly opposite to PR's role in curbing SOS activity in resting cells (**Fig. 5c**). Second, and more surprisingly, inclusion of the PR domain reduces SOS-Ras-pERK signals during the later phase of BCR stimulation (blue bars in **Fig. 5f and Supplementary Fig. 5g**). Thus the PR domain plays an unexpected role in late phase SOS signal attenuation.

In sum, p-FLOW results presented in **Figure 5** combined with single molecule measurements in our SLB assays (**Fig. 2** and **Fig. 3**) indicate that the flanking domains on both sides of SOS^{Cat} have evolved to dampen SOS activity in the basal state but enhance SOS activity upon receptor stimulation. The various positive regulatory events under conditions of receptor stimulation suggest a "point-of-no-return" mechanism for active SOS molecules and we hypothesized that the PR domain is critical for an active form of SOS signal attenuation, which we investigated in **Figure 6** and **Figure 7**.

Supplementary Note 4 - SOS1 functioning in signaling complexes and its relevance to SOS1 internalization

It has previously been shown that grafting a single SH2 domain of Grb2 to SOS^{HDPC} (SOS^{HDPC}-SH2 construct) can rescue certain ES cell fate decisions[15] and ERK activation in T cells[22]. On the other hand, additional reports have shown that multivalent SOS1-Grb2 is necessary for efficient

assembly of LAT signaling clusters[23], and induction of related signaling processes[22,24], downstream of the T cell receptor (TCR)[23 25] while TCR clustering takes place independently of SOS1-Grb2[22]. Reminiscent of this observation, in our B cell system devoid of any endogenous SOS expression, chimeric SOS^{HDPC}-SH2 did not colocalize with sites of BCR microclusters (**Fig. 6b** and **Supplementary Fig. 7b**) further indicating that the PR domain in SOS is critical for colocalization with the BCR and that this function can not be substituted by simply grafting Grb2's SH2 domain onto SOS^{HDPC}.

Our results corroborate the commonly accepted view that the Grb2 interacting PR domain of SOS mediates localization to receptor induced signaling clusters at the plasma membrane. More surprisingly, the same interactions that facilitate this recruitment effect also curb SOS activity in the resting state in unstimulated cells and mediate endocytosis of the activated SOS at later time-points of the signaling process. We propose that these three roles of the PR domain lends switch-like behavior to SOS, functioning as a timer for the activated SOS and thus providing a mechanism by which the cell can regulate the super-processive active membrane-bound state revealed by our single molecule experiments *in vitro*.

Supplementary Note 5 - SOS1 in a signaling complex destined for endocytosis

We observed that only full-length SOS constructs bearing multimeric Grb2-binding capacity get internalized following activation. The monomeric fusion of Grb2 SH2 domain to SOS^{HDPC} enhances SOS activation compared to SOS^{HDPC} in p-FLOW assay. SOS^{HDPC}-SH2 signals comparable to full length SOS at 10 min post-stimulation but supersedes full length at 20 min. Thus the kinetics of SOS signal downregulation are not fully restored by the monomeric SH2 fusion (**Supplementary Fig. 8a,b**). The arising question at this point is how multimeric Grb2 binding provides mechanistic connection to SOS internalization. Insights come from previously suggested scaffold function of SOS enabled by multivalent Grb2 binding[22-24]. Ample studies have established Grb2's role in endocytosis via coupling with Cbl[20,26], RN-tre[27] or Dynamin[28-30]. Especially, Grb2-Dynamin interaction is also conserved and operating in B lymphocytes expressing LAT-related B cell adapter protein LAB (linker of activated B cells)[31]. We postulated that SOS1 is likely to be targeted for the endocytic pathway in a similar manner based on its multivalent association with Grb2. Other known mechanisms terminating SOS1 activation include negative feedback phosphorylation of SOS1 C-terminus, that leads to dissocation of bound Grb2 [32-34]. However, this mode of SOS1 inactivation does not operate in Jurkat T cells[35]. In agreement, the amount of Grb2 associated with immunoprecipitated SOS1 does not decrease even when ERK activity is downregulated in antigen receptor stimulated mouse $CD4^+$ T cells and DT40 B cell line (**Supplementary Fig. 8c,d**). Additionally, a recent study indicates that Ras GTP loading continues in spite of feedback phosphorylation of SOS, thus questioning the importance of feedback phosphorylation for SOS1 inactivation [36].

Supplementary Note 6 - Ras activation at endomembranes

Historically, there has been considerable discussion regarding the possibility of Ras activation at endomembranes[37] although more recent reports conclude that Ras activation is indeed confined to the plasma membrane[38-40]. This view is further corroborated by a newly released study showing that Ras activation downstream of the EGFR is strictly localized to the plasma membrane at physiological Ras expression levels[41]. In this work it was further shown that Ras activation at endocytic vesicles takes place only in an overexpression scenario[41]. We cannot rule out that some minor degree of Ras activation takes place also at endocytic vesicles in our system. Nevertheless,

it should be noted that Ras activation at endomembranes is not per se conflicting with our main conclusion of endocytosis as a fundamental regulatory mechanism in SOS signaling.

Supplementary Note 7 - Data analysis

Quantifying the probability of SOS binding to Ras at the membrane

The mean time to capture a particle with diffusion coefficient D in a one-dimensional box of length a with an absorbing wall at one side is given by ref. [42]:

$$\tau = a^2/3D$$

Considering the same problem for a three-dimensional box with an absorbing wall at one side, the time to capture is the same, due to independence of diffusion in orthogonal directions, and the diffusive flux onto the capturing surface becomes

$$J = \frac{1}{a^2\tau}$$

If now we consider a box with particles at concentration C, each occupying the volume $1/a^3$, the total number of hits experienced by that surface in a time lapse Δt can be written as[43] :

$$N_{attempts} = \int_0^{\Delta t} 3DA_{SLB}C^{4/3}dt$$

Where A_{SLB} represents the area of the absorbing surface, here in the form of a supported lipid bilayer. Armed with this equation we can now obtain the number of binding attempts per field of view on the microscope during a SOS pulse of duration Δt:

$$\begin{aligned} N_{attempts}(\Delta t) &= \int_0^{\Delta t} 3D_3C(t)^{4/3}A_{SLB} * p_{HitRas}dt \\ p_{HitRas} &= \rho_{Ras}\pi R_{Ras}^2 \\ N_{attempts} &= 3D_3A_{SLB}p_{HitRas}C_0^{4/3}\int_o^{\Delta t}\hat{c}(t)^{4/3}dt => \\ N_{attempts} &\approx \frac{3k_bT\rho_{Ras}R_{Ras}^2A_{SLB}C_{SOS}^{4/3}}{6\,\eta R_{SOS}}\sum_{t_i=t_0}^{t_i=\Delta t}\hat{c}(t_i)^{4/3}\delta t \end{aligned}$$

The probability to hit Ras on the surface is approximated simply as the area fraction of the bilayer occupied by Ras. Recording the number of captured SOS as a function of Ras density confirmed a linear dependence as depicted above. The integral over the concentration profile during the flow was obtained simply as a sum over the flow profile recorded using a fluorescein solution. In the experiments here the sum evaluated as 5.6±0.5 s. The capture probability per encounter of Ras and SOS was finally obtained as:

$$p_{capture} = \frac{N_{bound}}{N_{attempts}}$$

Where N_{bound} denotes the number of SOS molecules remaining at the bilayer after the pulse.

Tracking of single molecules in fluorescence micrographs

All image analysis was performed using a software suite developed in Igor Pro ver. 6.22A (Wavemetrics, Oregon, OR). First step in the analysis involved localizing and quantifying fluorescence emission intensity spots in micrographs (from hereon referred to as “particles”). To

facilitate reliable and computationally efficient identification of single particles images were first transformed by smoothing using a 3x3 Gaussian filter, then calculating the gradient of each image and, finally, evaluating the divergence of the gradient vector field. This procedure yielded a new image where the approximate positions of particles could be readily determined by employing a threshold. A benefit of this approach, as opposed to direct intensity thresholding of the raw image, is that variation in optimal threshold among images is minimal and that closely positioned particles with partially overlapping intensity profiles are easily distinguished due to the presence of a saddle point at the inflection point of the two intensity profiles. This procedure yielded an initial list of x and y pixel coordinates for candidate particles, obtained as the centroid of pixel clusters with intensity above the defined threshold. To refine this initial guess, for each x and y position in the list a statistical test was conducted on that region of the original image (employing a 9x9 pixel region of interest) to evaluate the likelihood of the presence of a particle at this location. The statistical test was based on a Bayes' factor comparing the likelihood for the presence of a Gaussian peak against the null-hypothesis, i.e., that the intensity values in this region of the image represents only background with overlaid shot noise (the applied test is described in separate section below). If passing the test, the particle position and integrated intensity was quantified by fitting the intensity profile with a two-dimensional Gaussian function (generally, a cut off of 10^4 in the Bayes' factor was applied, corresponding to high confidence in fluorophore detection). An elliptical Gaussian was used for the fit to allow for slight asymmetry in the intensity profile, e.g., caused by diffusion in experiments with long exposure times. In this way, tables of particle coordinates and integrated emission intensities, calculated as the volume under the fitted Gaussian bell excluding background fluorescence, were produced for each frame in a time series.

For tracking experiments, single particle trajectories were obtained from time-series imaging data by linking identified particles in subsequent frames using a nearest neighbor algorithm. A maximum allowed travelled distance per frame, 15 or 20 pixels depending on the applied imaging conditions, set the bounds for particle connection. A filter was applied to remove immobile particles and unusually dim, or bright, particles. For diffusion analysis, tracks were broken whenever a particle had 2 possible links in the subsequent frame, thus ensuring the highest possible confidence in particle assignment. In residence time analysis, conflicts arising from multiple possible links were solved by calculating a likelihood score, $P_{connect}$, for particle linking based on the previous information on the displacement of each particle and each possible link. Inspired by the method introduced by Serge et al.[44] the score was defined as:

$$P_{connect}=1/[\sqrt{(2\pi)}\langle r_i\rangle]\exp[-r_{test}^2/(2\langle r_i\rangle^2)] ,$$

where $\langle r_i\rangle$ denotes the average of previously observed steps and r_{test} the current step under consideration for a given connection partner. Qualitatively, with the above score a particle moving slowly is considered more likely to take a short step in the next frame than a particle previously observed moving at a higher pace. In cases where less than 5 steps had previously been observed for a particular track (i.e., newly initiated tracks) a default value of $\langle r_i\rangle$=0.5 pixels was applied in the evaluation of the connection score.

The output of the tracking algorithm was visualized by constructing a movie showing identified particles and tracks. All results were inspected to verify proper particle connection. The employed single molecule tracking software was previously tested and benchmarked against fluorescence correlation spectroscopy measurements[45].

Bayes factor for detection of particles in micrographs

In this section, a Bayes' factor for testing for the presence of a particle with a Gaussian intensity profile within a ROI of an image is derived. Our test data are comprised by pixels of varying grey values from a square ROI. We seek a measure of the odds for this ROI containing an intensity hot

spot relative to the odds of the ROI displaying background with overlaid shot noise. We have two models for the sampling distribution of the pixel grey values inside the ROI:

M1 ("null hypothesis"): the data are sampled from a Gaussian with standard deviation σ and mean I_b (for intensity of background). With this hypothesis, the likelihood, $P(I_i)$, for observing pixels with grey values I_i is given by:

$$P_{M1}(I_i) = \prod_i^N (2\pi\sigma)^{-1/2} Exp\left[-\frac{(I_i - I_b)^2}{2\sigma^2}\right]$$

M2: we have a Gaussian intensity peak at position x_p, y_p with amplitude A and width, w, superimposed on a background with shot noise. In this case the likelihood for the data is given by:

$$P_{M2}(I_i) = \prod_i^N (2\pi\sigma)^{-1/2} Exp\left[-\frac{(I_i - I(x_i,y_i))^2}{2\sigma^2}\right] \quad ,$$

$$I(x_i,y_i) = AExp\left[\frac{-(x_i - x_p)^2 - (y_i - x_p)^2}{w^2}\right]$$

The desired Bayes factor for testing the presence of a particle in a ROI is:

$$BF_{Gaussian-peak} = \frac{P(M_2|data)}{P(M_1|data)} = \frac{P(data|M_2)}{P(data|M_1)}$$

The Bayes factor was derived using the likelihood functions above following a strategy equivalent to that taken by Ensign *et al.* [46] in the derivation of a Bayes factor for change point detection. For details of the method, the reader is referred to this work. The result is:

$$\begin{aligned} BF_{Gaussian-peak} &= \frac{1}{N}\sum_i \left[\frac{K^2(x_i,y_i)}{s^2}\right]^{1-\frac{N}{2}} \\ K^2 &= s^2 + g^2 - 2IIG \\ s^2 &= < I_i^2 > - < I_i >^2 \\ g^2 &= < I_G(x_i,y_i)^2 > - < I_G(x_i,y_i) >^2 \\ IIG &= < I_i I_G(x_i,y_i) > - < I_i >< I_G(x_i,y_i) > \\ I_G(x_i,y_i) &= AExp\left[\frac{-(x_i - x_p)^2 - (y_i - y_p)^2}{w^2}\right] \end{aligned}$$

Where the index i runs over the pixels inside the ROI with coordinates, (x_i,y_i). A denotes the amplitude of the Gaussian tested for and w the corresponding width. Instead of considering all possible values of A and w we evaluated $BF_{Gaussian\text{-}peak}$ as an average over a grid of selected values of these parameters. It should be noted that $BF_{Gaussian\text{-}peak}$ in this case should be viewed more as a relative, not an absolute, metric. By empirical calibration, the grid was chosen as w =(1,2,3) and A=(a/4,a/2,3a/4,a) where a denotes the difference between the maximum and minimum pixel grey value in the considered ROI ($a=I_{max}-I_{min}$). With these criteria, $BF_{Gaussian\text{-}peak}$ yielded excellent contrast in deciding between noise and particles. For our data, noise typically produced a $BF_{Gaussian\text{-}peak}$ of less than 1 while particles produced a $BF_{Gaussian\text{-}peak}$ of 100 or more.

Colocalization analysis for live cell experiments

The following algorithm implemented in Igor Pro ver. 6.22A (Wavemetrics, Oregon, OR) was employed to quantify colocalization of BCR clusters and SOS in micrographs. Prior to analysis a number of image transformations were applied to ease cluster identification: *(i)* subtraction of the

lowest pixel intensity in the image from the entire image, *(ii)* normalization of the image to the summed intensity and *(iii)* calculation of the divergence of the gradient of the image. For BCR micrographs, clusters were identified as groups of neighboring pixels in the image resulting from the above operations with an intensity above a user defined threshold. For SOS micrographs, initially, clusters were identified based on a threshold.

We found it necessary to implement a quality control step to effectively reject spurious intensity signals in the SOS channel, which was achieved as follows: *(i)* to gauge the significance of a detected intensity speckle, we first evaluated the ratio of the average pixel intensity at the site of a putative cluster to the average intensity signal of the cell body (excluding the sites of putative clusters identified from the threshold). A putative cluster was then accepted if the intensity ratio evaluated as 1.6 or higher (empirically optimized). If the intensity ratio fell below 1.6, an additional test was performed using the build in ImageSnake function in Igor Pro (command and applied flag settings: "ImageSnake /q /step=1 /alph=0.01 /beta=1 /delt=0.25 /gamm=200 /iter=1000 /eps=0 /sig=3 /sx=BoundX /sy=BoundY /updm=1" where "BoundX" and "BoundY" indicates the boundary pixels for a tested cluster). The ImageSnake function estimates the position of speckle boundaries in an image by iterative minimization of an energy function. The result of this operation is a new estimated cluster boundary. Based on this, we calculated two significance scores: (1) the percentage overlap of the originally estimated cluster boundary and the boundary estimated by the ImageSnake function and (2) the ratio of the pixel area of the cluster as estimated using the ImageSnake operation and the pixel area as determined from the original threshold. When the overlap was 20% or more and the area ratio was 2.4 or less (empirically optimized), a detected cluster was accepted as significant. The rationale behind this approach is that in the event of a significant speckle, the ImageSnake will converge to a boundary similar to that estimated by the simple threshold based approach.

The above operations resulted in two binary images, one indicating the position of BCR clusters and one indicating the position of SOS speckles. Finally, we evaluated the degree of colocalization as a modified Manders' coefficient[47], in our case given by the ratio of the total number of pixels within BCR clusters that overlap with pixels identified in the SOS image and the total number of pixels within all BCR clusters. This value is 100% if all pixels in identified BCR clusters overlap with pixels within identified SOS speckles. Only clusters located within the cell body was included in the colocalization analysis.

Supplementary Note References

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