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Spatial control of Draper receptor signaling initiates apoptotic cell engulfment

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The engulfment of apoptotic cells is essential for tissue homeostasis and recovering from damage. Engulfment is mediated by receptors that recognize ligands exposed on apoptotic cells such as phosphatidylserine (PS). In this study, we convert Drosophila melanogaster S2 cells into proficient phagocytes by transfecting the Draper engulfment receptor and replacing apoptotic cells with PS-coated beads. Similar to the T cell receptor (TCR), PS-ligated Draper forms dynamic microclusters that recruit cytosolic effector proteins and exclude a bulky transmembrane phosphatase, consistent with a kinetic segregation-based triggering mechanism. However, in contrast with the TCR, localized signaling at Draper microclusters results in time-dependent depletion of actin filaments, which facilitates engulfment. The Draper–PS extracellular module can be replaced with FRB and FKBP, respectively, resulting in a rapamycin-inducible engulfment system that can be programmed toward defined targets. Collectively, our results reveal mechanistic similarities and differences between the receptors involved in apoptotic corpse clearance and mammalian immunity and demonstrate that engulfment can be reprogrammed toward nonnative targets.

Introduction

The prompt clearance of dying cells and debris is essential for maintaining homeostasis and promoting tissue repair (Reddien and Horvitz, 2004; Arandjelovic and Ravichandran, 2015; Neumann et al., 2015). In healthy tissue, resident and infiltrating phagocytes clear cell corpses and debris through specific recognition, uptake, and digestion (Elliott and Ravichandran, 2010). Defects in clearance result in autoimmunity and further tissue damage (Elliott and Ravichandran, 2010; Iram et al., 2016; Kawano and Nagata, 2018). Despite the importance of efficient clearance across multicellular life, mechanisms of engulfment receptor activation remain poorly understood in comparison with other signaling systems. Defining the molecular basis of engulfment receptor activation could lead to new strategies for enhancing clearance under conditions of extreme injury or programming phagocytes to eliminate functionally relevant targets such as cancer cells or pathogens.

The initial event in apoptotic cell clearance involves interactions of “eat-me” ligands exposed on dying cells with receptors on the phagocyte. Phosphatidylserine (PS) exposed on the outer leaflet of the plasma membrane constitutes one such eat-me ligand, although several other protein ligands likely participate as well (Fadok et al., 1992; Ravichandran and Lorenz, 2007; Segawa and Nagata, 2015). Ligand binding causes receptor phosphorylation, a process known as receptor triggering, and this event leads to cytosolic signaling that ultimately promotes cytoskeletal rearrangements that power corpse internalization (Reddien and Horvitz, 2004; Ravichandran and Lorenz, 2007). For most transmembrane receptors, triggering proceeds via one of two mechanisms: (1) ligand-induced receptor conformational change to transmit the signal across plasma membranes or (2) kinetic segregation, in which spatially organized zones of ligated receptors physically exclude phosphatases to favor net receptor phosphorylation and activation. EGF receptor and G protein–coupled receptors are examples of conformation-induced activation (Dawson et al., 2005; Erlandson et al., 2018), while the mammalian immune receptors that promote T cell activation and Fc receptor (FcR)–dependent engulfment of opsonized targets are triggered via a kinetic segregation mechanism (Davis and van der Merwe, 2006; Freeman et al., 2016). It remains unclear which activation mechanism corpse clearance receptors use to transmit the eat-me signal across phagocyte plasma membranes. In this study, we use receptor triggering as an inlet to address two open questions about engulfment signaling initiation: Do apoptotic ligands transmit a signal across the plasma membrane via a receptor conformational change or a kinetic segregation mechanism? How are ligated receptors...
organized on phagocyte plasma membranes to potentiate engulfment signaling?

To gain insight into these questions, we focused on Draper, a *Drosophila melanogaster* engulfment receptor. Draper is expressed in glia, where it promotes clearance of damaged axons, and in the somatic epithelium, where it functions to remove dying cells from the follicle (Freeman et al., 2003; MacDonald et al., 2006; Etchegaray et al., 2012). Draper is similar in domain structure to the mammalian protein Megf10 and to CED-1, the first described apoptotic corpse receptor in *Caenorhabditis elegans*. All three receptors contain single-pass transmembrane domains that connect extracellular EGF repeats to intracellular tyrosine (Tyr) phosphorylation sites that are presumed to recruit one or more downstream effectors (Zhou et al., 2001; Ziegenfuss et al., 2008; Schelb et al., 2012). Draper function has been dissected genetically, especially in its damaged axon clearance role, which defined a “parts list” of other proteins involved in engulfment signaling including Draper’s cognate Src family kinase Src42a, the SH2-domain–containing kinase Shark, and the adapter protein Crk (Ziegenfuss et al., 2008, 2012; Lu et al., 2014). However, the mechanism by which receptor ligation leads to effector recruitment remains poorly understood.

In this study, we have reconstituted Draper-dependent engulfment in *Drosophila* S2 cells to dissect receptor triggering. We find that the lipid PS incorporated into lipid bilayers on beads is sufficient to induce receptor phosphorylation and a signaling cascade leading to engulfment. This system allows a dramatic reduction in the complexity of the apoptotic cell as the engulfment target. Similar to the T cell receptor (TCR), ligated Draper forms mobile microclusters that shift a kinase–phosphatase balance toward receptor phosphorylation. However, unlike TCR microclusters, Draper microclusters locally deplete F-actin. By mass spectrometry, we further demonstrate that phosphorylation of Draper is ordered and full activation of Draper requires an initial immunoreceptor Tyr-based activation motif (ITAM) phosphorylation followed by the modification of other Tyrs in its tail domain. We also reveal that the extracellular module of Draper and PS can be replaced with an artificial receptor and ligand that have an inducible interaction, providing another strategy for developing chimeric antigen receptors for phagocytosis (Morrissey et al., 2018). Our reconstitution of the initial steps of Draper signaling provides new insight into the mechanism of engulfment receptor triggering and a strategy to reprogram engulfment to new targets.

## Results

### Cellular reconstitution of Draper-dependent engulfment

We used cultured *Drosophila* S2 cells, which are derived from a hemocyte lineage (Schneider, 1972), as a cellular system for reconstituting corpse clearance. *Drosophila* S2 cells display a low level of engulfment of cell corpses (Fig. 1A). We hypothesized that the level of engulfment might be low because S2 cells lack sufficient levels of engulfment receptors. For example, Draper is expressed at low levels in S2 cells as measured by RNA sequencing (Cherbas et al., 2011). To test whether introducing an exogenous engulfment receptor was sufficient to promote engulfment, we transfected S2 cells with Draper and assayed internalization of cell corpses using microscopy. Draper transfection increased the uptake of apoptotic cell corpses by approximately fivefold (Fig. 1A). The overexpressed Draper-GFP and the endogenous Draper, which is expressed at approximately fivefold lower levels, show similar subcellular localization (Fig. S1A).

The synapse between Draper–transfected S2 cells and corpses also served as a site of local receptor activation. After engaging with a damaged axon, Draper expressed in glial cells becomes Tyr phosphorylated at an ITAM motif, which then enables it to recruit a Syk-related Tyr kinase called Shark (Ziegenfuss et al., 2008). Consistent with the studies of Ziegenfuss et al. (2008), we find a specific recruitment of Shark-mCherry to the plasma membrane to the interface between the corpse and S2 cell (Fig. 1B). Therefore, ligation of Draper to one or more ligands on a cell corpse is sufficient to trigger the local recruitment of the cytosolic tandem SH2-domain–containing kinase Shark and induce engulfment.

Apoptotic cells express an array of eat-me ligands (Ravichandran and Lorenz, 2007). As a next step, we sought to provide a homogenous synthetic target of uniform size. The lipid PS is exposed on dying cells and is considered to be an important eat-me signal (Fadok et al., 1992). PS was previously shown to cause Draper phosphorylation (Tung et al., 2013), but multiple protein ligands for Draper also have been suggested (Kurahishi et al., 2009; Okada et al., 2012). Based upon these prior studies, it was unclear whether one or multiple ligands were needed to execute full engulfment. To determine whether PS alone might be sufficient to trigger Draper-mediated engulfment, we replaced apoptotic cells with 6.5-μm-diameter silica beads coated with a lipid bilayer containing 10% PS and 0.5% atto390-phycerothyrin (PE) lipid for visualization by microscopy (Fig. 1C). Draper-GFP S2 cells engulfed ~0.8 PS beads per cell, on average, after a 30-min incubation (Fig. S1B), which was 50-fold higher than the ingestion of beads coated only with phosphatidylcholine (PC) alone. Control GFP transfected S2 cells also engulfed PS-coated beads approximately sevenfold less efficiently than the Draper–GFP transfected cells (Fig. S1B). As bead diameter decreased, Draper–GFP transfected cells internalized more beads (Fig. S1C). Overexpression of Shark-mCherry did not increase engulfment of 10% PS beads (Fig. S1D). Draper–GFP S2 cells engulfed beads at 2.5% PS lipid content and showed increasing engulfment up to 12.5% PS (Fig. 1C). The PS concentrations that promote engulfment of beads are within the physiological range present on dead and dying cells (Leventis and Grinstein, 2010). Thus, our S2 cell system is a simplified two-component platform that is dependent on (1) overexpression of the Draper receptor and (2) PS exposed on a lipid bilayer–coated bead.

Next, we examined the activation of Draper in the presence of 10% PS beads. Similar to the apoptotic cell, we found that Shark-mCherry colocalized with Draper-GFP at the interface between PS bead and the Draper-transfected S2 cell (Fig. 1D). A strong pTyr signal was also found at this interface as well (Fig. S1E). Collectively, these results demonstrate that PS ligation is sufficient to trigger Draper locally and transmit the signal from target to cytosol (Fig. 1D), where Draper receptor tails are phosphorylated and recruit the kinase Shark.
We next performed live-cell microscopy to visualize the process of Draper-mediated engulfment of PS beads (Fig. S1 F and Video 1). We cotransfected Draper-mCherry and LifeAct-GFP in S2 cells and imaged PS bead engulfment by time-lapse imaging. Draper-mCherry–expressing cells formed a synapse with PS beads and rapidly began forming a phagocytic cup; F-actin as assessed by LifeAct-GFP signal localized at the edges of the cup, and the leading edge of the cup extended gradually around the bead. When the phagocytic cup was complete, the PS bead was internalized. Thus, our system is useful for determining the kinetics of PS bead engulfment and following the dynamics of the cytoskeleton and membranes downstream of Draper activation.

Ligated Draper nucleates mobile microclusters that recruit effector proteins and remodel the actin cytoskeleton

To better visualize the behavior of Draper and the recruitment of proteins to the plasma membrane during the initiation of engulfment signaling, we used total internal reflection fluorescence microscopy (TIRF-M), a technique that confines illumination to within ~200 nm of the glass coverslip. We coated the glass coverslip with a supported lipid bilayer (SLB) containing 10% PS (Fig. 2 A). When Draper-GFP S2 cells settled on this PS SLB, the cells spread rapidly on the surface in a futile effort to engulf the planar surface. Strikingly, Draper-GFP formed microclusters typically near the leading edge of the cell; these microclusters then underwent retrograde flow from the leading edge toward a central synapse at a rate of 1.42 ± 0.45 µm/min (mean ± SD, n = 26; Fig. 2 A and Video 2). The velocity of Draper-GFP microclusters was comparable with actin retrograde flow in this system (1.38 ± 0.40 µm/min; mean ± SD; n = 25). The behavior of these Draper clusters is very similar to that reported for adaptive immune receptor microclusters that form in B and T cells and are thought to serve as sites of local signal activation (Kaizuka et al., 2007; Murugesan et al., 2016).

Next, we asked whether Draper-GFP clusters are sites of recruitment for downstream signaling molecules. Draper is proposed to interact with Shark, a dual SH2 protein, and work with the adapter protein Crk, which harbors one SH2 domain and two SH3 domains. To test whether these proteins are recruited to Draper microclusters, we allowed Draper-GFP–, Shark-TagBFP–,
and Crk-mCherry–transfected S2 cells to settle on 10% PS bilayers and imaged them by TIRF-M. Activated Draper colocalized with Shark kinase, the adapter Crk, and the activating subunit of PI3K, p85 (Fig. 2, B and C; Fig. S2; and Video 3). Cytosolic pTyr-binding proteins colocalized with Draper at peripheral microclusters immediately upon formation and persisted during migration toward the large central synapse (Fig. 2, B and C). Collectively, these results demonstrate that upon ligation to PS, Draper is phosphorylated and nucleates formation of signaling microclusters, mobile assemblies that recruit multiple SH2-domain-containing proteins.

To determine whether Draper clusters have local effects on the actin cytoskeleton, we coexpressed Draper-mCherry, Shark-GFP, and LifeAct-BFP and allowed these cells to settle on 10% PS bilayers. The appearance of Draper–mCherry microclusters was accompanied by an almost simultaneous colocalization signal with SharkGFP and LifeAct-BFP initially and appear to locally deplete F-actin over time as indicated by reduced LifeAct-BFP signal as the cluster matures over time. Time in minutes:seconds. LifeAct-BFP depletion at Draper-GFP clusters was observed in 26 of 27 spread cells examined. Bars, 5 µm.
process of engulfment, we treated cells with the synthetic actin nucleator jasplakinolide and scored assembly of phagocytic cups. Jasplakinolide-treated cells showed a striking defect in cup formation (Fig. S3 B), indicating that disruption of actin dynamics hinders early steps of Draper-dependent engulfment.

Exclusion of a transmembrane phosphatase at the ligated membrane–membrane interface

We next used our reconstitution system to define how PS triggers Draper phosphorylation. Both the TCRs and FcRs are believed to be triggered by the exclusion of the transmembrane phosphatase CD45, which has a bulky extracellular domain, from zones of close membrane–membrane contact created by the phosphorylated major histocompatibility complex (pMHC)-TCR or IgG–FcR binding (Choudhuri et al., 2005; Burroughs et al., 2006; James and Vale, 2012; Freeman et al., 2016). In contrast, Src family kinases associate with the inner leaflet of the plasma membrane via a palmitoylation and remain uniformly distributed after pMHC-TCR engagement. As a result, phosphatase exclusion shifts the kinase–phosphatase balance toward net receptor phosphorylation. In contrast, EGF receptor and G protein–coupled receptors are triggered via receptor conformational change (Dawson et al., 2005; Erlandson et al., 2018). We sought to test whether a segregation mechanism might function to trigger Draper phosphorylation. *Drosophila* S2 cells express several transmembrane phosphatases as measured by RNA sequencing (Fig. 3 A; Zhang et al., 2010). We tested the transmembrane phosphatase Ptp69D because of its high expression level and similar domain structure to the segregated mammalian phosphatase CD45 (extracellular fibronectin repeats and cytosolic Try phosphatase activity; Fig. 3 A).

To determine whether Ptp69D segregates away from Draper at the ligated interface, we cotransfected the two proteins with different fluorescent protein tags and assessed localization in living cells. Microscopy revealed that ligated Draper-mCherry concentrated at the synapse between 10% PS beads and the S2 cell. Draper-mCherry and Ptp69D-GFP were cotransfected into S2 cells and localization at the synapse with 10% PS beads assessed after a 15-min incubation. Ptp69D-GFP was excluded from 22 of 24 synapses examined. (C) Draper-mCherry clusters exclude Ptp69D-GFP on 10% PS SLBs. Draper-mCherry/Ptp69D-GFP–expressing S2 cells were allowed to settle and spread on bilayers and localization of receptor and phosphatase assessed after 15 min. Ptp69D-GFP exclusion from Draper-mCherry was observed in 22 of 25 spread cells examined. Bars, 5 µm.

**Figure 3.** Exclusion of a transmembrane phosphatase at the ligated membrane–membrane interface. (A) S2 transmembrane phosphatase expression data and schematic of Ptp69D-GFP domain structure highlighting features of its bulky extracellular domain. Phosphatase expression levels are indicated in reads per kilobase of transcript, per million mapped reads (RPKM). FN, fibronectin. (B) The transmembrane phosphatase Ptp69D segregates away from Draper at synapses between 10% PS beads and the S2 cell. Draper-mCherry and Ptp69D-GFP were cotransfected into S2 cells and localization at the synapse with 10% PS beads assessed after a 15-min incubation. Ptp69D-GFP was excluded from 22 of 24 synapses examined. (C) Draper-mCherry clusters exclude Ptp69D-GFP on 10% PS SLBs. Draper-mCherry/Ptp69D-GFP–expressing S2 cells were allowed to settle and spread on bilayers and localization of receptor and phosphatase assessed after 15 min. Ptp69D-GFP exclusion from Draper-mCherry was observed in 22 of 25 spread cells examined. Bars, 5 µm.
we tested whether the extracellular domain of Draper and its ligand PS could be replaced with an artificial receptor–ligand pair. Previous research on TCR triggering showed that the extracellular domain of the TCR and pMHC could be substituted with FKBP and FRB, which upon addition of rapamycin to bridge FKBP and FRB, trigger TCR phosphorylation \((\text{James and Vale, 2012}).\) We created a similar inducible system for Draper by replacing its endogenous extracellular domain with FRB (FRB-EXT-Draper-INT) and replacing PS with His\(_{10}\)-FKBP, which was bound to the lipid bilayer on the bead via a Ni-NTA–lipid \((\text{Fig. 4 A}).\) In the absence of rapamycin, beads were engulfed at very low levels \((0.003 \text{ beads per cell}).\) However, in the presence of rapamycin, the engineered receptor promoted robust engulfment of FKBP-bearing beads \((\text{Fig. 4 A and Video 5}).\) Live-cell microscopy revealed that the FRB-EXT-Draper-INT concentrated at the cell–bead interface and recruited Shark kinase, suggesting that the chimeric receptor, like Draper, is activated by Tyr phosphorylation \((\text{Fig. 4 B}).\) Like Draper-GFP, FRB-EXT-Draper-INT excluded the phosphatase Ptp69D-GFP \((\text{Fig. 4 C}),\) consistent with a kinetic segregation-based triggering mechanism. Thus, a chimeric receptor that is designed to interact with a nonphysiological ligand is functional for local receptor triggering, phosphatase exclusion, and engulfment.

Using LifeAct-GFP–expressing cells, we also examined whether microclusters nucleated by the FRB-EXT-Draper-INT receptor locally deplete F-actin. Like Draper, the FRB-EXT-Draper-INT receptor and LifeAct colocalized in the TIRF field, but over 1 min, the F-actin signal became depleted under the microcluster, creating the appearance of a small actin hole \((\text{Fig. 4 D and Video 6}).\) Collectively, our results using the engineered FRB-EXT-Draper-INT receptor indicate that formation of a ligated zone that excludes a transmembrane phosphatase is sufficient to trigger receptor phosphorylation, effector recruitment, and local F-actin depletion observed for Draper.

**Ordered multisite Tyr phosphorylation is required for full Draper activation**

We first sought to determine which Tyr residues are essential for Draper–dependent engulfment in our cellular constitution assay. The Draper tail domain contains an ITAM motif \((\text{Yxxl/Lx}_{-12}\text{Yxxl/L}),\) an NPxY motif thought to interact with CED-6 \((\text{Fujita et al., 2012}),\) and 10 other Tyr residues, two of which are in close proximity to the transmembrane domain. First, we created a construct in which 11 of the 13 Tyrs were mutated to alanine \((\text{Draper-ΔITAM-GFP}),\) leaving the two Tyrs that are immediately adjacent to the transmembrane segment intact, since they potentially could have a structural role. We then compared phagocytic proficiency of cells transfected with Draper-ΔITAM-GFP mutant with full-length Draper-GFP and GFP alone \((\text{endogenous background phagocytosis}).\) Consistent with a role for Tyr phosphorylation on Draper to promote phagocytosis, Draper-ΔITAM-GFP ingested PS beads at levels that were comparable with the GFP control and ~15-fold below Draper-GFP cells after a 45-min incubation \((\text{Fig. 5 A}).\)

We next sought to dissect the roles of Draper’s cytoplasmic Tyr residues directly using a purified biochemical system on liposomes. The cytoplasmic tail of Draper and its cognate Src family Drosophila kinase Src42a \((\text{Ziegenfuss et al., 2008})\) were purified and bound via a His10 tag to Ni-NTA–modified lipids in the liposome. To detect binding between Shark and Draper, we used a fluorescently labeled tandem SH2 Shark construct \((\text{BG505-tSH2-Shark}),\) whose signal quenches upon binding to Draper as it comes in proximity to a lipid-bound acceptor dye \((\text{Hui and Vale, 2014}).\) After ATP was added as substrate for Src42a-mediated phosphorylation, we observed a time-dependent quenching of Shark fluorescence as it bound to the phosphorylated receptor \((\text{Fig. 5 B, orange trace}).\) Next, we tested the Draper receptor with double Tyr to phenylalanine mutation in its ITAM motif \((\text{Fig. 5 B, Draper-ΔITAM}).\) Draper-ΔITAM showed little or no recruitment of Shark \((\text{Fig. 5 B, blue trace}).\) This biochemical result confirms that Shark binds Draper directly via Draper’s ITAM \((\text{Ziegenfuss et al., 2008}).\)

We next tested the role of the Draper ITAM in our cell-based system by mutating its two Tyr residues to Phe \((\text{Draper-ΔITAM; residues in Fig. 5 B})\) and assessed the ability of this mutant to promote phagocytosis. Draper-ΔITAM showed a kinetic delay and a lower number of beads ingested \((\text{Fig. 5 C})\) compared with WT Draper, revealing its critical role in signaling and Shark recruitment \((\text{Fig. S4}).\) However, Draper-ΔITAM still promoted phagocytosis to a greater extent than the effectively null Draper-ΔAla11 construct \((\text{Fig. 5 C}).\)

We also tested whether Draper’s ITAM is sufficient to signal by adding back the ITAM Tyrs \((\text{Y934 and Y949})\) to the null Ala11 mutant \((\text{Draper ITAM-only}).\) The Draper ITAM-only receptor promoted phagocytosis of PS beads, but it did so more slowly than WT and to a lesser extent than WT Draper \((\text{Fig. 5 C}).\) As expected, the Draper ITAM was necessary and sufficient to recruit Shark kinase to the synapse between S2 cell and PS bead \((\text{Fig. S4}).\) Collectively, our mutational analyses suggest that Draper’s ITAM plays a critical role in signaling but that non-ITAM residues also contribute to full receptor activity during engulfment.

To further understand the mechanism of Src42a–dependent activation of Draper, we tested the extent of Tyr phosphorylation on Draper’s cytoplasmic tail in the on-membrane biochemical assay. After a 30-min kinase reaction, immunoblotting with anti-pTyr antibodies revealed a noticeable shift in the electrophoretic mobility of Draper, which is suggestive of phosphorylation on multiple Tyr residues \((\text{Fig. 5 D}).\) Remarkably, even though the Draper-ΔITAM contains nine additional Tyr residues, its phosphorylation as detected by pTyr immunoblotting was minimal \((5\% \text{ of WT Draper})\) in the same kinase reaction \((\text{Fig. 5 D}).\) We observed a similar result using the Src family kinase Lck \((\text{Fig. S5 A}),\) suggesting that preferential modification through ITAM residues is a general mechanism of Draper receptor activation.

Greatly diminished phosphorylation of the Draper-ΔITAM mutant could arise because (1) only the ITAM residues are phosphorylated by Src family kinases or (2) phosphorylation of the ITAM motif is needed for the efficient phosphorylation of non-ITAM Tyrs in Draper’s tail. To distinguish between these possibilities, we repeated the Draper phosphorylation assay and quenched reactions at 30 and 60 s after addition of ATP. To determine whether any residues on Draper are phosphorylated in ordered fashion, we performed 2D mass spectrometry on the 30- and 60-s samples. This method yielded peptides covering Tyr residues on Draper and allowed us to differentiate between
phosphorylated and nonphosphorylated residues (Fig. 5 E). By comparing the proportion of phosphorylated peptides for each site at both 30 and 60 s, we could determine whether certain Tyr residues on Draper are preferentially modified over time. The ITAM Y934 residue was modified to a similar extent at both time points (pTyr ratio of 1.3; Fig. 5 E). However, the non-ITAM residues (except for Y888, which also had few phosphopeptides detected) were preferentially phosphorylated later as indicated by the 60:30-s ratios ranging from 1.9 to 5.2 (Fig. 5 E). Collectively, these results indicate that multiple Draper Tyrs are phosphorylated by Src42a and that phosphorylation of non-ITAM residues strongly depends on prior phosphorylation of the ITAM residues.
Discussion

In this study, we converted poor phagocytes (Drosophila S2 cells) into proficient eaters by expressing a single receptor, Draper. We also show that PS is sufficient to locally trigger receptor phosphorylation and engulfment signaling, building on previous work that demonstrated PS-liposome–dependent Draper phosphorylation (Tung et al., 2013). Thus, we developed a simplified assay platform to dissect a complex cell signaling process that requires multiple receptors and ligands in vivo (Freeman and Grinstein, 2014). Draper is also reported to recognize the protein ligands Pretaporter and DmCaBP1 (Kuraishi et al., 2009; Okada et al., 2012) and has been reported to work in collaboration with coreceptors in flies such as Six Microns Under (Kurant et al., 2008) and integrins (Nagaosa et al., 2011). Our results do not rule out a role for these proteins in vivo to tune sensitivity for triggering and/or allow engulfment under low concentrations of PS (Manaka et al., 2004). However, our study shows that Draper interaction with PS provides a minimal system for studying apoptotic cell engulfment.

Using this cellular platform, in conjunction with a liposome-based biochemical assay, we have been able to gain insight into the initial steps of the corpse clearance signaling pathway.
(see model in Fig. 6). Our results show that ligation to the lipid PS is sufficient to promote Draper clustering and segregation of a transmembrane phosphatase at the ligated interface. Spatial separation between ligated Draper clusters and transmembrane phosphatases shifts a kinase–phosphatase balance in favor of receptor phosphorylation by Src42a. Phosphorylation of Draper’s tail activates a downstream cytosolic signaling cascade, beginning with the recruitment of Shark and other pTyr-binding effector proteins, to Draper microclusters. After effector recruitment, early steps of Draper signaling include localized depolymerization of the actin cytoskeleton. Further downstream signaling likely leads to integrin activation and other events that lead to the formation of the phagocytic cup, although these more distal events in the pathway were not the focus of this study.

Comparison between Draper signaling and mammalian immune signaling

A potential similarity between Draper signaling and mammalian immune receptor activation was first suggested by the finding of an ITAM in the Draper cytoplasmic tail and the ability of this ITAM to bind Shark kinase in yeast two-hybrid and pulldown assays (Ziegenfuss et al., 2008). Our findings extend this work by providing evidence that Draper, like the T cell and Fc immune receptors, is triggered by a kinetic segregation mechanism. The kinetic segregation model, initially proposed as a mechanism for T cell activation, postulates that apposition of a membrane-membrane interface at the synapse between the T cell and antigen-presenting cell (APC) results in the exclusion of a bulky transmembrane phosphatase CD45, which shifts a kinase–phosphatase equilibrium in favor of receptor phosphorylation (Davis and van der Merwe, 2006). A segregation mechanism was recently proposed to regulate FcR activation, another mammalian immune signaling pathway, by creating an integrin-based diffusion barrier that excludes CD45 (Freeman et al., 2016).

Prior to our work, it remained unclear whether ligated corpse clearance receptors also use phosphatase exclusion to create zones of receptor triggering. In this study, we show that Draper ligation to PS on a second membrane excludes a transmembrane phosphatase (Ptp69D) from the synapse at the region of receptor phosphorylation and effector recruitment. Similar results showing exclusion were obtained with our chimeric receptor based on FKBP–rapamycin–FRB interaction. In the case of T cells, a single transmembrane phosphatase (CD45) is highly expressed on the surface and appears to be the dominant phosphatase in regulating signaling. However, phagocytic cells express several transmembrane phosphatases including Ptp69D, which together may contribute to regulating Draper and other phagocytic receptors. In the future, it will be illuminating to determine how the specific composition of phosphatases on the membrane (different sizes, localization patterns, and expression levels) are used to tune the sensitivity of engulfment and whether extracellular domains of differing sizes regulate engulfment as in other systems (Schmid et al., 2016; Carbone et al., 2017). It also will be interesting to explore whether Draper, like FcR (Freeman et al., 2016), activates integrin signaling to create a diffusion barrier for CD45.

Draper ligation to PS results in the formation of receptor microclusters, physical assemblies that organize engulfment effectors. The striking similarity extends to the recruitment of key signaling molecules to the microclusters. We show that similar to the TCR, Draper recruits several SH2-domain containing effector proteins, including Shark kinase, the adapter Crk, and the PI3K subunit p85. While the formation and retrograde transport of microclusters appears to be similar for the TCR and Draper, we observed a striking difference with regard to the effects of these clusters on F-actin. The TCR microclusters reside above actin-rich zones as they are transported from the periphery to the central supramolecular activation cluster (cSMAC) synapse (Kaizuka et al., 2007; DeMond et al., 2008; Yi et al., 2012; Murugesan et al., 2016). We also observed that Draper microclusters initially colocalize with the F-actin reporter LifeAct. However, as the clusters mature, but well before they reach the central synapse, the microclusters deplete F-actin to create “holes” in the actin cytoskeleton. We observed a similar anticorrelation between Draper and F-actin at microclusters of the synthetic FRB–EXT-Draper–INT receptor (Fig. 4 D) and at the large synapse between the Draper-expressing S2 cell and PS bead (Fig. S3 A). Our findings indicate that F-actin depletion over time both at peripheral microclusters and the central synapse is a distinct output of Draper receptor activation to actin regulators compared with the TCR signaling pathway.

Actin-depleted zones have been observed to form at the cSMAC at the mature synapse between a T cell and its target (Bunnell et al., 2001; Stinchcombe et al., 2001). For FcR-mediated engulfment, actin-depleted zones have been reported at the
interface between the phagocyte and a bead (Yamauchi et al., 2012; Schlam et al., 2015); actin depletion was suggested to be important for the phagocytosis of large, but not small, targets (Schlam et al., 2015). In addition, elegant recent work by Freeman et al. (2016) reported that macrophages interacting with micro-patterned, immobilized IgG will polymerize F-actin surrounding the ligated FcRs, thereby forming actin rings. This study focused on integrin-mediated actin assembly at the periphery outside of the zone of receptor–ligand engagement, but images in Freeman et al. (2016) also reveal actin depletion at the center of the receptor zones in the micropatterned array.

The kinetics of actin depletion observed in this study suggests that actin depletion is an active part of the Draper signaling pathway that follows receptor phosphorylation by ∼1 min and is restricted to the zone of ligated receptors. The molecular mechanism responsible for actin disassembly could involve (1) a repression of Arp2/3 actin nucleation near the phosphorylated Draper receptor, potentially in combination with a barrier that presents nearby actin filaments from polymerizing or moving into the receptor zone; (2) RhoGAP repression of Rac and Cdc42 as suggested by Schlam et al. (2015) for large phagocytic cups; or (3) the recruitment and/or activation of actin-depolymerizing factors such as cofilin at the Draper signaling zone. Additional work will be needed to distinguish between these models.

**Mechanism of Draper triggering upon ligation**

The current model for T cell activation and FcR-mediated phagocytosis suggests that triggering is driven entirely by phosphorylation of ITAM Tyrs. In those cases, there are not additional Tyr residues outside of the ITAM motifs. Shark kinase, like ZAP70 and Syk, binds specifically to the ITAM motif on Draper, and we show that ITAM phosphorylation plays an important role in engulfment signaling. However, unlike the TCR and FcR, we show that Draper contains non-ITAM Tyrs that are also phosphorylated during activation and are functionally important for engulfment. We find that non-ITAM Tyrs play a role in Draper activation, thus supporting a signaling function both for Draper’s ITAM and non-ITAM residues as suggested earlier (Ziegenfuss et al., 2008; Fujita et al., 2012). By measuring Draper Tyr phosphorylation directly via mass spectrometry, we report modification of residues outside of Draper’s ITAM. Importantly, Draper’s non-ITAM Tyr residues contribute to engulfment efficiency in cell-based assays. The phosphorylated non-ITAM Tyrs in Draper may bind additional SH2-containing adapter proteins or other effectors. Further studies will be needed to identify additional direct binding partners of the phosphorylated cytoplasmic tail domain of Draper and determine the higher-order structure of signaling microclusters that promote engulfment signaling.

Our biochemical reconstitution also reveals that Draper’s ITAM domain is required for efficient phosphorylation of the non-ITAM Tyrs. This result indicates a sequential mechanism for multisite phosphorylation on Draper. One model for how this might occur is through the direct recruitment of Src family kinases to the phosphorylated ITAM domain of Draper. We show that the Drosophila Src42a kinase has a higher specificity for the Tyrs in Draper’s ITAM domain and phosphorylates these residues first. We also confirmed ITAM specificity for Draper using Lck, another Src family kinase that phosphorylates ITAM domains on TCR cytosolic tails (Fig. S5 A). One possible mechanism for the sequential phosphorylation involves a mechanism in which a kinase creates its own binding site before modifying other residues. This idea has been suggested for Src-dependent phosphorylation of synthetic ITAM-containing substrate peptides (Pellicena et al., 1998) and phosphorylation of p130(CAS) by Abl kinase (Mayer et al., 1995). A similar phenomenon was observed for Lck-dependent modification of sequential ITAM residues on the TCR, which is required for full activation of this receptor (Lewis et al., 1997). Thus, our data could be explained by Src42a binding to the phosphorylated ITAM through its own SH2 domain and then phosphorylating non-ITAM residues on Draper (Fig. S5). Thus, in common with other signaling systems, Draper phosphorylation involves a hierarchal phosphorylation of Tyrs in its tail domain, and this ordered sequence of phosphorylation events is required for full receptor activation.

**Synthetic receptors to redirect engulfment signaling**

We show in this study that the extracellular Draper–PS receptor–ligand pair can be functionally replaced by a synthetic inducible module, FKBP–rapamycin–FRB. The ability of this synthetic receptor to promote engulfment is analogous to findings for the TCR, which can be triggered using the same extracellular FKBP–rapamycin–FRB module (James and Vale, 2012). These programmed receptors enable activation of intracellular signaling pathways independently of their native extracellular domains. These synthetic receptors create signaling zones for both Draper (Fig. 4 C) and TCR (James and Vale, 2012) that exclude a transmembrane phosphatase to favor phosphorylation of intracellular receptor signaling domains. Inducible engulfment receptors consisting of intracellular receptor signaling tails fused to non-native extracellular domains are conceptually similar to chimeric receptors that trigger phagocytosis (Morrissette et al., 2018) and chimeric TCRs that recognize cancer antigen to promote specific cell killing (Lim and June, 2017). Collectively, these programmed signaling systems highlight the future promise of redirecting cells to target cancer, harmful debris, and pathogens.

**Materials and methods**

**Reagents and recombinant DNA materials**

The complete catalog information for reagents and tools used in this study can be found in Table S1. Detailed information for all recombinant DNA materials can be found in Table S2.

**Drosophila S2 cell culture**

Drosophila S2U cells were cultured in Schneider’s media (Gibco) supplemented with 10% FBS (Atlanta) and 1× antibiotic/antimycotic (Gibco). S2 cells were grown in nonvented T25 or T75 flasks (Corning) and split every 3–6 d from high-density cultures (∼50–80%). S2U cells were not split to below ∼25% confluency as S2U cells are “unhappy” at low density. Healthy S2U cells adhere gently to the surface of the culture dish rather than floating but can be split by gently resuspending the adhered cells using a tissue culture pipet and a 10-mL sterile tissue culture pipet. Enzymatic dissociation using trypsin or by scraping...
is unnecessary for S2U cells. Frozen stocks were prepared regularly. S2U freeze media contains 45% fresh complete media, 45% sterile filtered conditioned media prepared after spinning the media for 5 min at 1,000 g, and 10% DMSO (Sigma-Aldrich). A confluent T75 dish of S2U cells was pelleted for 5 min at 1,000 g, resuspended in 10 ml freeze media (described above), split as 1-ml aliquots in externally threaded cryotubes (Corning), stored at −80°C in a cell-freezing apparatus containing isopropl alcohol, and transferred to long-term storage in dry nitrogen (vapor phase). Cells were regularly tested for mycoplasma using the MycoAlert kit (Lonza).

**Transfection of Drosophila S2U cells**

Cells were split to ~60% confluency into six-well tissue culture dishes 4–12 h before transfection. All transfection mixes were prepared in 5 ml low-retention polystyrene round-bottom tubes. To prepare transfection mixes, a maximum of 2.5 µg plasmid DNA was added to 300 µl serum-free Schneider’s media and gently mixed. 5 µl TransIT-Insect (Mirus) was added to diluted DNA and gently mixed. At this point, the solution appears cloudy. Transfection mixes were incubated at room temperature for 15 min and added dropwise gently to the 6-well plate using a p1000 pipet. After dropwise addition of transfection mixes, the plate was gently shaken laterally to ensure even distribution of TransIT-DNA particles. Transfection efficiencies of at least 50% and up to 90% were regularly obtained using this method, which is an improvement compared with other transient transfection methods employed for *Drosophila* S2U cells.

All constructs used in this study express proteins of interest in S2U cells were under control of a pMT copper-inducible promoter. To induce expression in transfected cells, CuSO₄ was added from a 50 mM stock in water to a final concentration of 250 µM (1:200 dilution from stock) 36–48 h after transfection. The timing of induction is important; at this later time point, we find expression levels to be lower and more uniform. At shorter time points (e.g., 12–24 h after transfection), transfection efficiency was lower, and expression levels in transfected cells were higher, as assessed by live-cell imaging of fluorescent proteins under control of the pMT promoter.

**Preparation of APC Annexin V–labeled apoptotic cell corpses**

To prepare labeled apoptotic cell corpses for use in internalization assays, 1 µM final concentration of actinomycin D (1 mg/ml in DMSO; 833 µM stock; Sigma-Aldrich) was added directly to 80% confluent cultures of *Drosophila* S2U cells in 6-well plates. Cells were incubated for 16 h in actinomycin D before harvesting by centrifugation for 5 min at 1,000 g in a 15-ml falcon tube. Actinomycin D was washed out by rinsing corpses twice in complete Schneider’s media in the same falcon tube; sequential washes, each with 10 ml complete media, were performed. Corpses from one well of a six-well dish were resuspended in 300 µl complete media after washes and transferred to a 1.7-ml Eppendorf tube for labeling. To label externalized PS, APC Annexin V (BioLegend) was added directly to the washed corpses to a final concentration of 50 ng/µl (from 5 µg/ml stock). The tube was wrapped in foil before labeling on a rotator at room temperature for 30 min. After labeling, cells were washed three times by sequential harvesting and resuspension in complete media. After washing, labeled corpses were resuspended in 300 µl complete media.

**Assay for internalization of apoptotic cell corpses**

To assess internalization of apoptotic cell corpses, washed Draper-GFP or mCherry-control transfected S2U cells were incubated with fresh complete media containing 10% FBS and labeled cell corpses prepared as above. Transfected cells were washed with complete S2U media, and culture media was replaced with 1 ml fresh complete culture media. Cells in fresh media were gently resuspended by pipetting up and down. 10 µl washed labeled cell corpses prepared as above was mixed in an Eppendorf tube with 300 µl washed transfected cells. The Eppendorf tube was flipped up and down four times, and complete mixture was plated in one well of a 96-well glass-bottom Matrigel imaging dish (Brooks). After a 45-min incubation at room temperature in the dark, high-content screening imaging and quantification were performed as described below.

**Preparation of 10% PS-coated silica beads**

10 molar percent PS beads were used for all experiments other than the PS titration in Fig. 1 C. To prepare 10 molar percent beads, chloroform suspended lipids sufficient for 1 ml of a 10 mM solution were mixed at the following proportions: 89.5% POPC, 10% DOPS, 0.5% PEG5000-PE, and 0.5% atto390-DOPC (label for visualization by microscopy). Lipids were transferred to a chloroform-washed glass vial using gas-tight Hamilton syringes and dried to a film under argon gas in a warm (~45°C) water bath. Dried lipids were stored under foil (if labeled lipids were used) and desiccated overnight at room temperature in a benchtop desiccator filled with Drierite. Dried lipids were suspended in 1 ml tissue culture-grade PBS by vortexing for 1 min under parafilm and gentle pipetting. Suspended lipids were transferred to a 1.7-ml Eppendorf tube and stored under argon gas. The PBS-lipid mix was freeze/thawed five times in liquid nitrogen and warm water and subjected to 2 × 5-min sonication in a Bioruptor Pico bath sonicator (Diagenode). Sonicated lipids were spun at 35,000 rpm for 35 min at 4°C in a TLA 120.1 rotor in a benchtop Beckman ultracentrifuge. Only a small pellet should be visible after spinning sonicated lipids. Small unilamellar vesicles (SUVs) prepared using this method were used immediately or stored under argon gas, flash frozen, and stored at ~80°C. Lipid mixes are best used within 2 wk of preparation. Undiluted 10 mM lipid mixes were used as described below to build bilayers on silica beads. For the PS titration in Fig. 1 C, differences in PS concentration were made up by balanced changes to the molar percentage of PC such that final lipid concentration in 1 ml PBS matched the 10 mM concentration described above.

50 µl of 6.46-µm silica beads (SS06N; Bangs Labs) were added to 300 µl Milli-Q water in 1.7 ml Eppendorf tubes for washing. Beads in suspension were pelleted three times in 300 µl Milli-Q water at 300 rcf and decanted. After the third wash, beads were resuspended in ~30 µl PBS until the pellet was barely covered. 300 µl of 2 mM final concentration of the desired lipid mix (diluted from 10 mM stock in PBS) was vortexed briefly, wrapped in foil, and rotated at room temperature for 45 min. Beads were pelleted and washed three times by sequential pelleting and
resuspension in 300 μl fresh PBS. Washed beads were resuspended in 300 μl fresh PBS for the bead assay (described below).

Assay for internalization of PS-coated silica beads
To assess internalization of PS-coated silica beads, 7 μl bead suspension was mixed with 300 μl washed transfected S2U cells resuspended in fresh complete media containing 10% FBS and flipped gently to mix four times, and then the complete mixture plated in one well of a 96-well glass bottomed MatriPlate imaging dish (Brooks). Cells were allowed to settle and imaged as described below. For endpoint assays, quantification was performed on images taken 30 or 45 min after plating. For kinetic experiments, imaging was started 5 min after plating.Engulfment experiments were performed using S2U cells cotransfected with GFP or a GFP-fusion receptor protein, mCherry-CAAX (full-length mCherry-LEKMSKDGGKKKKSSTKCVIM) for visualizing plasma membranes during quantification (described below), and 10% PS atto390-PE–labeled silica beads.

Assay for internalization of His₁₀-FKBP silica beads
Engulfment experiments using His₁₀-FKBP ligated beads were performed as above with the exception that FRB-EXT-Draper-INT-GFP–expressing S2 cells were cotransfected with mCherry-CAAX to serve as a specific receptor for the FKBP ligand. Silica beads were blocked for 15 min in 300 μl PBS + 0.1% wt/vol BSA, 2% DGS-NTA-nickel, 0.5% PEG5000-PE, and 0.5% atto390-DOPE. After washing, PBS was gently pipetted up and down over the surface of the bilayer to remove SUVs not part of the SLB. Bilayers were washed 10 times with Milli-Q water.

Building PS-containing SLBs
The night before the experiment, a 96-well MatriCal imaging chamber was gently removed and replaced with 150 μl of 6.4% paraformaldehyde. Cells were fixed under foil for 15 min, washed 3 × 5 min in PBS + 0.1% wt/vol BSA, and resuspended in PBS + 0.1% wt/vol BSA before the engulfment assay. 300 μl washed transfected cells were mixed with 7 μl His₁₀-FKBP ligated beads either in the presence of 1 μM rapamycin or a matching volume of DMSO (vehicle) and flipped four times to mix, and ingestion was quantified as described below.

Staining for pTyr and the synapse between S2 cell and bead
To fix and stain bead–cell synapses in chambers described above for pTyr localization, half the media (~150 μl) from imaging chambers was gently removed and replaced with 150 μl of 6.4% paraformaldehyde. Cells were fixed under foil for 15 min, washed 2 × 3 min in PBS, permeabilized with 0.5% vol/vol Triton X-100 in PBS for 10 min, and set overnight to block in PBS + 0.1% vol/vol Triton X-100 + 0.2% wt/vol BSA at 4°C wrapped in parafilm in the dark. Cells were stained in primary antibody (pY-20 mouse anti-pTyr, 2 μg/ml final concentration, 1:100 dilution; Santa Cruz Biotechnology) for 1 h at room temperature in the dark, washed 3 × 5 min at room temperature in PBS + 0.1% vol/vol Triton X-100 + 0.2% wt/vol BSA, and incubated in secondary antibody (anti-mouse IgG H+L Alexa Fluor 647 conjugated; Thermo Fisher Scientific) for 1 h at room temperature, and washed 3 × 5 min in PBS + 0.1% vol/vol Triton X-100 + 0.2% wt/vol BSA. Washed fixed stained cells were gently covered in 200 μl PBS before imaging. Cells were imaged immediately or stored for up to 2 d at 4°C in the dark while wrapped in parafilm before imaging by spinning-disk confocal microscopy (see below).

Staining for endogenous Draper and Draper-GFP
To fix and stain for endogenous Draper localization versus the Draper-GFP construct, half the media (~150 μl) from imaging chambers was gently removed and replaced with 150 μl of 6.4% paraformaldehyde. Cells were fixed under foil for 15 min, washed 2 × 3 min in PBS, permeabilized with 0.5% vol/vol Triton X-100 in PBS for 10 min, and blocked for 20 min in PBS + 0.1% vol/vol Triton X-100 + 0.2% wt/vol BSA at room temperature. Cells were stained in primary antibody (8A1 Hybridoma supernatant; 1:2 final dilution overnight at 4°C; DSHB) washed 3 × 5 min room temperature in PBS + 0.1% vol/vol Triton X-100 + 0.2% wt/vol BSA, incubated in secondary antibody (anti-mouse IgG H+L Alexa Fluor 647 conjugated; Thermo Fisher Scientific) for 1 h at room temperature, and washed 3 × 5 min in PBS + 0.1% vol/vol Triton X-100 + 0.2% wt/vol BSA. Washed, fixed, and stained cells were gently covered in 200 μl PBS before imaging. Cells were imaged immediately by spinning-disk confocal microscopy (see below).
temperature. Uncoupled protein was washed 3 × 5 min under foil at room temperature before adding cells as above with the exception that when FKBP ligand was used with receptors fused to FRB-extracellular domains, 1 µM rapamycin was added to form the 100 fM FRB–rapamycin–FKBP ternary complex.

Acute jasplakinolide treatment and quantification of phagocytic cup formation
S2 cells as above for bead internalization assay were treated with 1 µM final concentration jasplakinolide (a macrocyclic peptide that serves as an actin nucleator) or corresponding volume of DMSO (vehicle) for 20 min at room temperature. After acute drug treatment or control incubation, 7 µl of 10% PS bead suspension was mixed with the treated or control cells and flipped gently to mix four times, and then the complete mixture plated in one well of a 96-well glass-bottom MatriPlate imaging dish (Brooks). 15 min after plating, all cells exhibiting a synapse with one or more beads were scored for formation of phagocytic cups.

Liposome fluorescence resonance energy transfer assay for effector recruitment to phosphorylated receptor tails
This assay was based on one used to reconstitute mammalian T cell signaling (Hui and Vale, 2014). To present receptor tails in physiological geometry for phosphorylation by Src-family kinases, 1 µM His10 receptor tails (either His10-Draper-INT or His10-Draper-ΔITAM-INT) were ligated to liposomes comprised of the following molar percent: 74.5% POPC, 10% DGS-NTA, 0.5% rhodamine-PE, and 15% DOPS. Liposomes were prepared using an extruder with 200-nm filters (Avanti). Receptor tails and 1 nM His10-Lck-Y505F or 86 nM Src42a (soluble) were equilibrated with receptor tails in the presence of BG505-kinase-SH2 reporter proteins. Proteins were allowed to bind for 40 min at room temperature in the dark. At this point, Mg-ATP was added to a 1 mM final concentration, and BG505 signal was assessed at the indicated time points. Quenching of BG505 signal, dependent upon addition of ATP, indicates recruitment of reporters to phosphorylated receptor tails. Quenching of the BG505 dye was assessed using a Synergy H4 plate reader (BioTek).

Determining sites of Tyr phosphorylation by 2D mass spectrometry
To determine sites of Tyr phosphorylation on Draper (Fig. 5), 1 µM His10-Draper-INT was ligated to unlabeled liposomes (74.5% POPC, 10% DGS-NTA, and 15% DOPS) and phosphorylated by 1 nM His10-Lck-Y505F after 40 min of protein coupling to liposomes. 100-µl reactions were quenched with 100 µl of 8 M urea and 20 mM EDTA at the indicated time points (final concentrations of 4 M urea and 10 mM EDTA) and shock frozen in liquid nitrogen. To perform trypsinization, reactions were thawed by boiling, supplemented with a 5 mM final concentration of Tris(2-carboxyethyl)phosphine (TCEP), and incubated for 20 min at room temperature. After denaturation of samples, a 10 mM final concentration of iodoacetamide was added. Samples were then incubated in the dark for 15 min. Urea concentration was reduced twofold through addition of 200 µl PBS. A 1 mM final concentration of CaCl2 and 1 µg/ml Trypsin Gold (Promega) was added before samples were incubated overnight in the dark at 37°C. Tryptsinization was quenched through addition of 50 µl mass spectrometry-grade formic acid. Samples were then subjected to 2D mass spectrometry to remove contaminants, and peptides containing the characteristic shift for phosphorylation (+79.9663 D) were quantified and expressed as a percentage of all peptides covering the indicated residue.

Immunoblot analysis
To determine the degree of Tyr phosphorylation on His10-Draper intracellular domains, liposome phosphorylation assays were performed as above, quenched using SDS-PAGE buffer + DTT, and incubated for 5 min at 95°C. Reactions were run on 4–20% gradient SDS-PAGE gels and transferred onto nitrocellulose membranes using the iBlot system (Invitrogen). Membranes were blocked for 1 h at room temperature in PBS + 0.1% wt/vol BSA (Sigma-Aldrich) and incubated overnight at 4°C with anti-His probe (rabbit) and anti-pTyr (mouse) antibodies (Table S1). Primary antibodies were incubated with membranes simultaneously. Both were used at 1:1,000 final dilution PBS + 0.1% wt/vol BSA. Membranes were washed 3 × 5 min at room temperature in PBS + 0.1% wt/vol BSA and incubated with Li-Cor secondary antibodies at 1:10,000 dilution (700 nm anti-rabbit and 800 nm anti-mouse; Table S1) for 1 h at room temperature. Membranes were washed 3 × 5 min at room temperature in PBS + 0.1% wt/vol BSA and imaged using a Li-Cor Odyssey gel imager. Quantification of anti-pTyr and anti-His intensity was performed using the Gel Analysis feature of Fiji (ImageJ; National Institutes of Health).

Protein expression, purification, and labeling
His10-Draper and His10-Draper-ΔITAM were transformed into BL-21 DE3 RIPL cells (Agilent). Cells were grown in Terrific Broth, 12 g tryptone, 24 g yeast extract, and 4 ml glycerol up to 1 liter in Milli-Q water + 10% vol/vol sterile salt solution in Milli-Q water (0.17 M KH2PO4 and 0.72 M K2HPO4) until cells reached an OD260 of ~0.6–0.8, chilled in a 4°C room, and induced overnight by addition of 0.5 mM final concentration of IPTG. Protein expression was performed in shaker set to 18°C. Bacterial pellets were resuspended in lysis buffer (50 mM Hepes, pH 7.5, 300 mM NaCl, and 1 tablet Complete EDTA-free protease inhibitor tablet crushed per 100 ml buffer). Lysates were prepared by probe sonication and bound to Ni-NTA agarose (Qiagen). Beads were washed extensively with lysis buffer, and proteins were eluted in 500 mM imidazole in lysis buffer. Imidazole elutions were subjected to gel filtration chromatography using a Superdex 200 10/30 column (GE Healthcare) in-gel filtration buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, and 1 mM TCEP). Monomer fractions were pooled, shock frozen in liquid nitrogen, and stored in small aliquots at ~80°C. Soluble Src42a was purified from SF9 cells as a GST-(PP)-SNAP-Src42a fusion, where PP indicates a cut site for PreScission Protease. PreScission Protease cleavage was performed overnight, and postcleavage supernatants were gel filtered as described below. His10-ΔLck Y505F was purified from SF9 cells over nickel agarose (Qiagen) and eluted in 500 mM imidazole in lysis buffer. His10-Src42a was purified from Escherichia coli in the presence of GST-YopH to dephosphorylate the kinase. Imidazole elution was then run over GST resin (GE Healthcare) to remove the YopH phosphatase. All Src family
kinases were subjected to gel filtration chromatography using a Superdex 200 10/30 column (GE Healthcare) in gel filtration buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, and 1 mM TCEP). Monomer fractions were pooled, shock frozen in liquid nitrogen, and stored in small aliquots at −80°C. The tandem SH2 domain reporter for Shark kinase was cloned as a GST-(PP)-SNAP-Shark-tSH2 fusion, where PP indicates a cut site for PreScission Protease. The soluble SNAP-Shark-tSH2 reporter was generated by on-resin cleavage with PreScission Protease during purification. GST-PreScission Protease remained on the beads. Cleavage products were subjected to gel filtration chromatography using a Superdex 200 10/30 column (GE Healthcare) in gel filtration buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% glycerol, and 1 mM TCEP). To prepare BG505-labeled SNAP-Shark-tSH2 for the liposome fluorescence resonance energy transfer assay, 10 μM cleaved, gel-filtered monomer was incubated at a 1:2 molar ratio with SNAP-Cell 505 Star (NEB) overnight in the dark at 4°C and run over a PD MiniTrap G-25 column (GE Healthcare) to eliminate excess dye.

**Spinning-disk confocal microscopy**

All internalization and bead colocalization imaging performed for this study was done using a spinning-disk confocal microscope (Nikon Ti-Eclipse inverted microscope with a Yokogawa spinning disk). For bead internalization kinetic assays, images were acquired using a 40×/0.95 NA air objective. Live-image acquisition for internalization of corpses and beads was performed using the High Content Screening Site Generator plugin in µManager. All other images were acquired using a 100× 1.49 NA oil-immersion objective. Images were captured using an Andor iXon electron-multiplying charged-device camera. The open-source µManager software package was used to run the microscope and capture the images (Edelstein et al., 2010). All raw microscopy images were acquired as 16-bit TIFF stacks.

**TIRF-M**

All SLB imaging was performed using a TIRF microscope equipped with a motorized TIRF arm and a Hamamatsu Flash 4 camera. All TIRF-M imaging images were captured at 2 × 2 binning. The open-source µManager software package was used to run the microscope and capture the images (Edelstein et al., 2010).

**Imaging processing and analysis**

All image quantification was done on raw unprocessed images. All images in figures were opened in ImageJ. A middle z slice was extracted and the channels split. The image intensities were scaled to enhance contrast in Photoshop (Adobe) using appropriate levels of linear adjustment. Background correction was not performed. When mutants were compared in the same series, images were acquired using identical microscope and camera settings and scaled to equal intensity values.

**Quantification and statistical analysis: Corpse and bead internalization assays**

For both corpse internalization and bead internalization assays, targets were scored as internalized following complete engulfment of the object based on visible mCherry-CAAX or receptor-GFP signal visible completely around the target in stills from live imaging. Beads or corpses attached, but not internalized, were not scored as engulfed. Only targets with visible APC Annexin V (corpses) or attoPE lipid (beads) were scored as internalized. At least 100 cells per condition are shown. Mean ± 1 SD values are plotted (Prism 6.0; GraphPad Software).

**Quantification and statistical analysis: Draper microcluster and actin retrograde flow velocities**

Quantification of microcluster velocity was performed in MicroManager (Edelstein et al., 2010). Microcluster velocity was calculated by measuring the distance a single cluster traveled over a 100-s TIRF-M video captured at 5-s intervals. Quantification was performed on the same cell population used to calculate the velocity of actin retrograde flow (Draper-GFP, LifeAct-TagBFP transient transfection settling on 10% PS SLBs). A single channel (Draper-GFP) maximum-intensity z projection was created from a substack of 20 intervals. The freehand line tool was used to measure distance traveled by n = 26 microclusters on the maximum-intensity z projection. The reported velocity in the text is the mean for n = 26 individual microclusters; the microcluster velocity observed was 1.42 ± 0.45 μm/min (mean ± SD).

Quantification of actin retrograde flow velocity was performed in MicroManager (Edelstein et al., 2010). Quantification of actin retrograde flow was performed on the same cell population used to calculate the velocity of Draper microclusters above (Draper-GFP and LifeAct-TagBFP). To calculate actin retrograde flow, cells were spread on Concanavalin A so actin flow could be clearly visualized using TIRF-M. Actin retrograde flow velocities were calculated using a kymograph selected from the lamellar portion of the cell perpendicular to the leading edge. The reported velocity in the text is the mean for n = 25 individual measurements; the velocity observed was 1.38 ± 0.40 μm/min (mean ± SD).

**Quantification and statistical analysis: Quantification of Draper, Shark, and LifeAct intensity**

Quantification of normalized fluorescence intensity was performed in MicroManager (Edelstein et al., 2010). Intensity for each indicated channel was collected across a 2-pixel linescan for each indicated time point using the Plot Profile tool in ImageJ. Intensity measurements were performed in the same manner for TIRF-M and spinning-disk confocal imaging. Background was measured as the mean value for a 2-pixel linescan at a region adjacent to the cell. Background was subtracted from intensity at each position before normalization. Values were normalized to the maximum intensity across the linescan. Normalized intensity values are plotted using distance along the transect as the x axis. Transects are indicated on inset images for each cluster.

**Quantification and statistical analysis: Quantification of Draper immunofluorescence intensity**

Quantification of immunofluorescence intensity using the anti-Draper 81A antibody was performed in MicroManager (Edelstein et al., 2010). The data in Fig. S1 A represent the corrected total cell fluorescence (CTCF) values for each cell corresponding with the condition shown on the x axis.
Spatial control of engulfment signaling

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The supplemental figures contain supporting microscopy images and information directly in support of the main-text figures. Full legends accompany each supplemental figure. Fig. S1 supports Fig. 1 by comparing the subcellular localization between endogenous Draper and Draper-GFP and showing the fold overexpression in the Draper-GFP transfection system. We also characterize the system by investigating the bead diameter dependence and Shark requirement. This figure further supports Fig. 1 by demonstrating both a Draper and PS dependence for efficient engulfment as well as initiation of pTyr signaling and projections around a PS bead. Fig. S2 supports Fig. 2 by showing that the engulfment effectors that localize to signaling microclusters are also recruited to Draper at the synapse with a 10% PS bead. Fig. S3 supports Fig. 2 by showing that actin depletion behavior is observed in the bead assay. This figure also supports Fig. 2 by demonstrating that disruption of actin dynamics using the drug jasplakinolide disrupts early steps of Draper-dependent engulfment. Fig. S4 supports Fig. 5 by demonstrating that Draper’s ITAM is necessary and sufficient for Shark kinase recruitment in cells. Fig. S5 supports Fig. 5 by showing that like Src42a, the Src family kinase Lck preferentially modifies Draper based on the presence of an ITAM. This figure also supports Figs. 2 and 5 by showing that the N-terminal SH2 domain of p85 does not directly bind Draper’s cytoplasmic tail in an on-liposome biochemical assay. The complete catalog information for reagents and tools used in this study can be found in Table S1. Detailed information for all recombinant DNA materials can be found in Table S2. Video 1 shows cellular reconstitution of Draper signaling. Video 2 shows formation of mobile Draper-GFP microclusters on 10% PS bilayers. Video 3 shows visualization of signaling microclusters on 10% PS bilayers. Video 4 shows split channel visualization of Draper, Shark, and F-actin on 10% PS bilayers. Video 5 shows visualization of FKBP-bead engulfment by a chimeric FRB-Draper receptor. Video 6 shows visualization of FRB-EXT-Draper-INT and LifeAct in an FKBP SLB.

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CCTF = integrated density − (area of cell × mean background signal for the same image) for the 647 anti–mouse secondary antibody. We chose CTCF because it is an area-corrected analysis method that takes into account the background in each image.

Online supplemental material

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