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2014-08-26

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DRK/DOS/SOS converge with Crk/Mbc/dCed-12 to activate Rac1 during glial engulfment of axonal debris

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Nervous system injury or disease leads to activation of glia, which govern postinjury responses in the nervous system. Axonal injury in *Drosophila* results in transcriptional up-regulation of the glial engulfment receptor Draper; there is extension of glial membranes to the injury site (termed activation), and then axonal debris is internalized and degraded. Loss of the small GTPase Rac1 from glia completely suppresses glial responses to injury, but upstream activators remain poorly defined. Loss of the Rac guanine nucleotide exchange factor (GEF) Crk/myoblast city (Mbc)/dCed-12 has no effect on glial activation, but blocks internalization and degradation of debris. Here we show that the signaling molecules downstream of receptor kinase (DRK) and daughter of sevenless (DOS) (mammalian homologs, Grb2 and Gab2, respectively) and the GEF son of sevenless (SOS) (mammalian homolog, mSOS) are required for efficient activation of glia after axotomy and internalization/degradation of axonal debris. At the earliest steps of glial activation, DRK/DOS/SOS function in a partially redundant manner with Crk/Mbc/dCed-12, with blockade of both complexes strongly suppressing all glial responses, similar to loss of Rac1. This work identifies DRK/DOS/SOS as the upstream Rac GEF complex required for glial responses to axonal injury, and demonstrates a critical requirement for multiple GEFs in efficient glial activation after injury and internalization/degradation of axonal debris.

reactive glia | engulfment signaling | Draper pathway | Wallerian degeneration

Activation of glia is a hallmark of nearly all neurodegenerative diseases and neural injuries. When brain insults have occurred, glia rapidly change their morphology and gene expression profiles to invade the injury site and clear pathogens and/or neuronal debris by phagocytic engulfment (1, 2). Failure to clear debris from the CNS can result in prolonged neuroinflammation and hamper the recovery of the CNS (3, 4). However, the genetic pathways promoting glial activation after neural injuries remain poorly defined.

Genetic studies of Wallerian degeneration in *Drosophila* have provided important insights into glial responses to axotomy (5). Olfactory neuron axotomy results in the degeneration of axons projecting into the antennal lobe of the fly brain, where local glia sense degenerating axons, and initiate a multistep process of reactivity. Reactive glia up-regulate the transcription of the engulfment receptor Draper (*drpr*) and extend membranes to degenerating axons (5, 6). When at the injury site, glia internalize axonal debris and degrade it through the phagolysosomal pathway (7). Finally, glia terminate their responses by withdrawing from the injury site and down-regulating Draper, and finally return to a resting state (6).

Draper is essential for all glial responses to axonal injury. In *drpr*-null mutants, glia fail to respond morphologically to axonal injury, and axonal debris lingers in the brain for weeks after axotomy (5). Downstream of Draper, the small GTPase Rac1 appears to be critical in executing glial activation to axon injury, as loss of Rac1 phenocopies *drpr*-null mutants (7). The only Rac guanine nucleotide exchange factor (GEF) known to be required for glial engulfment of axonal debris is the noncanonical GEF Crk/myoblast city (Mbc)/dCed-12. However, in contrast to loss of

Rac1, animals lacking Crk/Mbc/dCed-12 signaling exhibit relatively normal activation of glia after axotomy, with glia increasing Draper expression and extending membranes to degenerating axons, but glia then fail to internalize and degrade axonal debris (7). These data argue for a specific role for the Crk/Mbc/dCed-12 complex at the internalization/degradation phase of the glial response, and suggest that an additional Rac1 GEF must act earlier during initial activation of glial responses to axonal injury.

In an RNAi-based screen for new engulfment genes, we identified downstream of receptor kinase (*drk*) as a gene required for efficient glial clearance of degenerating axons. *drk* is best known for its role in signaling downstream of the Sevenless (Sev) receptor tyrosine kinase (RTK), where it functions with daughter of sevenless (*dos*) and son of sevenless (*sos*) to activate the small GTPase Ras (8–13). More recent studies have also linked SOS to the activation of Rac1 in the regulation of axon guidance during *Drosophila* embryonic CNS development (14), indicating that DRK/DOS/SOS can act upstream of multiple small GTPases. Here we show that the DRK/DOS/SOS complex plays a critical role in activation of glial responses to injury and internalization of axonal debris. Moreover, we provide genetic evidence that, at the earliest stage of glial activation, DRK/DOS/SOS function redundantly with Crk/Mbc/dCed-12 to promote Rac1 activation and initiate all steps in glial responses to axonal injury.

Results

DRK, DOS, and SOS Are Required for Glial Engulfment of Axonal Debris. To identify new pathways required for glial engulfment of degenerating axons, we performed an RNAi-based screen for genes required in glia for clearing axonal debris after axotomy. We expressed each of ~500 UAS-RNAi constructs from the Vienna *Drosophila* Resource center (15) by using the pan-glial driver *repo-Gal4* (16). For animals that did not survive to adulthood, we

> **Significance**
>
> Neuronal cell death or injury leads to the production of neuronal cell corpses and cellular debris that must be cleared from the nervous system to avoid inflammation or toxicity. Glia are the primary cell type responsible for clearing neuronal debris, but precisely how these cells recognize, phagocytose, and destroy this material remains poorly defined. Here we use a simple nerve injury assay to identify genes downstream of the engulfment receptor Draper that are required for efficient glial engulfment of degenerating axons. Based on the molecular and functional conservation of this pathway in mammals, this study sheds new light on pathways potentially used by mammalian glia to react to brain injury or neurodegeneration.

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further incorporated a temperature-sensitive version of Gal80 (*Gal80^ts*) (17) in the background to temporally control the induction of RNAi exclusively in the adult glia (*SI Materials and Methods*). We ablated maxillary palps (mps) in which a subset of olfactory receptor neurons (ORNs) were labeled with membrane-tethered GFP (*OR85e-mCD8::GFP*) (18) and scored axonal debris clearance 5 d after axotomy by quantifying GFP immunoreactivity of OR85e$^+$ glomerulus in the antennal lobe, as previously reported (5).

In our primary, screen we found that an RNAi construct (*drk^RNAi#105498*) targeting DRK suppressed glial clearance of axonal debris. In control animals, the vast majority of axonal debris was cleared 5 d after axotomy (Fig. 1*A*). However, a significant amount of the axonal debris still lingered in the brain of *drk^RNAi* animals at day 5, and ultimately cleared in 20 d after axotomy (Fig. S1*A*; quantified in Fig. S1*B*), which argues for a glial role for DRK in engulfment of axonal debris. DRK is known to physically interact with the adaptor protein DOS (19) and the GEF SOS (20), which can activate downstream small GTPase such as Ras and Rac1 (8, 9, 14, 21). We therefore designed a UAS-RNAi construct (*dos^RNAi#3*) to knock down DOS in glia and assayed for engulfment defect. Adult glia expressing *dos^RNAi#3* also exhibited a significant delay in engulfment of axonal debris (Fig. 1*A*), arguing that DOS also plays an important role in glial engulfment of degenerating axons. To further validate this result, we repeated this experiment with an additional RNAi line (*dos^1044R-3*), which targets different region of *dos* mRNA from *dos^RNAi#3*, and found similar engulfment defects 5 d after injury (Fig. S2), arguing that the phenotype was not caused by the off-target effects of RNAi. We next knocked down glial SOS by using RNAi (*sos^RNAi#42849*) and found that axonal debris remained uncleared in the CNS for as long as 20 d (Fig. 1*A* and Fig. S1*A*). We did not observe significant change in the expression of Draper when DRK, DOS, and SOS were knocked down respectively (Fig. S1 *E* and *F*), implying that the delayed clearance is not caused by a lower level of Draper expression. Together, these data suggest that, during glial engulfment, similar to Sev RTK signaling, DRK, DOS, and SOS interact with each other to regulate downstream small GTPase activity.

During Sev signaling, DRK and DOS couple RTK activation to stimulation of downstream small GTPase activity through SOS. We therefore speculated that increasing SOS activity could potentially compensate for the depletion of DRK and DOS. To test this hypothesis, we explored the effect of a gain-of-function (GOF) SOS allele (*Sos^JC2*) (8, 22) on the ability of glia to clear axonal debris when DRK or DOS was knocked down. *Sos^JC2*/+ animals did not exhibit any discernible clearance defect (Fig. 1*A*), nor did they clear debris faster than controls (Fig. S3*A*; quantified in Fig. S3*B*). However, we found the delay in clearance of axonal debris caused by *drk^RNAi* and *dos^RNAi* was completely rescued by *Sos^JC2*/+ (Fig. 1*A*; quantified in Fig. 1*B*). These data indicates that the DRK/DOS/SOS complex is required for efficient engulfment of axonal debris by glial cells, and that activation of SOS is sufficient to drive glia to engulf axonal debris when DRK or DOS are depleted, consistent with the notion that SOS acts downstream of DRK and DOS.

Glial DRK Is Recruited to Degenerating Axons After Injury. After axon injury, Draper is up-regulated in glial cell and recruited to sites where glia actively engulf axonal debris (5, 6). We sought to determine whether DRK was also expressed in glia, as our RNAi data would suggest, and whether it was recruited to injury sites during glial engulfment. We used α-DRK polyclonal antibodies (11) to detect DRK expression in the adult brain. We first examined DRK localization along the maxillary nerve in the subesophageal ganglion (SOG), through which GFP-labeled mp ORN axons are projected to the antennal lobe. In control brains, we observed widespread DRK expression and, interestingly, 1 d after mp ablation, DRK was enriched along the maxillary nerve,
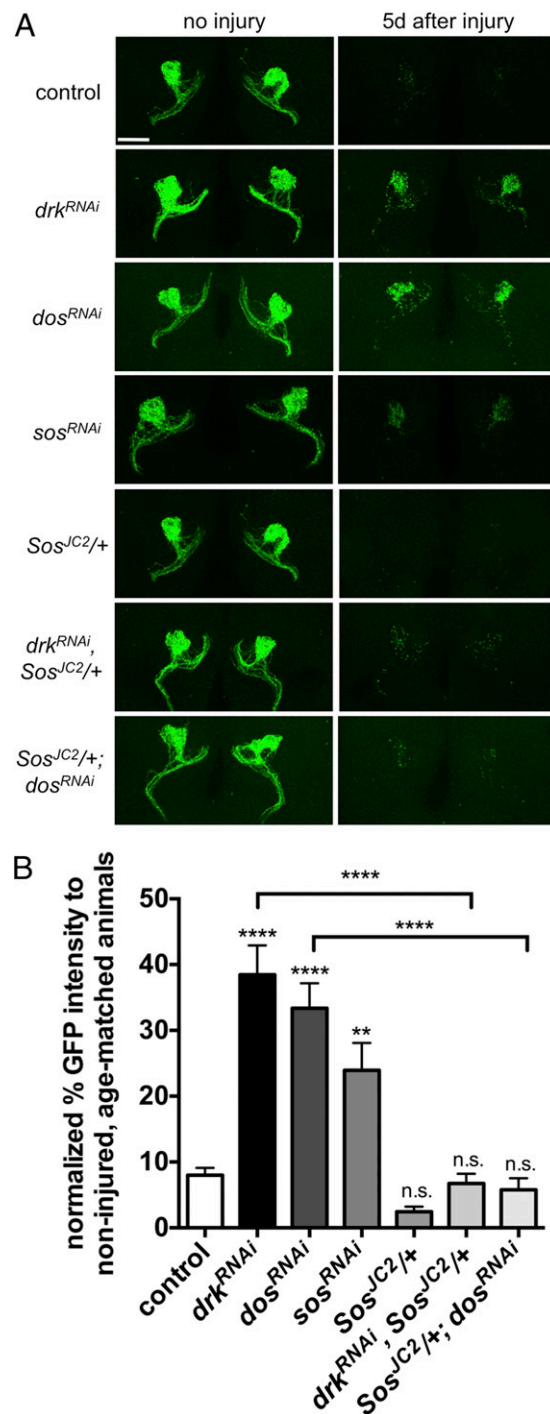

Fig. 1. Glial clearance of axonal debris requires DRK, DOS, and SOS. (*A*) Glia failed to clear OR85e$^+$ axonal debris in 5 d after axotomy in *drk* RNAi (*drk^RNAi*), *dos* RNAi (*dos^RNAi*), and *sos* RNAi (*sos^RNAi*) animals and the clearance defects in *drk^RNAi* and *dos^RNAi* animals were completely rescued by a GOF allele of *sos, Sos^JC2*. Axons from a subset of mp ORNs were labeled by *OR85e-mCD8::GFP* and the integrity of axons 5 d after mp ablation (5 d after injury) was examined under confocal microscope. Representative images (*z*-stack) are shown. (Scale bar: 30 μm.) *SI Materials and Methods* provides all genotypes throughout the figures. (*B*) Quantification of OR85e$^+$ axonal debris remaining in the brain 5 d after injury. GFP immunoreactivity of each OR85e$^+$ glomerulus was measured and normalized to uninjured, age-matched cohorts (as 100%). Error bars represent SEM throughout. n.s., not significant. (*P < 0.05, **P < 0.005, and ***P < 0.0001; one-way ANOVA and Bonferroni post hoc throughout unless otherwise mentioned.)

which was absent when we drove drk^{RNAi} in glia (Fig. 2A), indicating that glial DRK is recruited to severed axons. Moreover, we found that Draper is required for the recruitment of DRK after injury because we could not detect up-regulation of DRK 1 d after axotomy in $drpr$-null ($drpr^{\Delta5}$) animals (Fig. S4), suggesting that the activation of DRK/DOS/SOS requires Draper.

We next sought to determine if DRK expression was increased after antennal ablation, which leads to a dramatic increase in

Fig. 2. DRK is recruited to glial membranes surrounding the injured axons. (A) Endogenous DRK (red, α-DRK) in glia was recruited to the severed mp nerves labeled with *OR85e-mCD8::GFP* (green, α-GFP) 1 d after axotomy. Before injury, DRK expression was evenly distributed in the SOG but not colocalized with the OR85e+ maxillary nerve. One day after mp injury, DRK expression was increased around the degenerating maxillary nerves (arrowheads), which was not seen in drk^{RNAi} animals. Representative images (single slice) are shown. (Scale bar: 10 μm.) (B) Endogenous DRK expression in glia was increased 1 d after axotomy. Glial membranes were labeled with mCD8::GFP by glia-specific *repo-Gal4* driver. Antennal ablation (removal of the third segment of antennae) was performed to induce a greater extent of axon degeneration in the antennal lobe (AL). Normally, thin glial membranes ensheath (arrows) the antennal lobe and each glomerulus (no injury, control). However, 1 d after antennal ablation, ensheathing glia became hypertrophy (1 d after injury, dashed lines), and strong DRK immunoreactivity (red) was found in hypertrophic region of ensheathing glia (yellow), but not when drk^{RNAi} was expressed by *repo-Gal4*, indicating that the increase of DRK is glia-specific. Representative images (single slice) are shown. C, cortex. (Scale bar: 10 μm.)

Draper expression and hypertrophy of glial membranes (5). We labeled glial membranes with mCD8::GFP by using the *repo-Gal4* driver, and assayed glial morphology and DRK expression in control animals and animals where the third antennal segments had been ablated 1 d earlier. Consistent with our findings in the SOG, DRK immunoreactivity was dramatically increased around the antennal lobe 1 d after antennal ablation in control flies (Fig. 2B). The increase in DRK is likely a result of recruitment of DRK to glial membranes at sites of axon injury rather than up-regulation of *drk* gene transcription and/or translation, as DRK protein levels did not increase significantly after ablation of olfactory organs (Fig. S5). When we drove drk^{RNAi} in glia, there was a significant decrease of DRK immunoreactivity in the hypertrophic ensheathing glia (Fig. 2B, arrows) but not in neurons, confirming that the increase of DRK immunoreactivity comes from glia. Together, these results are consistent with the model that DRK acts downstream of Draper to promote engulfment of axonal debris.

DRK-SOS Activation Helps Glia to Internalize Axonal Debris and Activate the Phagolysosomal Program. After axotomy, glial membranes are recruited to severed axons, where they internalize and degrade axonal debris (7). As the first step to determine where DRK impinged on glial engulfment of axonal debris, we examined glial membrane dynamics after mp injury. In healthy, uninjured animals, glia usually wrap each glomerulus with their fibrous membranes. After axon injury, glia form large numbers of membranous vesicles throughout the field of degenerating axons (Fig. 3A); these vesicles often contain internalized axonal debris, presumably to become acidic thereafter for degradation (7). However, we found that, unlike in control animals, knocking down DRK in glia by using *repo-GAL4* resulted in a failure of glia to efficiently form enclosed vesicles 1 d after injury, with total vesicle number in drk^{RNAi} animals being reduced to ~30% of that found in the controls (Fig. 3B). DRK activity in glial membrane vesicle formation appears to act through SOS, as the $Sos^{JC2}/+$ was sufficient to suppress the loss of glial membrane vesicles in the drk^{RNAi} background.

We next sought to determine whether reduced DRK function affected the ability of glia to internalize axonal debris. We labeled ensheathing glia with *UAS-mCD4::tdTomato* by using the *TIFR-Gal4* driver (7) and compared internalization of *OR85e-mCD8::GFP*-labeled ORN axons in control and drk^{RNAi} animals. Internalized axonal debris was scored when GFP+ axonal debris was found within tdTomato+ membrane-enclosed vesicles. Before injury, tdTomato-labeled glial membrane vesicles were not detectable in the OR85e+ glomerulus. However, in controls, 1 d after mp ablation, glial membranes invaded the glomerulus and tdTomato-labeled vesicles containing mCD8::GFP+ axonal materials were found throughout this glomerulus (Fig. 3C, arrows). Expression of drk^{RNAi} in TIFR+ glia significantly reduced the number of glial vesicles containing axonal debris compared with the control (Fig. 3D). Activating SOS signaling in the drk^{RNAi} background through GOF Sos^{JC2} resulted in glial internalization of axonal debris at WT levels. Sos^{JC2} itself did not increase the total number of glial vesicles, nor did it alter the number of vesicles internalizing degenerating axons, arguing that the rescuing effect did not result from a Sos^{JC2}-dependent general increase in vesicle number. We therefore concluded that DRK-SOS signaling plays an important role in forming phagocytic vesicles during glial engulfment of axonal debris.

After internalization of axonal debris, glia activate the phagolysosomal pathway to degrade engulfed axonal materials (23). To explore if activation of the phagolysosomal pathway was also affected by altered DRK-SOS signaling, we examined the formation of acidified phagolysosomes surrounding severed axons by using LysoTracker red DND-99. In control uninjured brains, the area surrounding OR85e+ axons did not exhibit detectable
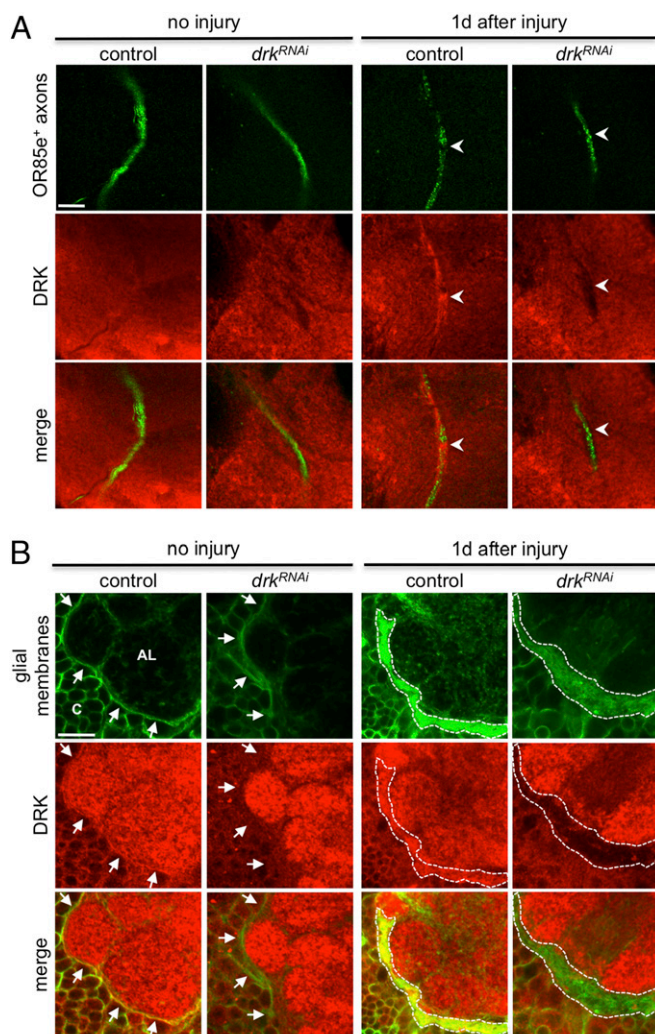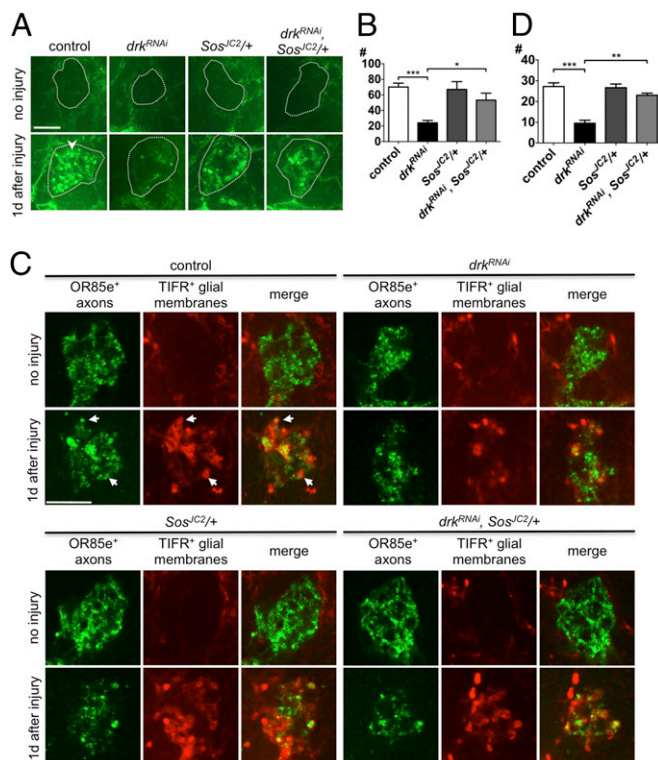
Fig. 3. DRK-SOS activation is required for glia to internalize axonal debris. (*A*) Glia failed to form membrane vesicles 1 d after axotomy in *drk* RNAi animals, which was recued by *Sos^JC2^*. Glial membranes were labeled by *repo-Gal4* driving mCD8::GFP. Without injury, glial membranes normally loosely wrap each glomerulus (delineated by dotted lines) in the antennal lobe. However, 1 d after axon injury, glial membranes invaded the field of injured glomerulus and formed enclosed membrane vesicles (arrowhead). These membrane vesicles were largely absent in animals in which DRK expression was knocked down by *repo-Gal4* (*drk^RNAi^*). *Sos^JC2^* alone did not increase the number of vesicles formed 1 d after injury, but rescued the defect of glial membrane vesicle formation in *drk^RNAi^*. Representative images (single-slice) are shown. (Scale bar: 10 μm.) (*B*) Quantification of the number of glial membrane vesicles in *A*. Because there were hardly any detectable glial membrane vesicles without axon injury, we quantified only the number of vesicles in animals at day 1 after axon injury (*n* ≥ 3 for all). (*C*) Internalization of OR85e-mCD8::GFP-labeled axonal debris was reduced when *drk* was knocked down in ensheathing glia but restored by *Sos^JC2^*. Ensheathing glial membranes were labeled with mCD4::tdTomato (red) driven by *TIFR-Gal4*. OR85e^+^ axons were labeled with mCD8::GFP (green). Degenerating OR85e^+^ axons were often found inside glial membrane vesicles (arrows). Representative images (single-slice) are shown. (Scale bar: 10 μm.) (*D*) Quantification of the number of glial vesicles containing GFP-labeled axonal debris 1 d after axotomy in *C* (*n* ≥ 3 for all).

LysoTracker^+^ puncta (Fig. S6*A*). One day after injury, Lyso-Tracker^+^ puncta appeared robustly along the degenerating OR85e^+^ axons. Knocking down DRK in ensheathing glia resulted in ∼75% of reduction in the number of LysoTracker^+^ puncta (Fig. S6*B*), indicating that the loss of DRK function severely impedes glial activation of phagolysosomal pathway. We detected a slight increase in the efficiency of phagolysosome maturation with *Sos^JC2^*, as revealed by a ∼1.5-fold increase in the number of LysoTracker^+^ puncta compared with the controls, suggesting that activation of SOS promotes phagolysosome formation. However, we found that *Sos^JC2^* was not sufficient to rescue the phagolysosome maturation defect caused by *drk^RNAi^*. This lack of rescue by *Sos^JC2^* could indicate that the *Sos^JC2^* allele is not sufficiently strong to overcome the absence of DRK activity during phagolysosome formation, or that another molecule

might act in a redundant fashion with DRK during this specific signaling step.

In summary, these data argue that DRK-SOS signaling plays a critical role during glial internalization of axonal debris and activation of the phagolysosomal program for degradation of axonal materials.

Rac1, but Not Ras, Is the Main Small GTPase Effector Downstream of SOS in Activating the Engulfing Glia. SOS is an evolutionarily conserved GEF for the small GTPase Ras, and DRK/DOS/SOS were initially identified as key molecules coupling the Sev RTK to Ras activation (8–11, 13, 24). We therefore explored the possibility that Ras might act as the downstream effector after SOS activation to promote glial clearance of axonal debris. We expressed a dominant-negative (DN) form of Ras (*Ras85D^N17^*) (25) specifically in adult glia and assayed glial engulfment after axotomy. In these animals, 5 d after mp ablation, we found that the vast majority of axonal debris was cleared (Fig. S7*A*), as <10% of axonal debris was left 5 d after injury (Fig. S7*B*). This phenotype is extremely mild compared with what we observed with *dos*, *drk*, and *sos* RNAi. Although we could not exclude the possibility that Ras is activated by SOS after injury and plays a minor role in clearance of axonal debris, we suspected additional GTPases might be the key downstream effectors of SOS during glial engulfment.

The small GTPase Rac1 is a potent regulator of membrane ruffling and lamellipodia formation, and is required for glial phagocytosis of degenerating axons (7). Interestingly, SOS possesses Rac1-specific GEF activity in mammals and *Drosophila* through its conserved N-terminal Dbl homology domain (14, 21). We therefore assayed for genetic interactions between Rac1 and SOS. In *Drosophila* eye development, overexpression of a DN Rac1 (*Rac1^N17^*) results in a rough eye phenotype that can be suppressed by supplying excess upstream GEF complex proteins, Mbc and dCed-12 (26). We therefore reasoned that overactivation of SOS by use of *Sos^JC2^* might suppress *Rac1^N17^* phenotypes during glial engulfment of axonal debris. Adult-specific overexpression of *Rac1^N17^* in glia resulted in a potent suppression of axonal debris clearance as previously described (7) (Fig. 4*A*). However, when we overexpressed *Rac1^N17^* in *Sos^JC2^*/+ background, we observed a ∼50% suppression of clearance defect compared with animals expressing *Rac1^N17^* alone (Fig. 4*B*). As *Rac1^N17^* also blocks glial membrane extension to the site of injury (7), we also examined whether *Sos^JC2^* could rescue this defect by scoring recruitment of Draper to severed axons 1 d after axotomy (Fig. 4 *C* and *D*). We found that, consistent with our previous study, glial expression of *Rac1^N17^* completely suppressed the recruitment of Draper to severed axons, but overactivation of SOS using *Sos^JC2^* partially restored (to ∼30% control levels) glial recruitment of Draper to axonal debris. The simplest interpretation of these data, based on interactions between SOS and RAC1 in other signaling contexts such as the Sevenless pathway, is that the GEF component SOS acts upstream of the small GTPase RAC1. However, we cannot exclude the formal possibility that SOS acts downstream of RAC1 to activate another small GTPase during glial engulfment of axonal debris.

Both DRK/DOS/SOS and Crk/Mbc/dCed-12 Activation Contribute to Rac1 Activity in Glial Response to Axon Injury. Our previous finding argued that the GEF complex Crk/Mbc/dCed-12 acts upstream of Rac1 in glial clearance of axonal debris (7), but there were significant differences in the requirements for Rac1 vs. Crk/Mbc/dCed-12. Specifically, depletion of Rac1 activity by RNAi or use of DN constructs completely blocked glial activation: there was no recruitment of glial membranes or Draper to sites of axon injury, along with severe defects in axonal debris clearance. In contrast, depletion of Crk, Mbc, or dCed-12 resulted in a slight delay in glial recruitment to severed axons, suggesting
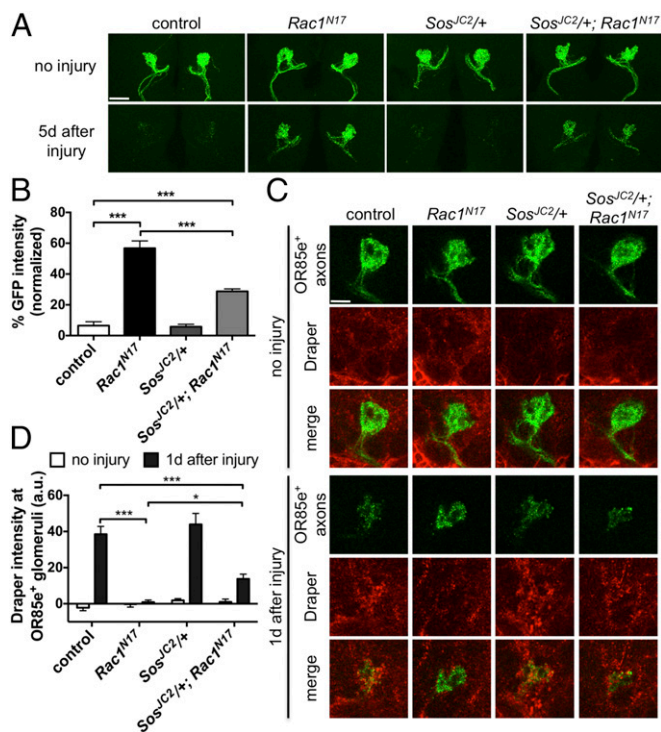
Fig. 4. SOS modulates Rac1 activity in glial response to axonal injury. (A) Sos^{JC2} suppressed the inhibitory effects of DN Rac1 ($Rac1^{N17}$) on glial clearance of axonal debris. The clearance assay was performed as described in Fig. 1. (Scale bar: 30 μm.) (B) Quantification of data in A (n = 10 for all). (C) Sos^{JC2} partially rescued $Rac1^{N17}$ and increase glial membrane (red, α-Draper) recruitment to severed OR85e$^+$ glomeruli (green, α-GFP) 1 d after axotomy. (Scale bar: 10 μm.) (D) Quantification of Draper immunoreactivity at OR85e$^+$ glomerulus in C. a.u., arbitrary unit. n = 10 for all.

a complementary GEF acting upstream of Rac1 might also play a role in glial response to axon injury. Based on the similarity of the phenotypes associated with inhibition of Crk/Mbc/dCed-12 and DRK/DOS/SOS (i.e., reduced vesicle formation, failure to activate the phagolysosomal pathway, and an axonal debris clearance defect), we speculated that Crk/Mbc/dCed-12 and DRK/DOS/SOS activation might act redundantly downstream of Draper to activate Rac1 not only at the early phases of the glial response (when glial membranes are recruited to severed axons), but also at later phases (during internalization and degradation of axonal debris). This predicts that simultaneous depletion of Crk/Mbc/dCed-12 and DRK/DOS/SOS should result in a complete suppression of glial activation. To test this model, we simultaneously knocked down SOS and MBC, both of which possess the enzymatic GEF activity for Rac1, specifically in adult glia, and examined the recruitment of glial membranes to the severed OR85e$^+$ axons 1 d after injury (Fig. 5A). Interestingly, we found that sos^{RNAi} or mbc^{RNAi} resulted in a slightly reduced recruitment of Draper-decorated glial membranes to severed axons. However, when sos^{RNAi} and mbc^{RNAi} were simultaneously expressed, the recruitment of glial membrane to injured OR85e$^+$ glomerulus was completely blocked 1 d after injury. The additive nature of the sos^{RNAi} and mbc^{RNAi} phenotypes also extended to clearance of axonal debris as well as glial hypertrophy response (Fig. S8). Thus, simultaneous blockade of DRK/DOS/SOS and Crk/Mbc/dCed-12 signaling phenocopied *drpr*-null mutants (5), and inhibition of Rac1 signaling (7).

We further explored if SOS activation could partially substitute for Crk/Mbc/dCed-12 during glial clearance of axonal debris by crossing Sos^{JC2} into $dCed-12^{RNAi}$ background. As

previously shown (7), glial knockdown of dCed-12 function strongly suppressed glial clearance of axonal debris 5 d after injury (Fig. 5C). However, in a Sos^{JC2}/+ background, the effect of $dCed-12^{RNAi}$ was attenuated (Fig. 5D), indicating that activation of SOS can partially compensate for the reduced activity from Crk/Mbc/dCed-12 to promote glial clearance of axonal debris. We conclude that Crk/Mbc/dCed-12 and DRK/DOS/SOS act in a partially redundant fashion downstream of Draper to activate Rac1 and thereby glia, and promote glial clearance of axonal debris.

Discussion

In this work, we identify *Drosophila* DRK, DOS, and SOS as new molecules required for glial responses to axonal injury. We show that glial depletion of DRK, DOS, or SOS results in a delay in glial responses to ORN axotomy and reduced efficiency of glial internalization and digestion of axonal debris. We observe no obvious alterations in glial morphology or expression of engulfment machinery (e.g., Draper), and demonstrate that adult-specific knockdown of DRK/DOS/SOS leads to defects in glial clearance of degenerating axons. These data indicate that DRK/DOS/SOS promote engulfment signaling in mature glia, and argues against a developmental defect causing the phenotypes we observe. Based on our observation that a dominant GOF allele of SOS (Sos^{JC2}) can partially suppress depletion of DRK and DOS, we propose that SOS acts genetically downstream of DRK and DOS.

Our previous work demonstrated a key role for the *Drosophila* GEF Crk/Mbc/dCed-12 in glial engulfment activity, with elimination of this signaling complex from glia resulting in normal
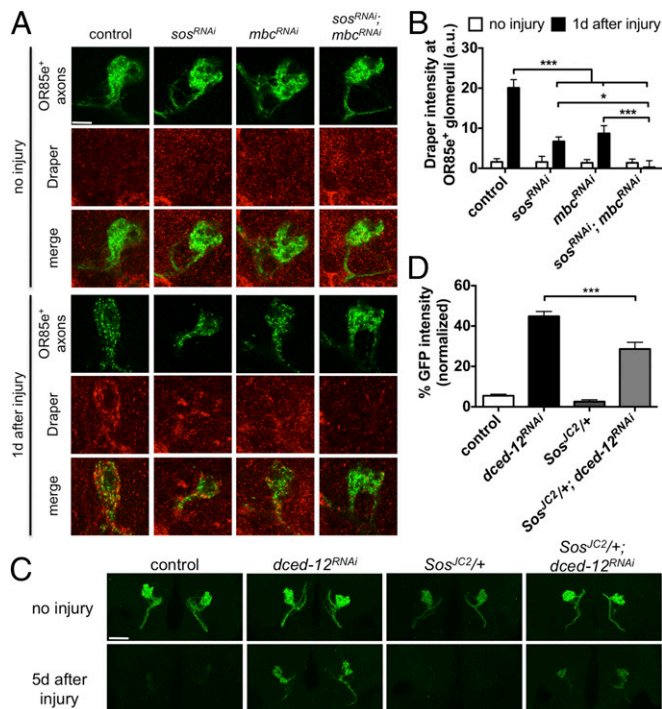

Fig. 5. DRK/DOS/SOS and Crk/Mbc/dCed-12 are redundant pathways in activating glial clearance of axonal debris. (A) Simultaneous knockdown of SOS and Mbc completely blocked glial membrane (red, α-Draper) recruitment to injured OR85e$^+$ glomerulus (green, α-GFP) 1 d after injury, whereas individual RNAi expression showed mild inhibitory effects. (Scale bar: 30 μm.) (B) Quantification of data in A (n = 10 for all). (C) Sos^{JC2} partially rescued the clearance defect cause by $dCed-12^{RNAi}$. The clearance assay was performed as described in the other figures, and the results were quantified in D (n = 10 for all).

glial activation (e.g., recruitment of glial membranes to axonal debris), but a failure to engulf axonal debris (7). Here we provide strong evidence that DRK/DOS/SOS and Crk/Mbc/dCed-12 act redundantly downstream of Draper at two key steps in the engulfment process (Fig. S9). First, based on the fact that simultaneous depletion of both signaling complexes phenocopies Rac1 loss of function, we propose that these complexes act redundantly to activate Rac1 and glial responses, including Draper up-regulation and extension of glial membranes to degenerating axons. Second, after glia have arrived at axonal debris, both complexes are required for the elimination of axonal debris. At this step, DRK/DOS/SOS and Crk/Mbc/dCed-12 appear to act in a nonredundant fashion to promote glial internalization of axonal debris and activation of the phagolysosomal program for degradation of internalized axonal material.

DRK/DOS/SOS signaling has been studied most intensively for its role downstream of the RTK Sev, where it acts to activate small GTPase Ras (8–13). However, consistent with our findings, in vitro and in vivo studies have also demonstrated a role for SOS in activating Rac1. In cell culture studies, SOS stimulates guanine nucleotide dissociation from Rac1 but not Cdc42 (21). In some cases, such as axon guidance in the *Drosophila* embryo, SOS action as a Rac1 GEF is independent of Ras activation (14), but, in other situations, SOS activation of Rac1 is coupled to stimulation of Ras (21). Based on our observation that glial expression of a DN Ras only very weakly suppresses clearance of degenerating axons, SOS activation of Rac1 during glial responses to axonal injury is likely largely independent of Ras activation.

The increase of DRK localization to glial membrane processes engulfing axonal debris, and the consequences of DRK depletion by RNAi all argue for an early role for DRK (and likely DOS and SOS) in engulfment events. Although DRK is expressed in neurons and glia (see Fig. 2), our glial-specific RNAi clearly demonstrates that DRK function is required in glia during engulfment. Western blot analysis of the brain lysates before and after axotomy suggests the increase of DRK in the glial membrane processes is likely a result of recruitment of DRK other than up-regulation of gene transcription/translation (Fig. S5). We also found that Draper is responsible for DRK localization to the site of injury, as DRK does not localize to severed nerves in *drpr*-null animals (Fig. S4), suggesting DRK/DOS/SOS activation requires Draper. It seems Draper activation or Draper-mediated recruitment of DRK is necessary for DRK/DOS/SOS to execute their functions, as we found Sos^{JC2} failed to rescue the debris clearance defect in *drpr*-null animals (Fig. S10). Does DRK interact directly with Draper? Despite considerable efforts, we were unable to detect physical interactions between DRK and Draper. DRK may therefore interact very transiently or indirectly with Draper or DRK might associate with another yet unknown glial receptor that localizes to degenerating axonal materials.

The requirements we describe for DRK/DOS/SOS appear to be conserved in mammals in the context of phagocytic activity. Mammalian DRK (growth factor receptor-bound protein 2) is accumulated at the phagocytic cup during leukocyte phagocytosis (27). Mammalian DOS (Grb2-associated binding protein 2) also plays a critical role during Fcγ receptor-mediated phagocytosis (28). Recently, MEGF10 (mouse Draper) is expressed in mammalian astrocytes and essential for synaptic pruning during refinement of dorsal lateral geniculate nucleus connectivity (29). The work we present here also argues that DRK/DOS/SOS and Crk/Mbc/dCed-12 are exciting candidates downstream of MEGF10 to promote synaptic pruning.

Materials and Methods

Olfactory axon injury, adult brain dissection, sample preparation, and image analysis were previously described in ref. 5. Lysotracker staining in *Drosophila* adult brain was previously described in ref. 7. Fly strains and antibodies used in this study are provided in *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Fly Strains and Antibodies. The following *Drosophila* strains were used: *OR85e-mCD8::GFP/CyO* (1) (gift from B. Dickson, Research Institute of Molecular Biology, Vienna, Austria), *UAS-mCD8::GFP* (2), *UAS-mCD4::tdTomato* (3), *repo-Gal4/TM3* (4), *tub-Gal80^{ts}* (5) (gift from S. Waddell, University of Oxford, Oxford, United Kingdom), *TIFR-Gal4/TM3* (gift from H. Hing, University of Illinois, Urbana, IL), *Sos^{JC2}/CyO* (gift from G. M. Rubin, Janelia Farm, Ashburn, VA), *UAS-Rac1^{N17}* (gift from L. Luo, Stanford University, Stanford, CA), and *UAS-Ras85D^{N17}* (purchased from Bloomington *Drosophila* Stock Center, Bloomington, IN). The following UAS-RNAi lines were from Vienna *Drosophila* Resource Center (Vienna, Austria): *UAS-drk$^{RNAi#105498}$*, *UAS-sos$^{RNAi#42849i}$*, *UAS-mbc$^{RNAi#16044}$*, and *UAS-dCed-12$^{RNAi#10455}$*. *UAS-dos$^{RNAi#3}$* was generated by cloning daughter of sevenless (dos) cDNA fragment (nucleotide 1,629–2,112; SD02517; Drosophila Genomics Resource Center cDNA Stock Center) into pWIZ vector. Injection of pWIZ-dos^{RNAi} into fly embryos was performed by BestGene (Chino Hills, CA) to make transgenic flies. The following fly stocks were obtained from NIG-FLY Stock Center: *UAS-drk$^{6033R-2}$* and *UAS-dos$^{1044R-3}$*. To study genetic interactions, the following strains were generated following standard procedure: *tub-Gal80^{ts}, OR85e-mCD8::GFP/CyO*; *repo-Gal4/TM3, tub-Gal80^{ts}*; *repo-Gal4, UAS-mCD8::GFP/TM3*, *OR85e-mCD8::GFP, UAS-mCD4::tdTomato/CyO*, and *UAS-drk$^{RNAi#105498}$, Sos^{JC2}/CyO*.

Rabbit anti-downstream of receptor kinase (DRK) polyclonal antibodies (1:500) was a gift from M. A. Simon (Stanford University, Stanford, CA). Rabbit anti-Draper antisera (1:500) was raised as previously described (6). Mouse anti-GFP monoclonal antibody (1:200) was purchased from Molecular Probes (A11120). Cy3 anti-rabbit IgG and FITC anti-mouse IgG were purchased from Jackson ImmunoResearch and used at 1:100.

Injury Protocols and Adult Fly Brain Dissection. Standard maxillary palp (mp) and antennal ablations were performed as previously described (7). For experiments that required *tub-Gal80^{ts}*, flies were raised at 18 °C before eclosion and then transferred to 29 °C at least 5 d before injury. After injury, flies were grown at 29 °C until the day of dissection. Standard methods were used for dissection, fixation, and antibody staining of *Drosophila* adult brain (7). Fly brains were eventually mounted in Vectashield Mounting Medium (H-1000; Vector Labs) and stored at 4 °C in the dark before confocal microscopy analysis within 2 wk.

Lysotracker Staining in *Drosophila* Adult Brains. Flies were aged and injured as described in *Injury Protocols and Adult Fly Brain Dissection*. Heads, after having been removed from the bodies, were immediately immersed and dissected in chilled PBS solution. Dissected brains were stained by LysoTracker Red DND-99 (L-7528; Molecular Probes)/PBS solution at a dilution of 1:500 at room temperature for 15 min with constant rocking, followed by five quick washes in PBS solution within 15 min, and then fixed for another 30 min at room temperature with 4% formaldehyde/PBS solution/0.1% Triton X-100. To visualize OR85e$^+$ axons, fixed brains were further stained with mouse anti-GFP antibody at 1:200. Mounted brains were kept in the dark for 1 h before confocal analysis and imaged on the same day to minimize the decay of LysoTracker signals.

Confocal Microscopy and Image Analysis. Confocal microscopy settings were always kept constant throughout the same set of experiments. For axon debris clearance, brains were imaged in 0.85-μm steps with a Zeiss LSM5 Pascal confocal microscope under 63× oil objective lens. Pixel intensity of GFP or Draper immunoreactivity at each OR85e$^+$ glomerulus was measured by using ImageJ (National Institutes of Health) as previously described (7). Glial membrane vesicles and LysoTracker$^+$ puncta were detected by spinning-disk confocal microscope (Carl Zeiss) under 63× oil objective lens and analyzed in Volocity (PerkinElmer). Statistics were all carried out in GraphPad Prism 6 (GraphPad Software).

Detailed Listing of Animal Genotypes. *Figure 1.* Control: *OR85e-mCD8::GFP, tub-Gal80^{ts}/+*; *repo-Gal4/+*. *drk^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/UAS-drk$^{RNAi#105498}$*; *repo-Gal4/+*. *dos^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/+*; *repo-Gal4/UAS-dos$^{RNAi#3}$*. *sos^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/UAS-sos$^{RNAi#42849}$*; *repo-Gal4/+*. *Sos^{JC2}/+*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/Sos^{JC2}*; *repo-Gal4/+*. *Sos^{JC2}/+; dos^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/Sos^{JC2}*; *repo-Gal4/ UAS-dos$^{RNAi#3}$*. *drk^{RNAi}, Sos^{JC2}/+*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/Sos^{JC2}, UAS-drk$^{RNAi#105498}$*; *repo-Gal4/+*.
Figure 2. (A) Control: *OR85e-mCD8::GFP, tub-Gal80^{ts}/+*; *repo-Gal4/+*. *drk^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/UAS-drk$^{RNAi#105498}$*; *repo-Gal4/+*. (B) Control: *tub-Gal80^{ts}/+*; *repo-Gal4, UAS-mCD8::GFP/+*. *drk^{RNAi}*: *tub-Gal80^{ts}/UAS-drk$^{RNAi#105498}$*; *repo-Gal4, UAS-mCD8::GFP/+*.
Figure 3. (A and B) Control: *tub-Gal80^{ts}/+*; *repo-Gal4, UAS-mCD8::GFP/+*. *drk^{RNAi}*: *tub-Gal80^{ts}/UAS-drk$^{RNAi#105498}$*; *repo-Gal4, UAS-mCD8::GFP/+*. *Sos^{JC2}/+*: *tub-Gal80^{ts}/Sos^{JC2}*; *repo-Gal4, UAS-mCD8::GFP/+*. *drk^{RNAi}, Sos^{JC2}/+*: *tub-Gal80^{ts}/UAS-drk$^{RNAi#105498}$, Sos^{JC2}*; *repo-Gal4, UAS-mCD8::GFP/+*. (C and D) Control: *OR85e-mCD8::GFP, UAS-mCD4::tdTomato/+*; *TIFR-Gal4/+*. *drk^{RNAi}*: *OR85e-mCD8::GFP, UAS-mCD4::tdTomato/UAS-drk$^{RNAi#105498}$*; *TIFR-Gal4/+*. *Sos^{JC2}/+*: *OR85e-mCD8::GFP, UAS-mCD4::tdTomato/Sos^{JC2}*; *TIFR-Gal4/+*. *drk^{RNAi}, Sos^{JC2}/+*: *OR85e-mCD8::GFP, UAS-mCD4::tdTomato/UAS-drk$^{RNAi#105498}$, Sos^{JC2}*; *TIFR-Gal4/+*.
Figure 4. Control: *OR85e-mCD8::GFP, tub-Gal80^{ts}/+*; *repo-Gal4/+*. *Rac1^{N17}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/+*; *repo-Gal4/UAS-Rac1^{N17}*. *Sos^{JC2}/+*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/Sos^{JC2}*; *repo-Gal4/+*. *Sos^{JC2}/+; Rac1^{N17}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/Sos^{JC2}*; *repo-Gal4/UAS-Rac1^{N17}*.
Figure 5. (A and B) Control: *OR85e-mCD8::GFP, tub-Gal80^{ts}/+*; *repo-Gal4/+*. *sos^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/UAS-sos$^{RNAi#42849}$*; *repo-Gal4/+*. *mbc^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/+*; *repo-Gal4/UAS-mbc$^{RNAi#16044}$*. *sos^{RNAi}; mbc^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/UAS-sos$^{RNAi#42849}$*; *repo-Gal4/UAS-mbc$^{RNAi#16044}$*. (C and D) Control: *OR85e-mCD8::GFP, tub-Gal80^{ts}/+*; *repo-Gal4/+*. *dCed-12^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/+*; *repo-Gal4/UAS-dCed-12$^{RNAi#10455}$*. *Sos^{JC2}/+*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/Sos^{JC2}*; *repo-Gal4/+*. *Sos^{JC2}/+; dCed-12^{RNAi}*: *OR85e-mCD8::GFP, tub-Gal80^{ts}/Sos^{JC2}*; *repo-Gal4/UAS-dCed-12$^{RNAi#10455}$*.

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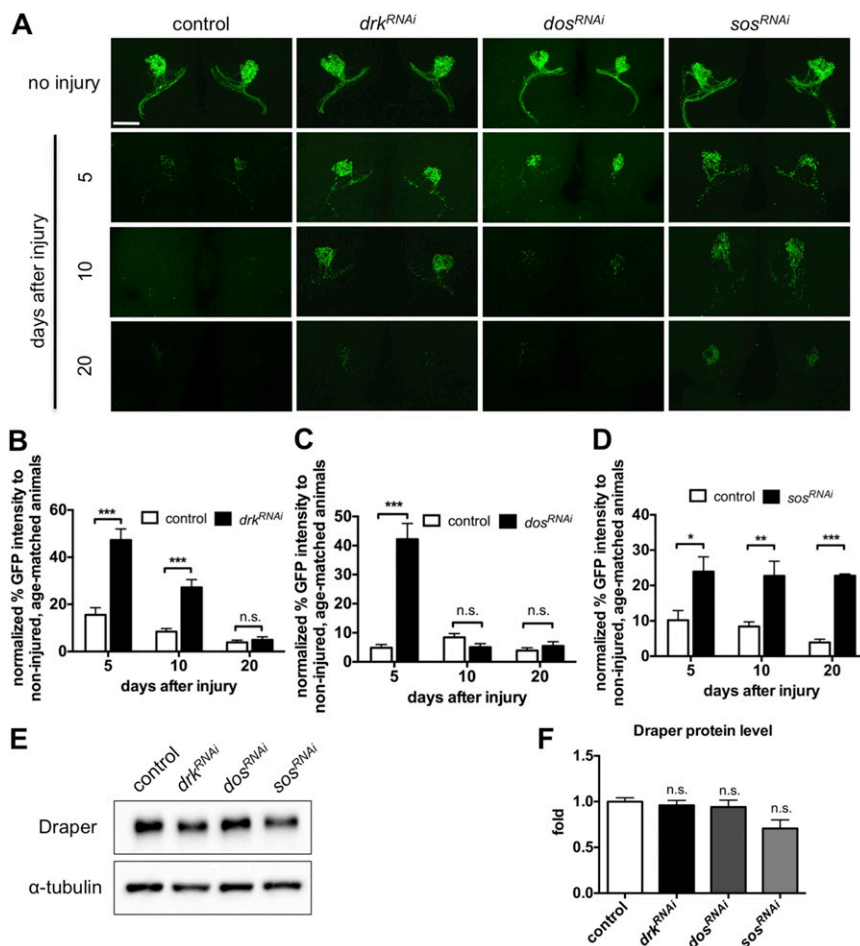
Fig. S1. The depletion of DRK, DOS, and son of sevenless (SOS) in glia results in delay of axonal debris clearance after axotomy. (*A*) Representative images (*z*-stack) of OR85e$^+$ axons from control, *drk* RNAi, *dos* RNAi, and *sos* RNAi animals before and after mp ablation (at days 5, 10, and 20). (Scale bar: 30 μm.) (*B–D*) Quantification of axonal debris remaining in the antennal lobe after axotomy in *drk* RNAi (*B*), *dos* RNAi (*C*), and *sos* RNAi (*D*) animals (*n* = 10 for all except *sos*^{RNAi} at day 20, *n* = 4 because of poor survival of animals). Student *t* test. Control: OR85e-mCD8::GFP, *tub-Gal80*^{ts}/+; *repo-Gal4*/+. *drk*^{RNAi}: OR85e-mCD8::GFP, *tub-Gal80*^{ts}/UAS-drk$^{RNAi\#105498}$; *repo-Gal4*/+. *dos*^{RNAi}: OR85e-mCD8::GFP, *tub-Gal80*^{ts}/+; *repo-Gal4*/UAS-dos$^{RNAi\#3}$. *sos*^{RNAi}: OR85e-mCD8::GFP, *tub-Gal80*^{ts}/UAS-sos$^{RNAi\#42849}$; *repo-Gal4*/+. (*E*) Draper protein expression does not differ between control, *drk*^{RNAi}, *dos*^{RNAi}, and *sos*^{RNAi} animals. Anti-Draper antibody was used to detect the protein level of Draper in Western blot with approximately five dissected brains per lane. RNAi was induced for at least 5 d at 29 °C before dissection. α-Tubulin was used as the internal control. Representative images are shown. The intensity of anti-Draper signals were quantified and normalized and are shown in *F* (*n* = 3 for all). Control: *tub-Gal80*^{ts}/+; *repo-Gal4*/+. *drk*^{RNAi}: *tub-Gal80*^{ts}/UAS-drk$^{RNAi\#105498}$; *repo-Gal4*/+. *dos*^{RNAi}: *tub-Gal80*^{ts}/+; *repo-Gal4*/UAS-dos$^{RNAi\#3}$. *sos*^{RNAi}: *tub-Gal80*^{ts}/UAS-sos$^{RNAi\#42849}$; *repo-Gal4*/+.
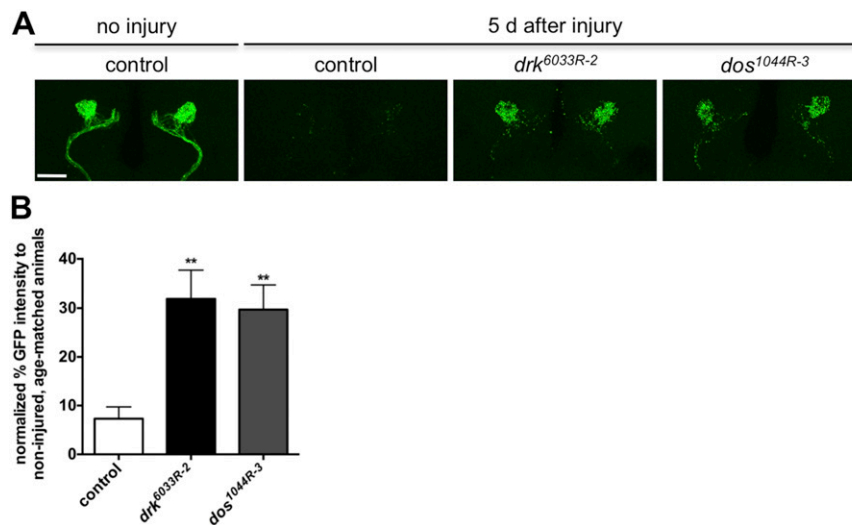
Fig. S2. Alternative RNAi of *drk* and *dos* caused similar engulfment defects in adult glia. (A) The clearance assay was performed as described in Fig. 1. (Scale bar: 30 μm.) (B) Quantification of data in A. GFP immunoreactivity of injured OR85e^{+} glomerulus in RNAi animals was normalized to uninjured, age-matched controls (as 100%) as a result of insufficient number of RNAi animals collected during experiment (*n* = 10 for all). Control: *OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/+*. *drk6033R-2*: *OR85e-mCD8::GFP, tub-Gal80ts/UAS-drk6033R-2; repo-Gal4/+*. *dos1044R-3*: *OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/UAS-dos1044R-3*.
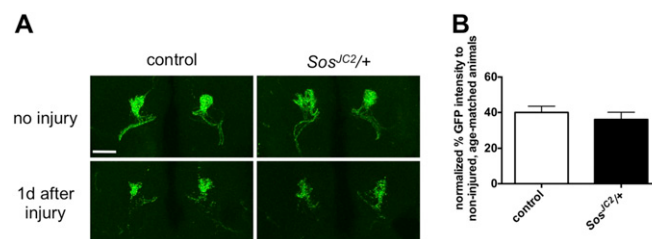

Fig. S3. Gain-of-function allele of *sos* (*SosJC2*) alone does not change the efficiency of glial clearance of axonal debris. (A) Representative images of OR85e^{+} axons in control and *SosJC2*/+ animals before and 1 d after mp ablation. (Scale bar: 30 μm.) (B) Quantification of A (*n* = 10 for all). Student *t* test. Control: *OR85e-mCD8::GFP, tub-Gal80ts/+; repo-Gal4/+*. *SosJC2*/+: *OR85e-mCD8::GFP, tub-Gal80ts/SosJC2; repo-Gal4/+*.
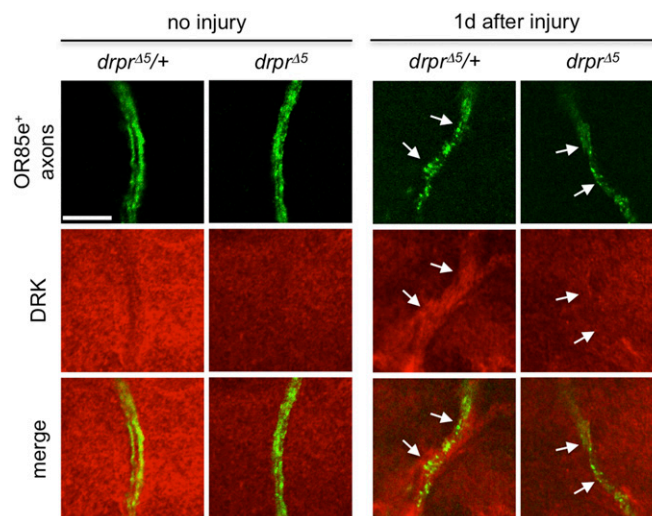

Fig. S4. Draper is required for DRK to be recruited to the severed axons. Maxillary nerves were labeled with mCD8::GFP (green) and DRK immunoreactivity before and after injury was determined by anti-DRK polyclonal antibodies (red). Compared with the increase of DRK around the severed maxillary nerves (arrows) 1 d after injury in Draper (*drpr*) heterozygous null animal (*drprΔ5*/+), no detectable increase of DRK was found around the severed maxillary nerves in *drpr*-null animals. Representative images are shown (single slice). *drprΔ5*/+: *OR85e-mCD8::GFP/CyO, drprΔ5/TM6*; *drprΔ5*: *OR85e-mCD8::GFP/CyO; drprΔ5*. (Scale bar: 10 μm.)
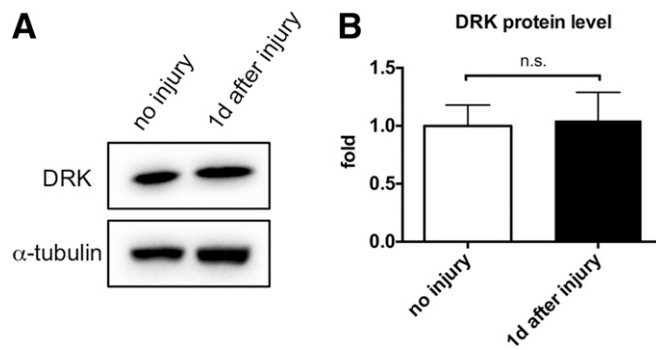
Fig. S5. DRK protein level in the brain is unaltered 1 d after axonal injury. (*A*) Western blots of adult brain lysates (yw) without injury (no injury) or 1 d after removal of the third segments of the antenna and mps (1 d after injury). No significant change of DRK level was observed. α-Tubulin was used as the internal control. Approximately five brains were used per lane. (*B*) Quantification of *A* (n = 3; Student *t* test).
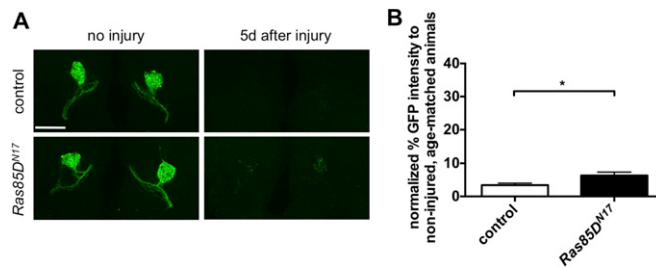
Fig. S6. DRK-SOS is required for glia to activate phagolysosomal program. (*A*) Phagolysosome formation 1 d after axotomy was suppressed when *drk* was knocked down in ensheathing glia (*drk*^RNAi^), as assayed by LysoTracker staining. *Sos*^JC2^ enhanced phagolysosomal activities (*Sos*^JC2^/+), although it was unable to rescue the effect of *drk*^RNAi^ on phagolysosome formation (*drk*^RNAi^, *Sos*^JC2^/+). Representative images (*z*-stack) are shown. (Scale bar: 10 μm.) (*B*) Quantification of the amount of LysoTracker⁺ puncta formed in *A* (*n* ≥ 5 for all). Control: *OR85e-mCD8::GFP/+; TIFR-Gal4/+*. *drk*^RNAi^: *OR85e-mCD8::GFP/UAS-drk*^RNAi#105498^; *TIFR-Gal4/+*. *Sos*^JC2^/+: *OR85e-mCD8::GFP/Sos*^JC2^; *TIFR-Gal4/+*. *drk*^RNAi^, *Sos*^JC2^/+: *OR85e-mCD8::GFP/UAS-drk*^RNAi#105498^, *Sos*^JC2^; *TIFR-Gal4/+*.

Fig. S7. Ras has little effect on glial clearance of axonal debris. (*A*) Overexpression of dominant-negative Ras85D (*Ras85D*^{N17}) in adult glia only mildly affected axonal debris clearance. Representative images (z-stack) are shown. Control: $^{+/+}$; *OR85e-mCD8::GFP, tub-Gal80*^{ts}/+; *repo-Gal4*/+. *Ras85D*^{N17}: *UAS-Ras85D*^{N17}/+; *OR85e-mCD8::GFP, tub-Gal80*^{ts}/+; *repo-Gal4*/+. (Scale bar: 30 μm.) (*B*) Quantification of data in *A* (n = 10 for all; Student *t* test).
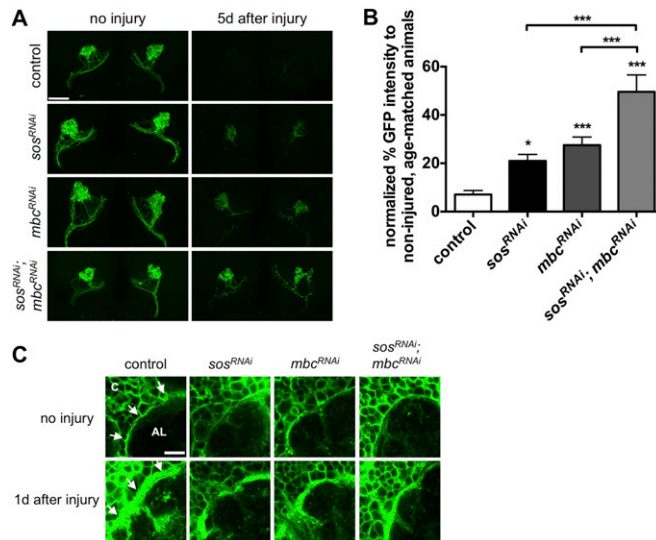

Fig. S8. Glial activation and the clearance of axonal debris require SOS and myoblast city (Mbc). (*A*) Knocking down *sos* and *mbc* simultaneously in adult glia resulted in more severe axonal debris clearance defects compared with single RNAi. Control: *OR85e-mCD8::GFP, tub-Gal80*^{ts}/+; *repo-Gal4*/+. *sos*^{RNAi}: *OR85e-mCD8::GFP, tub-Gal80*^{ts}/*UAS-sos*$^{RNAi\#42849}$; *repo-Gal4*/+. *mbc*^{RNAi}: *OR85e-mCD8::GFP, tub-Gal80*^{ts}/+; *repo-Gal4*/*UAS-mbc*$^{RNAi\#16044}$. *sos*^{RNAi}; *mbc*^{RNAi}: *OR85e-mCD8::GFP, tub-Gal80*^{ts}/*UAS-sos*$^{RNAi\#42849}$; *repo-Gal4*/*UAS-mbc*$^{RNAi\#16044}$. (Scale bar: 30 μm.) (*B*) Quantification of data in *A* (n ≥ 10 for all). (*C*) Glial hypertrophy was blocked in *sos*^{RNAi} and *mbc*^{RNAi} double RNAi animals. Glial membranes (labeled with mCD8::GFP) surrounding the antennal lobe (AL) undergo hypertrophy 1 d after antennal ablation, as indicated by arrows in control. However, glial knockdown of SOS and Mbc slightly reduced the hypertrophy, respectively, and the reduction was more severe when SOS and Mbc were knocked known simultaneously in adult glia. Representative images (single slice) are shown. C, cortex. (Scale bar: 10 μm.) Control: *tub-Gal80*^{ts}/+; *repo-Gal4, UAS-mCD8::GFP*/+. *sos*^{RNAi}: *tub-Gal80*^{ts}/*UAS-sos*$^{RNAi\#42849}$; *repo-Gal4, UAS-mCD8::GFP*/+. *mbc*^{RNAi}: *tub-Gal80*^{ts}/+; *repo-Gal4, uas-mCD8::GFP*/*UAS-mbc*$^{RNAi\#16044}$. *sos*^{RNAi}; *mbc*^{RNAi}: *tub-Gal80*^{ts}/*UAS-sos*$^{RNAi\#42849}$; *repo-Gal4, UAS-mCD8::GFP*/*UAS-mbc*$^{RNAi\#16044}$.
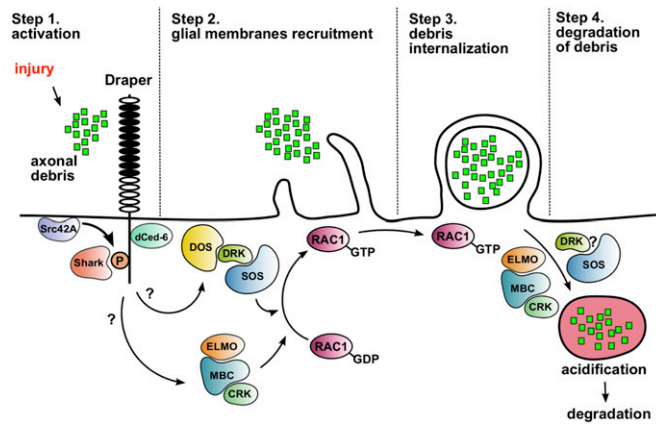
Fig. S9. The proposed model of glial response to and clearance of axonal debris after axon injury in *Drosophila* adult CNS. Upon axon injury, glia receive a yet unknown signal(s) to activate a series of events to clear axonal debris from the CNS, which requires Src42A to phosphorylate tyrosine residue(s) in the intracellular domain of Draper, allowing Shark to bind (1) (step 1). dCed-6, which is required for clearance of axonal debris through an undetermined mechanism, can also be recruited (2). After activation, glial membranes become hypertrophic and Draper is up-regulated. Meanwhile, glia start to extend their processes to the injured axons (3) (step 2). At this step, glia activate DRK/DOS/SOS and Crk/Mbc/dCed-12 pathways to efficiently drive RAC1 function, as knocking down each pathway results in delay of glial membrane recruitment toward the injured axons (Fig. 5) (4). The clearance of axonal debris is mediated by glial membrane vesicles, which enclose and internalize axonal debris (step 3). DRK/DOS/SOS and Crk/Mbc/dCed-12 contribute to the formation of glial membrane vesicles (Fig. 3) (4), possibly through regulating Rac1 activity. Later, the internalized axonal debris is degraded through phagolysosomal pathway (step 4), which also requires DRK, SOS, and Crk/Mbc/dCed12.

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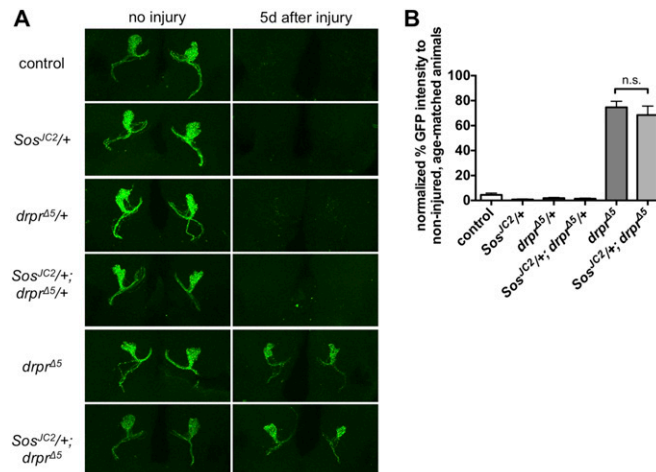
Fig. S10. DRK/DOS/SOS requires Draper to function. (*A*) *Sos^{JC2}* failed to rescue the clearance defect in *drpr*-null animals. Representative images (*z*-stack) are shown. (Scale bar: 10 μm.) (*B*) Quantification of *A* (*n* = 10 for all). Control: *OR85e-mCD8::GFP/+*. *Sos^{JC2}/+*: *OR85e-mCD8::GFP/Sos^{JC2}*. *drpr^{Δ5}/+*: *OR85e-mCD8::GFP/+; drpr^{Δ5}/+*. *Sos^{JC2}/+; drpr^{Δ5}/+*: *OR85e-mCD8::GFP/Sos^{JC2}; drpr^{Δ5}/+*. *drpr^{Δ5}*: *OR85e-mCD8::GFP/+; drpr^{Δ5}*. *Sos^{JC2}/+; drpr^{Δ5}*: *OR85e-mCD8::GFP/Sos^{JC2}; drpr^{Δ5}*.