

The adaptor protein GULP promotes Jedi-1–mediated phagocytosis through a clathrin-dependent mechanism

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ABSTRACT During the development of the peripheral nervous system, the large number of apoptotic neurons generated are phagocytosed by glial precursor cells. This clearance is mediated, in part, through the mammalian engulfment receptor Jedi-1. However, the mechanisms by which Jedi-1 mediates phagocytosis are poorly understood. Here we demonstrate that Jedi-1 associates with GULP, the mammalian homologue of CED-6, an adaptor protein required for phagocytosis mediated by the nematode engulfment receptor CED-1. Silencing GULP or mutating the NPXY motif in Jedi-1, which is required for GULP binding, prevents Jedi-1–mediated phagocytosis. How GULP promotes engulfment is not known. Of interest, we find that Jedi-1–induced phagocytosis requires GULP binding to clathrin heavy chain (CHC). During engulfment, CHC is tyrosine phosphorylated, which is required for Jedi-mediated engulfment. Both phosphoclathrin and actin accumulate around engulfed microspheres. Furthermore, knockdown of CHC in HeLa cells prevents Jedi-1–mediated engulfment of microspheres, and knockdown in glial precursors prevents the engulfment of apoptotic neurons. Taken together, these results reveal that Jedi-1 signals through recruitment of GULP, which promotes phagocytosis through a noncanonical phosphoclathrin-dependent mechanism.

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INTRODUCTION

Apoptosis is a normal part of development for all multicellular organisms, as it is a means of eliminating unnecessary or defective cells, establishing proper cell numbers, and sculpting tissues. In vertebrates, ~50% of the neurons generated undergo apoptosis (Burek and Oppenheim, 1996), and removal of these corpses is a vital step in preventing secondary necrosis, which can lead to inflammatory

response and possibly autoimmunity (Elliott and Ravichandran, 2010; Nagata *et al.*, 2010). We previously reported that glial cell precursors act as “nonprofessional” phagocytes to clear the heavy burden of apoptotic neurons during the development of the peripheral nervous system (Wu *et al.*, 2009). This phagocytosis is mediated, in part, through the engulfment receptor Jedi-1 (Wu *et al.*, 2009), a mammalian homologue of the *Drosophila melanogaster* receptor Draper and the *Caenorhabditis elegans* receptor CED-1. Jedi-1 and Draper signal engulfment through recruitment of the tyrosine kinase Syk (Scheib *et al.*, 2012) or Shark (Ziegenfuss *et al.*, 2008), respectively, which binds to an immunoreceptor tyrosine-based activation motif (ITAM) within the intracellular domain of the receptor.

Draper (Awasaki *et al.*, 2006) and CED-1–mediated (Zhou *et al.*, 2001; Su *et al.*, 2002) engulfment also depends on the adaptor protein CED-6, which binds to the NPXY motif in the receptor’s intracellular domain. Jedi-1, like CED-1 and Draper, contains an NPXY sequence, but the importance of this motif in Jedi-1 signaling has not been established. The mammalian homologue of CED-6, GULP,

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Abbreviations used: CHC, clathrin heavy chain; DRG, dorsal root ganglia; GFP, green fluorescent protein; GST, glutathione S-transferase; HA, human influenza hemagglutinin; ITAM, immunoreceptor tyrosine-based activation motif; MEF, mouse embryonic fibroblast; NGF, nerve growth factor; PI, phagocytic index; PNS, peripheral nervous system; PTB, phosphotyrosine-binding domain.

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has been shown to bind to the NPXY motif of other mammalian engulfment receptors, including stabilin-1 (Park *et al.*, 2010), stabilin-2 (Park *et al.*, 2008), and LRP-1 (Su *et al.*, 2002). However, how CED-6/GULP facilitates engulfment is largely unknown.

Recently CED-6 was reported to act as a clathrin adaptor protein necessary for yolk uptake in *Drosophila* egg chambers (Jha *et al.*, 2012). CED-6 associated with the vitellogenin receptor Yolkless and was also found to bind to clathrin heavy chain (CHC), thereby facilitating clathrin-dependent endocytosis. GULP was also shown to be a positive regulator of Arf6, a GTPase implicated in promoting clathrin-mediated endocytosis by recruiting AP-2 to the cell membrane (Ma *et al.*, 2007). Despite these observations, clathrin is generally not considered to contribute to phagocytosis due to the size limitation of clathrin-coated vesicles. However, clathrin was recently reported to act as a scaffold for actin remodeling, which was required for the internalization of pathogenic bacteria (Bonazzi *et al.*, 2011). Bacterial adhesion to the host cell induced the phosphorylation of CHC, which was required for the recruitment of actin. The formation of this clathrin-actin network was necessary for bacterial internalization. Similarly, clathrin has been implicated in the internalization of certain large viruses (Ehrlich *et al.*, 2004; Rust *et al.*, 2004) and fungi (Moreno-Ruiz *et al.*, 2009). Taken together, these results led us to hypothesize that Jedi-1 mediates engulfment through the recruitment of GULP, which then associates with clathrin to facilitate phagocytosis.

RESULTS

GULP interacts with the NPXY motif of Jedi-1

Previous studies demonstrated that CED-6/GULP binds to the NPXY motif of CED-1 (Su *et al.*, 2002) and Draper (Awasaki *et al.*, 2006), as well as to a number of mammalian receptors involved in phagocytosis (Su *et al.*, 2002; Hamon *et al.*, 2006; Park *et al.*, 2008, 2010). Therefore we investigated whether Jedi-1 also associates with GULP. Using lysates from mouse dorsal root ganglia and spinal cord at embryonic day 13.5 (E13.5), a time at which apoptotic cell death of neurons occurs during development (Farinas *et al.*, 1996), we found that endogenous GULP coimmunoprecipitated with Jedi-1 (Figure 1A). To determine whether the NPXY motif of Jedi-1 was required for GULP binding, we generated a Jedi-1 construct with

the NPXY motif (⁷⁸⁸NPSY) mutated to APXA. Wild-type Jedi-green fluorescent protein (GFP) or APXA mutant Jedi-GFP was transiently transfected into mouse embryonic fibroblasts (MEFs) or MEFs that stably express GST-GULP. On pull down of GST-GULP, an interaction with wild-type Jedi-1 but not the APXA mutant was observed by Western blot (Figure 1B). We also tested a Jedi-1 construct with the tyrosine residues (923, 941, 1004, 1016) in the ITAM domains, where Syk binds (Scheib *et al.*, 2012), mutated to phenylalanine to determine whether this domain was required for association with GULP. In contrast to the NPXY sequence in Jedi-1, mutation of the ITAM motifs did not affect interaction with GULP.

The NPXY motif of Jedi-1 is required for engulfment

To assess the functional significance of the NPXY motif during phagocytosis, we used a microsphere engulfment assay using HeLa cells. GFP-tagged wild-type Jedi-1 or Jedi-1 with the NPXY domain mutated (either to APXA or NPXF) was expressed in HeLa cells, and cells were exposed to 2- μ m carboxylate-modified fluorescent microspheres, which mimic certain features of apoptotic cells, for 2 h. Uptake of microspheres was analyzed by confocal microscopy (Figure 2A), and the percentage of transfected cells with at least one microsphere fully engulfed (as determined based on a confocal z-stack) was determined. In comparison to GFP-transfected control cells, Jedi-1 expression significantly increased the engulfment of microspheres (Figure 2, B and C). We also calculated the phagocytic index (PI) of the HeLa cells using the formula $PI = (\text{total number of engulfed beads} / \text{total number of counted cells}) \times (\text{number of cells containing engulfed beads} / \text{total number of counted cells}) \times 100$. GFP-transfected HeLa cells had a PI of 0.49, whereas Jedi-GFP-transfected cells had a PI of 15.70. As previously observed (Scheib *et al.*, 2012), Jedi-GFP-transfected cells typically engulf no more than one to three beads. However, neither of the NPXY mutants exhibited any engulfment capability (Figure 2, B and C). These findings indicate that the NPXY motif is essential for Jedi-mediated engulfment of microspheres, consistent with its requirement for GULP binding.

To determine the importance of the NPXY motif in Jedi-1-mediated engulfment of apoptotic neurons, we cocultured sensory neurons and glial precursors from E13.5 mouse dorsal root ganglia (DRG) and transfected wild-type Jedi-1-GFP or APXA mutant Jedi-1-GFP into the glial precursor cells. Nerve growth factor (NGF), initially added to promote neuronal survival, was then removed to induce neuronal apoptosis, and after 2 d, confocal microscopy was used to determine the percentage of GFP-positive glial cells that were engulfing at least one apoptotic body. In accordance with our previous results (Wu *et al.*, 2009; Scheib *et al.*, 2012), overexpression of Jedi-1 in glial precursor cells increased the engulfment of dead neurons. In contrast, expression of the APXA mutant Jedi-1 did not increase engulfment above basal levels, demonstrating an essential role for the NPXY motif in apoptotic cell clearance (Figure 2D).

GULP is essential for Jedi-1-mediated engulfment

The requirement for GULP in Jedi-1-mediated engulfment was investigated by transfecting Jedi-1 or the APXA mutant of Jedi-1

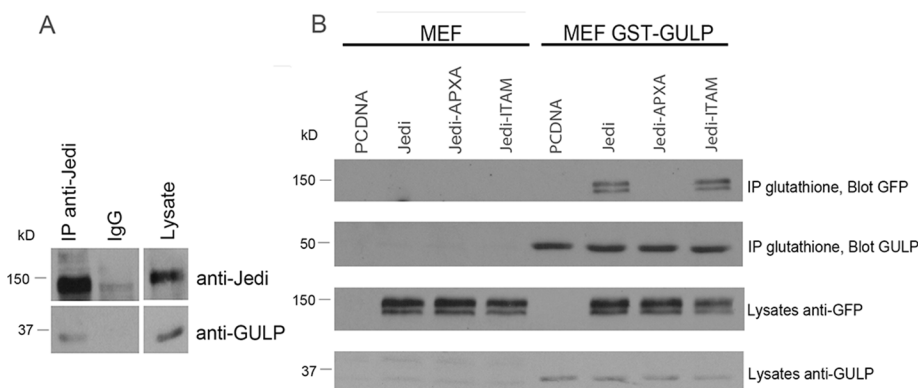


FIGURE 1: Jedi-1 interacts with GULP through its NPXY motif. (A) Endogenous Jedi was immunoprecipitated from lysates of E13.5 dorsal root ganglia and spinal cord, and the proteins were separated by SDS-PAGE and immunoblotted with an antibody to Jedi-1 or CED-6 (GULP). Representative blot of three experiments. (B) Jedi-1-GFP and Jedi-1-GFP with the NPXY motif mutated to APXA or the four ITAM tyrosines mutated to phenylalanine were transiently transfected into control MEF cells or MEF cells stably expressing GST-GULP. GST-GULP was pulled down with glutathione beads, and anti-GFP was used to detect coimmunoprecipitation of Jedi-1 by Western blot ($n = 3$).

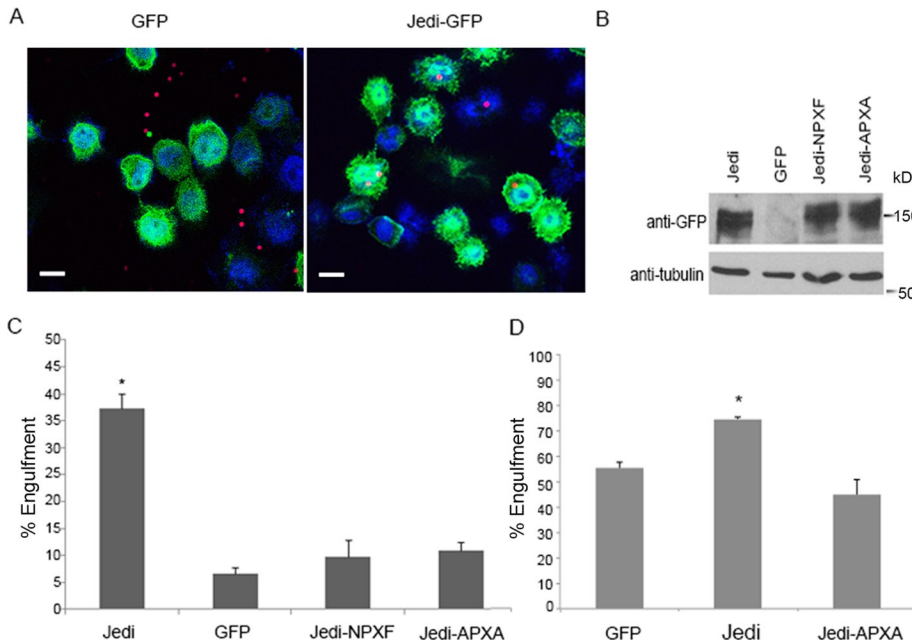


FIGURE 2: The NPXY motif of Jedi-1 is required for engulfment. Jedi-1-GFP, Jedi-1-GFP with the NPXY mutated to NPXF or APXA, or GFP only was transfected into HeLa cells, and 2 d later, fluorescent microspheres were added for 2 h before rinsing and fixing the cells. The cells were imaged by confocal microscopy to ensure complete internalization. (A) Confocal images with Jedi-1-GFP- or GFP-expressing cells (green) with microspheres (red). Cell nuclei are shown in blue. Scale bars, 10 μ m. (B) Expression of Jedi-1-GFP and mutant Jedi-1-GFP confirmed by immunoblotting. (C) Percentage of transfected HeLa cells engulfing at least one microsphere was quantified (Student's *t* test, $p = 0.00015$ for Jedi-1 relative to GFP; $p = 0.0003$ for Jedi-1 NPXF and $p = 0.0004$ for Jedi-1 APXA relative to wild type Jedi-1; $n = 3$). (D) GFP, Jedi-1-GFP, or APXA mutant Jedi-1-GFP was transfected into glial cells in cocultures of E13.5 DRG neurons and glia in the presence of NGF. NGF was withdrawn to induce neuronal death, and 48 h later the cultures were fixed and immunostained with anti-GFP and nuclei labeled with TO-PRO-3. The percentage of transfected glia (GFP positive) engulfing at least one apoptotic body (based on condensed TO-PRO-3 staining) was quantified by confocal analysis (Student's *t* test, $p = 0.002$ for Jedi-1 relative to GFP, $p = 0.007$ for Jedi-1-APXA relative to wild-type Jedi-1; $n = 3$).

into control MEFs, MEFs with GULP constitutively knocked down, or MEFs with GULP knocked down but stably transfected with a knock-down-resistant glutathione *S*-transferase (GST)–GULP construct. The transfected cells were incubated with carboxylated fluorescent microspheres and evaluated by confocal microscopy to determine the percentage of cells that had engulfed the spheres. As observed in HeLa cells, expression of the APXA mutant of Jedi-1 did not increase engulfment above the GFP control. Of note, knockdown of GULP in cells expressing wild-type Jedi-1 significantly decreased the engulfment of spheres, consistent with an essential role for GULP in Jedi-1 signaling, and this decrease in engulfment could be rescued by expression of GST-GULP (Figure 3, A and B).

To determine whether GULP is required for phagocytosis of apoptotic neurons by glial cells, which depends on endogenous Jedi-1 (Wu *et al.*, 2009), we knocked down GULP in glial cells cocultured with DRG neurons. After NGF removal, the percentage of transfected glia that engulfed an apoptotic body was quantified. We found that GULP knockdown significantly reduced engulfment of the apoptotic neurons by 43% in comparison to cells transfected with GFP (Figure 4). In addition, we calculated the PI of the glial cells. Control short hairpin RNA (shRNA)–transfected glial cells had a PI of 44.64, whereas GULP shRNA transfected cells had a PI of 12.24. This is consistent with previous studies (Scheib *et al.*, 2012), in which we observed that glial cells typically engulf between one and three apoptotic cell fragments.

GULP is required for Jedi-1 internalization

Phagocytosis is a complex, multistep process involving recognition of the body to be engulfed, binding, internalization, maturation of the phagosome, and eventual lysosomal degradation of the engulfed material. Because NPXY motifs are often involved in the internalization of cell surface proteins (Bonifacino and Traub, 2003), we hypothesized that the NPXY motif in Jedi-1 and the association with GULP are required for the internalization process. To monitor internalization of Jedi-1 in response to exposure to the microspheres, we used a reversible biotinylation system in which surface proteins were biotinylated. After addition of the microspheres for various times, the surface-bound biotin was removed by treatment with the reducing agent dithiothreitol (DTT). The internalized, biotinylated proteins were then detected by avidin pull down and Western blotting. We found that within 30 min of exposing HeLa cells that express Jedi-1 to the microspheres, internalized Jedi-1 could be detected; however, there was only marginal internalization of the APXA mutant detected, even after 90 min (Figure 5, A and B). These results indicate that the NPXY motif is essential for internalization of Jedi-1 in response to stimulation with carboxylated microspheres.

Because the NPXY motif in Jedi-1 is required for association with GULP, we sought to investigate directly whether GULP is necessary for Jedi-1 internalization, using the same reversible biotinylation assay with

control MEFs or MEFs with GULP knocked down. The MEFs were transfected with Jedi-1 and exposed to microspheres for 90 min. Jedi-1 was internalized in the microsphere-exposed control MEFs, but no internalization was detected in the cells with GULP knocked down (Figure 5C), indicating that GULP is required for Jedi-1 internalization.

GULP association with clathrin is required for Jedi-1–mediated engulfment

Increasing evidence suggests that NPXY motifs are recognized by phosphotyrosine-binding domain (PTB)–containing proteins, such as Numb, ARH, Dab1, and Dab2, which work with AP-2 to promote clathrin recruitment and assembly (Traub, 2003). Furthermore, CED-6, which has a PTB domain, was shown to be a clathrin adaptor protein in *Drosophila* required for yolk endocytosis (Jha *et al.*, 2012). It was also demonstrated that mammalian GULP could bind clathrin heavy chain (CHC). Therefore we hypothesized that GULP may be an essential adaptor protein to promote Jedi-1–mediated phagocytosis by recruiting clathrin. By transfecting hemagglutinin (HA)–GULP into HeLa cells, we confirmed the interaction with CHC by coimmunoprecipitation (Figure 6A). The interaction between CED-6 and CHC required the C-terminal portion of CED-6, outside of the PTB domain, where there is a conserved DLF sequence required for CHC binding (Jha *et al.*, 2012). GULP also contains a DLF triplet in its C-terminal region, and deletion of the C-terminal 71 amino acids,

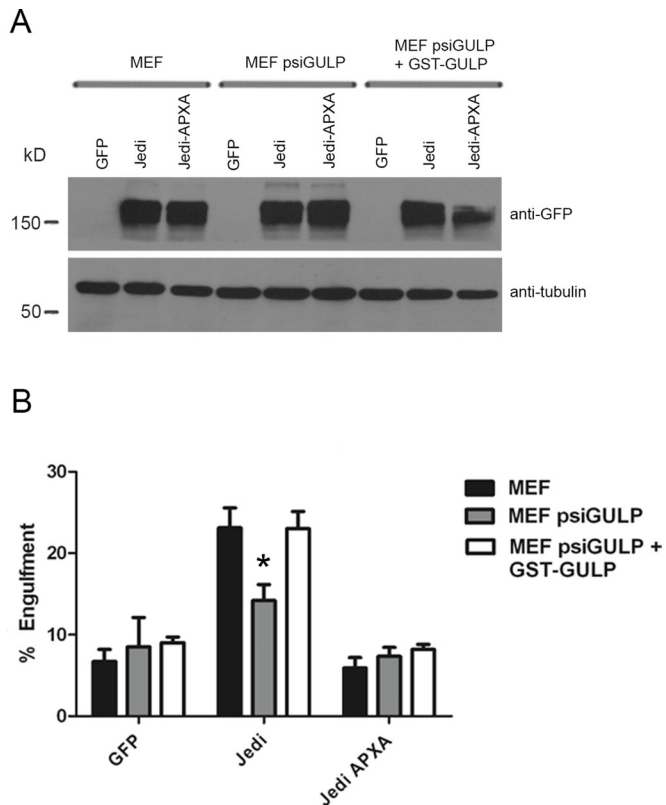


FIGURE 3: GULP is required for Jedi-1–mediated engulfment. Control MEFs, MEFs with GULP stably knocked down (psiGULP), or MEFs with endogenous GULP silenced but stably transfected with a knockdown-resistant GULP (psiGULP+GST-GULP) were transfected with GFP, Jedi-1-GFP, or the NPXY/APXA mutant of Jedi-GFP. (A) The cells were lysed, and the proteins were separated by SDS–PAGE and Western blotted for GFP (to detect Jedi-1) or tubulin. (B) Fluorescent microspheres were added to the cells, and after 2 h the cells were fixed and engulfment of microspheres was analyzed by confocal microscopy and quantified to determine the percentage of transfected cells (GFP positive) engulfing at least one microsphere (by two-way analysis of variance with a Bonferroni posthoc analysis; $p < 0.001$ for MEFs with wild-type Jedi-1 relative to MEFs with GULP knockdown–expressing wild-type Jedi-1; $p < 0.001$ for MEFs with GULP knocked down with wild-type Jedi-1 relative to MEFs with GULP knocked down and rescued with GST-GULP with wild-type Jedi).

including this sequence, abolished the association with CHC (Figure 6A). Of importance, deletion of the C-terminal domain did not disrupt GULP binding to Jedi-1 (Figure 6B), thereby allowing use of this mutant to explore the functional importance of GULP association with CHC.

To determine whether the interaction between GULP and CHC was necessary for Jedi-1–mediated engulfment, we expressed the C-terminal deletion mutant of GULP in MEFs with GULP constitutively knocked down and measured Jedi-1–mediated phagocytosis of microspheres. Control MEFs or MEFs with GULP knocked down were transfected with GFP or Jedi-1-GFP with or without HA-GULP or the C-terminal GULP mutant. Full-length GULP rescued the engulfment, but the clathrin-binding–deficient mutant of GULP did not (Figure 6C).

To further explore the requirement for clathrin in Jedi-1-mediated engulfment, we assessed the effects of knocking down CHC. HeLa cells expressing Jedi-1 were transfected with shRNA to CHC,

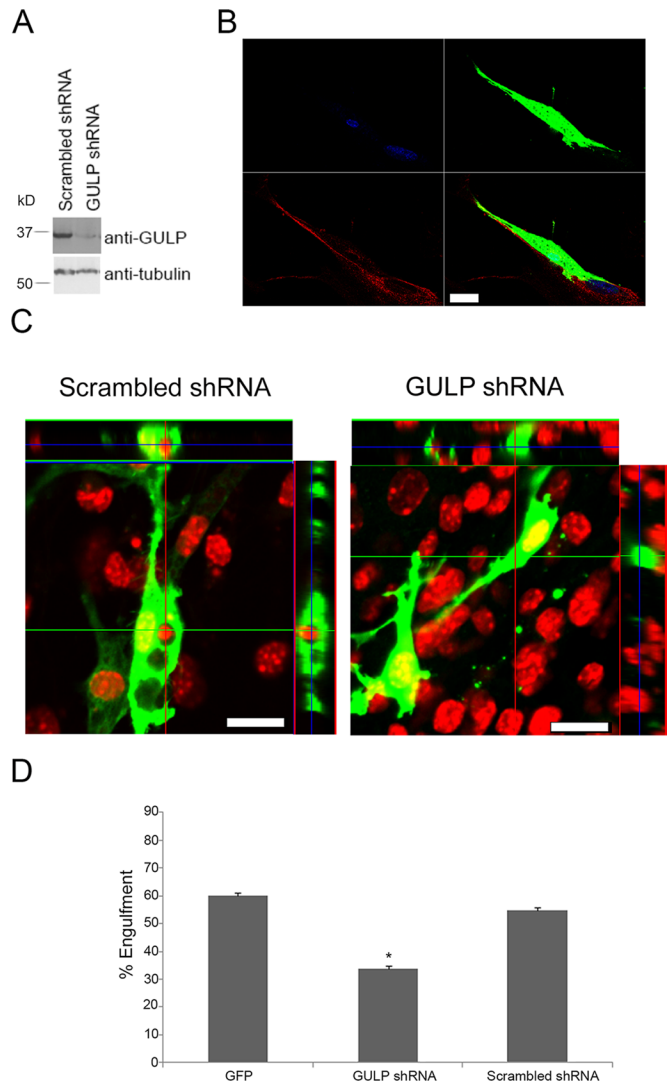


FIGURE 4: Knockdown of GULP reduces the engulfment of neurons by glial precursors. (A) Nontargeting or GULP shRNA was transfected into 3T3 cells. Lysates were subjected to Western blotting with antibodies to GULP and tubulin. (B) Knockdown of GULP was also verified by immunostaining glial cell precursors transfected with GULP shRNA for GFP to identify transfected cells (green) and GULP (red). Nuclei were stained with TO-PRO-3. (C) Representative confocal images of cocultures of E13.5 DRG neurons and glial precursor cells transfected with nontargeting or GULP shRNA (coexpressing GFP). On the day of transfection, neuronal death was induced by removing NGF. After 48 h, the cultures were fixed and immunostained with anti-GFP and nuclei labeled with TO-PRO3. Transfected glia are depicted in green and nuclei in red. Scale bars, 20 μm . The cross-hair in the orthogonal view of the cell transfected with scrambled shRNA is positioned on an engulfed apoptotic nuclear fragment, and the cross-hair in the GULP shRNA–transfected image is positioned on the cell body of a glial cell containing no apoptotic nuclear fragments. (The rectangle on top of the images depicts the xz-plane, and to the right is the yz-plane.) (D) Images from transfected cocultures were analyzed by confocal microscopy. The percentage of transfected glia engulfing TO-PRO-3 stained apoptotic nuclear fragments was quantified ($p = 0.0005$ for GULP shRNA relative to GFP only, by Student's *t* test; $n = 3$).

and the engulfment of microspheres was analyzed. There was a significant 39.9% reduction in engulfment in cells with reduced levels of CHC (Figure 7C). Similarly, silencing CHC in glial cells reduced

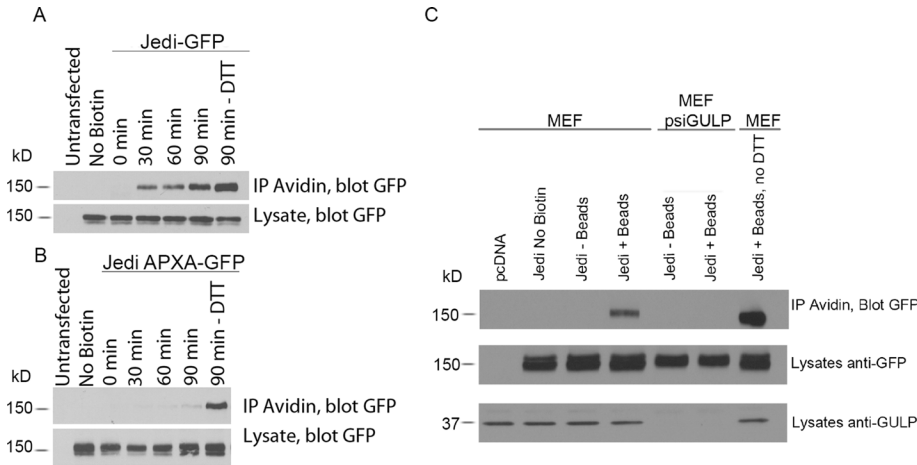


FIGURE 5: The NPXY motif and GULP are required for internalization of Jedi-1. Time course for internalization of Jedi-1-GFP (A) or Jedi-1-GFP APXA mutant (B) after addition of microspheres. HeLa cells were transfected with wild-type or APXA mutant Jedi-1-GFP. Surface proteins were biotinylated with EZ-Link Sulfo-NHS-SS-Biotin at 4°C and then exposed to fluorescent microspheres and warmed to 37°C for the time indicated. The biotinylation of surface proteins was then reversed using the reducing agent DTT. The cells were lysed, and internalized, biotinylated Jedi-1 or mutant Jedi-1 was pulled down with avidin-conjugated agarose beads and detected by immunoblotting with a GFP antibody. Total levels of Jedi-1 are shown in lysates ($n = 3$). (C) Control MEFs or MEFs with GULP knocked down (MEF psiGULP) were transfected with Jedi-1-GFP. The cells were then exposed to microspheres or left untreated for 90 min, and the internalized Jedi-1 was detected as in A and B. Levels of Jedi-1-GFP and GULP are shown in the lysates.

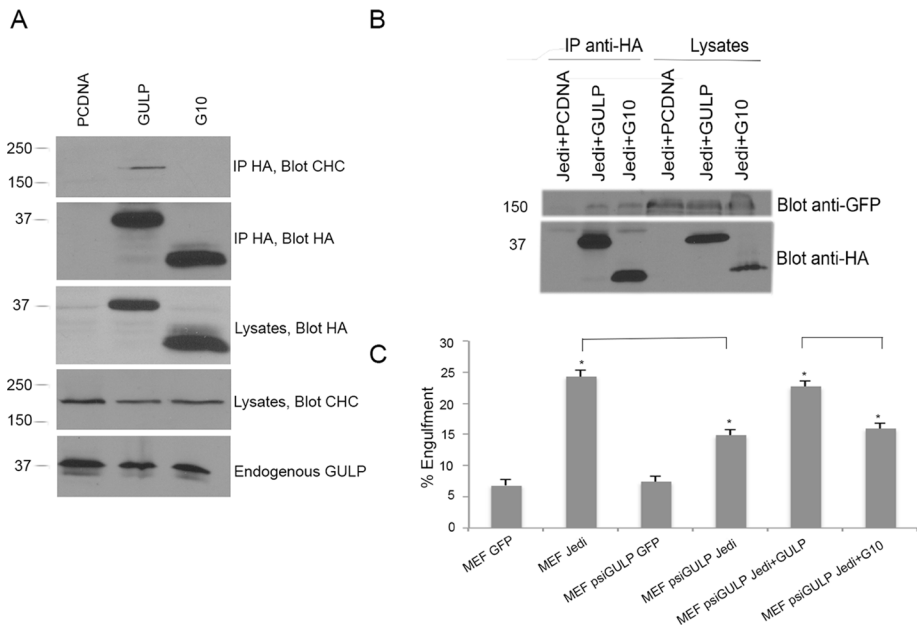


FIGURE 6: GULP interacts with clathrin, and this interaction is required for Jedi-1-mediated engulfment. (A) HeLa cells were transfected with pcDNA3, HA-GULP, or HA-GULP lacking the C-terminal 71 amino acids (G10). After 2 d, the cells were lysed, immunoprecipitated with HA antibody, and subjected to Western blotting with antibodies to HA or CHC. Endogenous GULP levels were analyzed by immunodepleting the lysates of HA-GULP using two subsequent rounds of immunoprecipitation with HA antibody and blotting the supernatant lysate with an antibody that recognizes endogenous GULP. A representative blot ($n = 3$). (B) HeLa cells transfected with Jedi-1-GFP and pcDNA3, HA-GULP or HA-G10 were lysed and immunoprecipitated with an antibody to HA and blotted with an antibody to GFP to detect coimmunoprecipitated Jedi-1. The lysates were subjected to Western blotting using antibodies to HA (to detect GULP) or GFP (to detect Jedi-1). (C) Control MEFs or MEFs with GULP constitutively knocked down (MEF psiGULP) were transfected with GFP, Jedi-1-GFP, or Jedi-1-GFP and HA-GULP or HA-G10. Two

their ability to phagocytose dead neurons in cocultures of DRG cells (Figure 7, D and E).

Jedi-1 colocalizes with phosphorylated clathrin and actin during engulfment

It was reported that CHC becomes tyrosine phosphorylated and accumulates around sites of bacterial adhesion. This phosphorylation promotes internalization by acting as a scaffold for actin polymerization (Bonazzi *et al.*, 2011). After discovering that CHC was required for Jedi-1-mediated engulfment, we examined its phosphorylation status during Jedi-1-mediated phagocytosis using a phospho-CHC antibody (Bonazzi *et al.*, 2011; Figure 8, A and B). MEFs expressing Jedi-1 were exposed to microspheres, and after 2 h, we observed a significant increase in phospho-CHC (Figure 8). However, when GULP was knocked down, there was a marked decrease in the level of CHC phosphorylation. Reexpression of a knockdown-resistant GULP rescued the inducible phosphorylation of CHC. The results were confirmed by immunoprecipitating CHC and blotting for total phosphotyrosine (Figure 8C).

To determine whether phospho-CHC and actin accumulated around engulfed microspheres, we assessed their localization in MEFs expressing Jedi-1 during phagocytosis. After confirming the specificity of the phospho-CHC antibody for immunostaining (Supplemental Figure S1), we found that Jedi-1 and phospho-CHC colocalized around internalized microspheres (Figure 9A). Bonazzi *et al.* (2011) found that phosphorylation of clathrin during bacterial internalization promoted recruitment of an actin scaffold. We found that in MEFs transfected with Jedi-1, both Jedi-1 and actin accumulated around internalized microspheres (Figure 9B). We also observed colocalization of total clathrin heavy chain and actin around microspheres in Jedi-1-expressing MEF cells, although total CHC was more broadly distributed than phospho-CHC, as expected due to the many roles clathrin has in the cell

days later, microspheres were added for 2 h. The cells were then fixed, and the engulfment of the microspheres was quantified by confocal microscopy ($p = 0.0001$ for MEFs with GFP relative to MEFs expressing Jedi-1; $p = 0.0009$ for MEFs expressing Jedi-1 relative to MEFs expressing Jedi-1 with GULP knocked down; $p = 0.0009$ for MEFs expressing Jedi-1 with GULP knocked down and resistant GULP added back relative to MEFs expressing Jedi-1 with GULP knocked down and transfected with G10, by Student's *t* test).

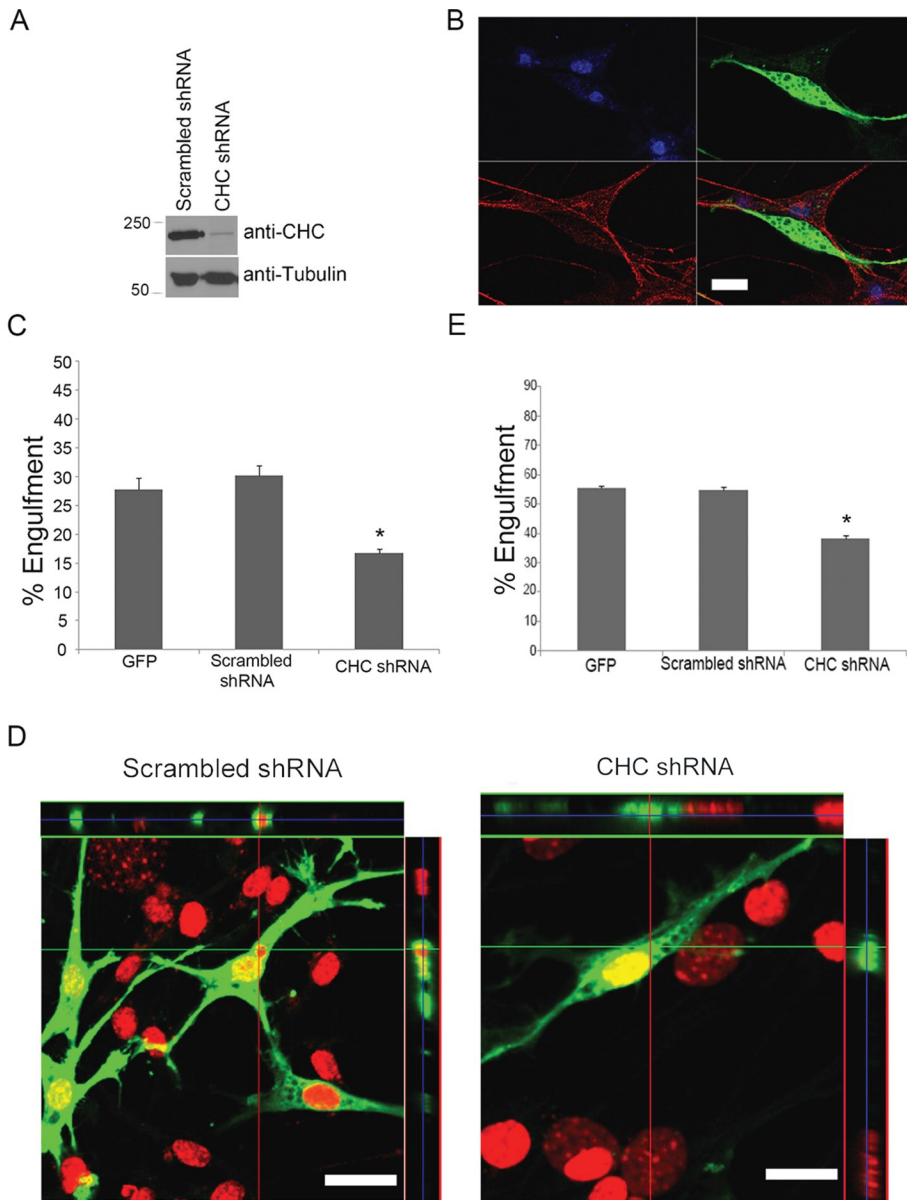


FIGURE 7: Clathrin is required for Jedi-1-mediated engulfment. (A) Nontargeting or CHC shRNA were transfected into HeLa cells. Lysates were subjected to Western blotting with antibodies to CHC and tubulin. (B) Knockdown of CHC was also verified by immunostaining glial cell precursors transfected with CHC shRNA for GFP to identify transfected cells (green) and CHC (red). Nuclei were stained with TO-PRO-3. (C) HeLa cells were cotransfected with Jedi-1-FLAG and GFP, nontargeting shRNA, or CHC shRNA that coexpresses GFP. After 48 h, fluorescent microspheres were incubated with the HeLa cells for 2 h before rinsing and fixation. The percentage of GFP positive cells that had engulfed one or more spheres was determined by confocal microscopy ($n = 3$; $p = 0.006$ for CHC shRNA relative to Jedi-1-FLAG-only control by Student's *t* test). (D) Confocal images depicting cocultures of E13.5 neurons and glial precursor cells transfected with GFP or CHC shRNA coexpressing GFP. On the day of transfection, neuronal death was induced by removal of NGF, and after 48 h the cocultures were fixed and immunostained with antibodies to GFP and nuclei were labeled with TO-PRO-3. Transfected glia are depicted in green and nuclei in red. The cross-hair in the orthogonal view of the cell transfected with scrambled shRNA is positioned on an engulfed apoptotic nuclear fragment, and the cross-hair in the CHC shRNA-transfected image is positioned on the cell body of a glial cell containing no apoptotic nuclear fragments. (The rectangle on top of the images depicts the *xz*-plane, and to the right is the *yz*-plane.) Scale bars, 20 μm . (E) Images from transfected cocultures were analyzed by confocal microscopy, and the percentage of GFP-positive glia that were engulfing apoptotic nuclear fragments was quantified ($n = 3$; $p = 0.0002$ for CHC shRNA relative to nontargeting control, by Student's *t* test).

(Figure 9C). To quantify the localization of phospho-CHC and actin around the microspheres, we scored the percentage of fully internalized microspheres that showed positive staining within 0.5 μm and surrounding at least 50% of the sphere. We observed that $70.3 \pm 6.2\%$ of engulfed microspheres were actin positive and $41.7 \pm 4.8\%$ were phospho-CHC positive (Figure 9D). Because we hypothesized that actin and phospho-CHC were necessary for the internalization step of engulfment, we also divided the population of internalized beads into those distal ($>10 \mu\text{m}$) to the nucleus, which were closer to the cell surface, and those proximal ($<10 \mu\text{m}$). The majority of internalized microspheres that were positive for actin or phospho-CHC were localized distally, suggesting that accumulation of actin and phospho-CHC occurs during early engulfment but dissipates as the phagosome matures and progresses deeper into the cell.

After finding that clathrin is required for Jedi-1-mediated engulfment, we wanted to determine whether CHC plays a role in recruitment of Jedi-1 and/or actin to the phagocytic cup. We performed a kinetic analysis of localization of Jedi-GFP and actin using a microsphere engulfment assay (Figure 10). We found that in early stages of engulfment, Jedi-GFP accumulates near microspheres in contact with the cells, even when CHC has been knocked down. However, recruitment of actin was delayed or markedly decreased when CHC was silenced. This suggests that the inability of Jedi-1-expressing cells to internalize microspheres when CHC is knocked down could be due to a defect in actin localization. Jedi-1 could be observed accumulating near beads in contact with the surface of the cells, even in the absence of clathrin (e.g., notice the 45-min time point in Figure 10), but complete phagocytic cups were not formed. These results were consistent with the results of Bonazzi *et al.* (2011) and suggest that CHC mediates actin scaffolding during engulfment.

To test the importance of clathrin phosphorylation in engulfment, we knocked down endogenous CHC in Jedi-1-transfected HeLa cells and rescued with either small interfering RNA (siRNA)-resistant wild type (WT) CHC-GFP or a phosphomutant CHC (CHC-Y1477F/Y1487F-GFP) in a microsphere engulfment assay. The phosphorylation of CHC at Y1477 and Y1487 was previously shown to be required for internalization of bacteria (Bonazzi *et al.*, 2011). The WT-CHC-GFP was able to rescue the defect in Jedi-mediated engulfment observed after knocking down endogenous CHC;

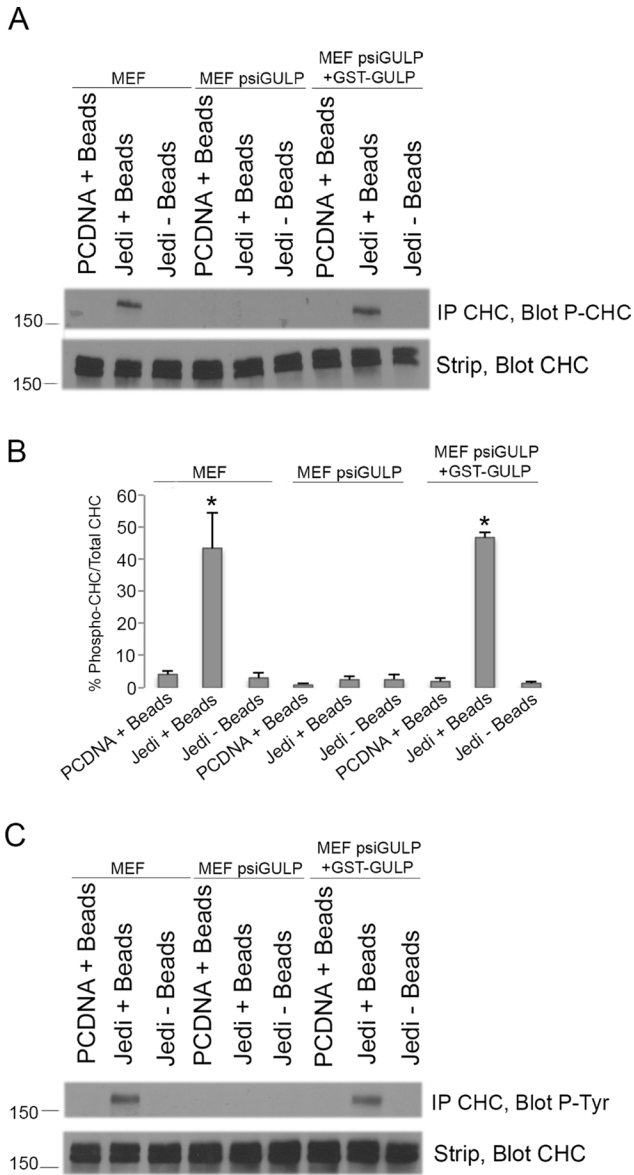


FIGURE 8: CHC is phosphorylated during Jedi-1-mediated engulfment in a GULP-dependent manner. Control MEFs, MEFs with GULP stably knocked down (psiGULP), or MEFs with endogenous GULP silenced but stably transfected with a knockdown-resistant GST-GULP (psiGULP+GST-GULP) were transfected with pcDNA3 or Jedi-1-GFP and then treated with or without fluorescent microspheres for 90 min. (A) The cells were lysed and immunoprecipitated with anti-CHC. The protein was subjected to Western blotting using anti-phospho-CHC and total immunoprecipitated CHC ($n = 3$). (B) Densitometry of phospho-CHC divided by the total CHC signal is shown $n = 3$ experiments ($p = 0.02$ for MEFs transfected with Jedi-1 with bead stimulation relative to Jedi-1-transfected MEFs with no bead stimulation, and $p = 0.0009$ for MEFs with GULP knockdown and GST-GULP rescue with Jedi-1 and bead stimulation relative to MEFs with GULP knockdown and GST-GULP rescue with Jedi-1 and no bead stimulation, by Student's t test). (C) The experiment in A was repeated, but total CHC was immunoprecipitated from the lysates and Western blotted for phosphotyrosine ($n = 3$).

however, the phosphomutant CHC-GFP did not provide any significant rescue (Figure 11). These results indicate that Jedi-1-mediated engulfment requires recruitment and phosphorylation of CHC.

DISCUSSION

Recognition and removal of apoptotic cells generated in the course of development is essential to prevent progression into secondary necrosis and exposure of intracellular contents that have the potential to be immunogenic or toxic. Indeed, there is evidence that suppressing the timely clearance of cell corpses results in autoimmune phenotypes in mouse models (Elliott and Ravichandran, 2010; Nagata *et al.*, 2010). Relatively little is known about mechanisms of neuronal corpse removal, especially in the PNS. We recently demonstrated that glial precursors in the developing DRG are the primary phagocytes responsible for clearing apoptotic sensory neurons (Wu *et al.*, 2009). In addition, we identified MEGF10 and Jedi-1 as engulfment receptors expressed by the glial precursors and required for phagocytosis of the dead neurons. MEGF10 and Jedi-1 are both mammalian homologues of the *D. melanogaster* receptor Draper and the *C. elegans* receptor CED-1, which signal at least in part via recruitment of the adaptor protein CED-6 (GULP in mammals; Su *et al.*, 2002; Awasaki *et al.*, 2006). Several mammalian engulfment receptors, including stabilin-1 (Park *et al.*, 2010), stabilin-2 (Park *et al.*, 2008), LRP-1 (Su *et al.*, 2002), and MEGF10 (Hamon *et al.*, 2006), interact with GULP; however, the role of GULP in the phagocytic process was largely unknown. Here we demonstrate that Jedi-1 interacts with GULP, resulting in phosphorylation and recruitment of clathrin, which is required for phagocytosis.

CED6/GULP is an adaptor protein without catalytic activity that was first identified in a screen for genes regulating the clearance of apoptotic cells in *C. elegans* (Ellis *et al.*, 1991). It was subsequently shown to bind to the nematode engulfment receptor CED-1 (Su *et al.*, 2002) and the fly receptor Draper (Awasaki *et al.*, 2006); however, only recently has its functional role in the phagocytic process been considered. Osada *et al.* (2009) suggested that GULP is involved in activation of Rac by scavenger receptor B1. They demonstrated that upon exposure of a macrophage cell line to phosphatidylyserine (PS)-containing liposomes, there was an increase in GTP-bound Rac. In addition, PS treatment increased p38 and ERK phosphorylation, which was reduced by silencing GULP. Addition of p38 and ERK inhibitors prevented Rac activation by PS; however, GULP was not directly linked to Rac activation (Osada *et al.*, 2009). CED-6 was also suggested to function upstream of CED-10, the worm homologue of Rac, based on an epistatic relationship in genetic studies. Moreover, both CED-1 and CED-6 were required for the accumulation of "actin halos" around the engulfed apoptotic cells (Kinchen *et al.*, 2005); however, the mechanism underlying the actin recruitment was not addressed.

Our results demonstrate that GULP facilitates engulfment through interaction with clathrin. Several previous findings suggested a role for clathrin in Jedi-1-GULP-mediated phagocytosis. First, GULP was shown to sequester ACAP1 (a GTPase-activating protein) and thereby act as a positive regulator of Arf6, a GTPase implicated in promoting clathrin-mediated endocytosis (Ma *et al.*, 2007). The regulation of Arf6 by GULP was assessed during cell migration; however Arf6 is known to act in a multitude of cellular functions, including cell migration, cell adhesion, endocytosis, and phagocytosis (Donaldson and Honda, 2005; Casanova, 2007). Second, CED-6 associates with CED-1 (Su *et al.*, 2002) and Draper (Awasaki *et al.*, 2006) through an interaction between the PTB domain of CED-6/GULP and an NPXY motif in the intracellular domain of the receptors. Similarly, we found that the NPXY domain in Jedi-1 was required for binding to GULP. Mutation of the NPXY in Jedi-1 or knockdown of GULP prevented Jedi-mediated engulfment, indicating that Jedi-1 interaction with GULP is essential for this process. NPXY motifs play a role in the internalization of

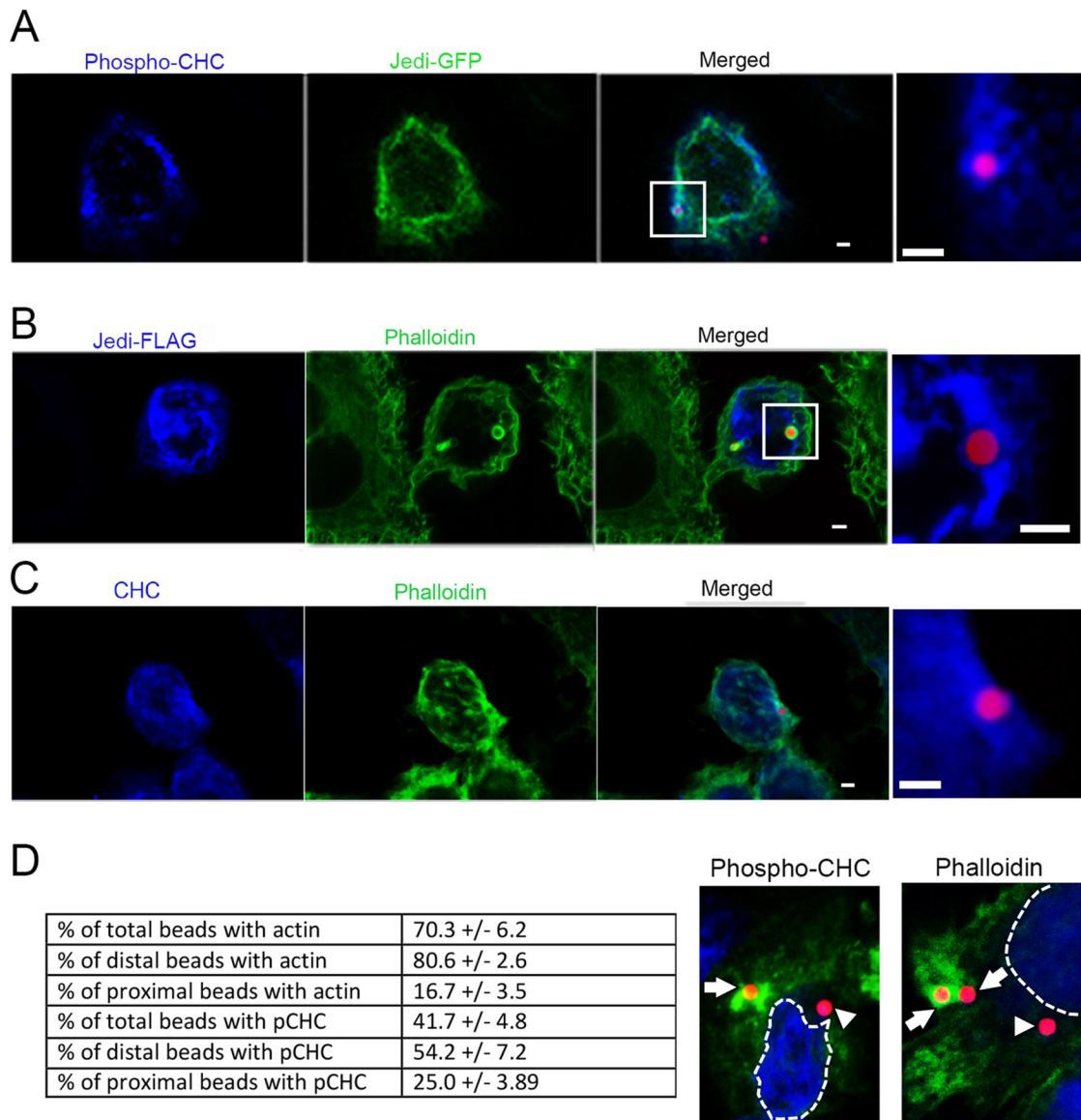


FIGURE 9: Phospho-CHC and actin colocalize with Jedi-1 and accumulate around engulfed microspheres. (A) MEFs transfected with Jedi-1-GFP (green) and treated with fluorescent microspheres (red) for 2 h were fixed and immunostained with anti-phospho-CHC (blue). The cells were imaged using confocal microscopy to ensure full internalization of microspheres. Note the accumulation of Jedi-1 and phospho-CHC around the internalized microsphere. (B) MEFs transfected with Jedi-1-FLAG (blue) were incubated with fluorescent microspheres and then fixed and stained with anti-FLAG and Alexa 488-phalloidin (green) to label actin. Note the ring of actin and Jedi-1-FLAG around the internalized microsphere. (C) Jedi-1-transfected MEF cells were incubated with fluorescent microspheres (red) and then fixed and stained with an antibody recognizing all CHC and phalloidin. Note the accumulation of phalloidin and CHC near the internalized microsphere. Scale bars, 5 μ m. The regions boxed in white in A–C are enlarged and shown to the right of the merged image (only the blue and red channels are shown). (D) Table representing results of quantification of accumulation of actin or phospho-CHC within 0.5 μ m of internalized beads and surrounding them by at least 50%. Distal beads are beads >10 μ m from the nearest edge of the nucleus, and proximal beads are <10 μ m from the nucleus. Representative images used for this quantification are shown to the right of the table. The nucleus is in blue and outlined with a dotted white line, and anti-phospho-CHC or phalloidin labeling is depicted in green, as indicated. Note that microspheres distal to the nucleus (arrows) have more phosphoclatrin or actin staining than beads that are proximal to the nucleus (arrowheads).

proteins from the plasma membrane through clathrin-coated vesicles (Bonazzi *et al.*, 2011). Furthermore, clathrin and AP-2 directly bind to peptides containing an FXNPXY sequence (Kibbey *et al.*, 1998; Boll *et al.*, 2002), but increasing evidence suggests that this motif is actually recognized by PTB-containing proteins, such as Numb, ARH, Dab1, and Dab2, which work with AP-2 to promote

clathrin recruitment and assembly (Traub, 2003); therefore GULP could function as such an adaptor. Third, GULP itself was shown to bind to clathrin by yeast two-hybrid and GST pull down, and it colocalized with clathrin in SN56 cells (Martins-Silva *et al.*, 2006). In addition, the *Drosophila* vitellogenin receptor Yolkless was recently demonstrated to associate with CED-6/GULP through a

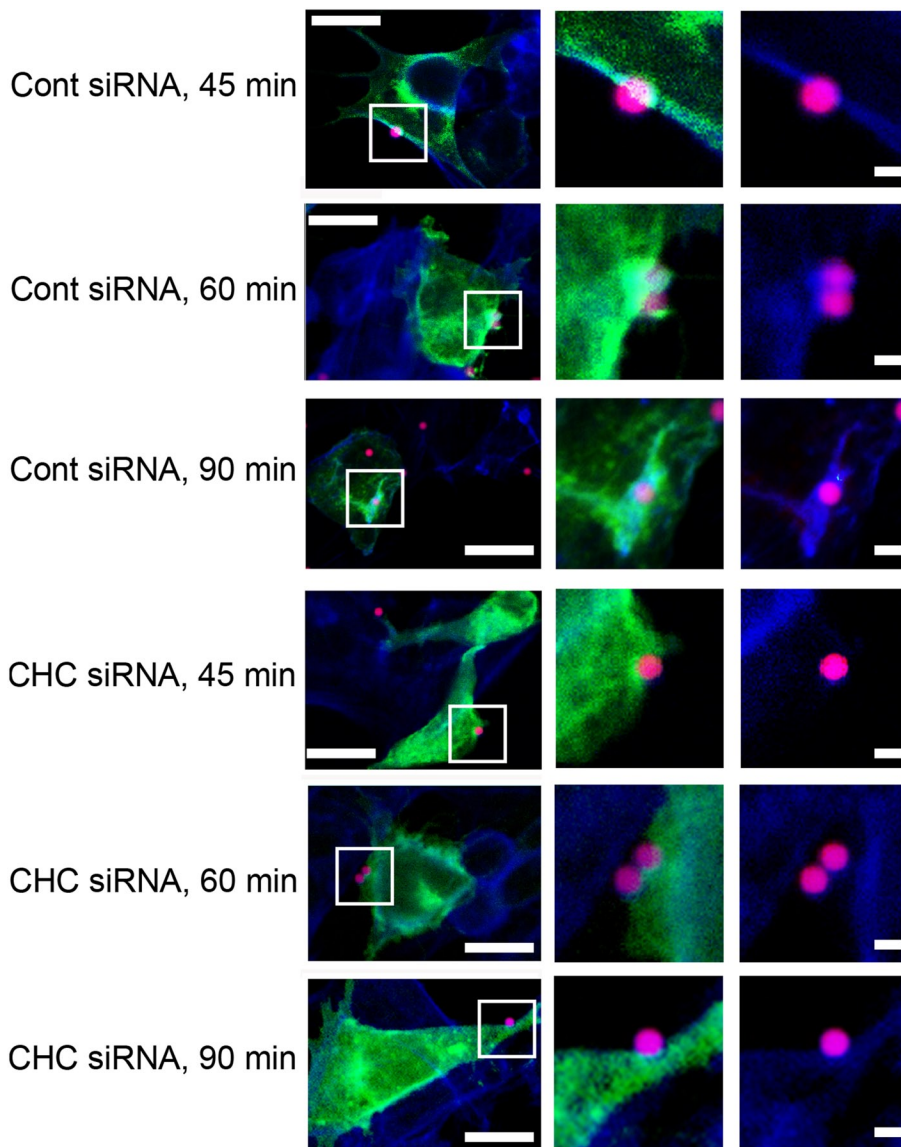


FIGURE 10: Clathrin is required for recruitment of actin to the phagocytic cup. HeLa cells were transfected with Jedi-GFP and control siRNA or CHC siRNA. Cells were incubated for 45, 60, or 90 min with fluorescent microspheres. Localization of Jedi-GFP (green) and actin (blue) was analyzed using confocal microscopy. Scale bars, 5 μ m (low-magnification images), 2 μ m (enlarged images). Regions boxed in white are enlarged and shown to the right of the original image. Far right, only the blue and red channels. Note that beads are still in contact with Jedi-GFP even with CHC siRNA treatment, but the recruitment of actin is delayed and less robust. Representative images from three experiments.

FXNPXA sequence in Yolkless, and internalization of the receptor required CED-6 expression (Jha *et al.*, 2012). Similarly, we found that Jedi-1 internalization in response to addition of microspheres was GULP dependent (Figure 5). Furthermore, Jha *et al.* (2012) found an interaction between CED-6 and CHC that depended on a C-terminal DLF/DPF bipartite sequence in CED-6. We also demonstrated interaction between GULP and CHC that depended on the C-terminal portion of GULP containing this bipartite motif (Figure 6). Of importance, in cells with GULP silenced, Jedi-1-mediated phagocytosis could be rescued by wild-type GULP but not a mutant lacking the CHC-binding domain (Figure 6), indicating that GULP association with clathrin is necessary for Jedi-1-induced engulfment.

is formed in response to Jedi-1 activation.

CHC undergoes tyrosine phosphorylation by Src family kinases during internalization of a number of cell surface receptors, such as the epidermal growth factor (Wilde *et al.*, 1999), NGF (Beattie *et al.*, 2000), T-cell (Crotzer *et al.*, 2004), and interleukin-7 (Jiang *et al.*, 2004) receptors. In addition, upon binding to its receptor Gb3, the bacterial Shiga toxin was shown to undergo endocytosis by promoting interaction between the tyrosine kinase Syk and clathrin, which results in a Syk-dependent phosphorylation of CHC (Lauvrak *et al.*, 2006). Similarly, human rhinovirus binding to cells resulted in Syk recruitment to the cell surface and association with clathrin (Lau *et al.*, 2008). Of interest, we recently demonstrated that during engulfment, Jedi-1 recruits and activates the tyrosine kinase Syk

The requirement for clathrin in phagocytosis was rather surprising, given the small size of a clathrin-coated vesicle (typically <200 nm; McMahon and Boucrot, 2011) and the large size of an apoptotic cell or microsphere (2 μ m) engulfed via a Jedi-1-dependent mechanism. The triskelion clathrin cage that forms around vesicles would be too small to accommodate a phagocytosed particle as large as the microspheres; nevertheless, clathrin was required for this process. To the best of our knowledge, this is the first report to identify a direct role for mammalian clathrin in phagocytosis of apoptotic cells. However, clathrin was previously implicated in internalization of other large bodies, such as bacteria (Veiga and Cossart, 2005), fungi (Moreno-Ruiz *et al.*, 2009), and some viruses (Ehrlich *et al.*, 2004; Rust *et al.*, 2004). The Cossart group reported that pathogenic bacteria associate with their host cells and are internalized through a clathrin-dependent mechanism (Veiga and Cossart, 2005; Bonazzi *et al.*, 2011). They demonstrated that bacterial adhesion stimulated localized formation of clathrin-coated pits, which served as docking sites for actin accumulation rather than forming conventional clathrin-coated vesicles. The actin recruitment and bacterial internalization required phosphorylation of CHC (Bonazzi *et al.*, 2011). Exactly how phosphorylation facilitated the recruitment of actin is not clear; however, it was necessary for formation of a complex that included phospho-CHC and clathrin light chain, as well as the actin-binding protein Hip1R. We similarly found that CHC is phosphorylated in response to microsphere addition to cells expressing Jedi-1 and that the phospho-clathrin, as well as actin, accumulated around the engulfed spheres and colocalized with Jedi-1. Furthermore, silencing GULP inhibited CHC phosphorylation (Figure 8) and microsphere internalization (Figure 3), and the phosphorylation of CHC was required for engulfment mediated by Jedi-1 (Figure 11). It will be interesting to determine whether a similar Hip1R complex

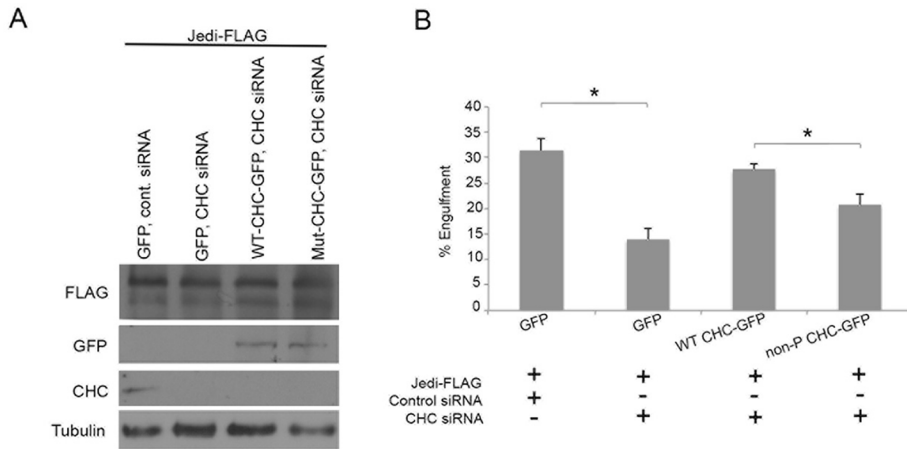


FIGURE 11: Phospho-CHC is required for Jedi-1-mediated engulfment of microspheres. (A) HeLa cells were transfected with Jedi-FLAG, control or CHC targeting siRNA, and GFP, WT-CHC-GFP, or phosphomutant CHC-GFP and verified for expression and knockdown by immunoblotting. WT-CHC-GFP and phosphomutant-CHC-GFP are siRNA resistant. (B) Cells were plated for a microsphere engulfment assay, and the number of GFP/FLAG double-positive cells engulfing at least one microsphere was determined ($n = 3$; $p = 0.006$ for Jedi-FLAG control siRNA vs. Jedi-FLAG with CHC siRNA; $p = 0.04$ for Jedi-FLAG CHC siRNA WT-CHC-GFP vs. Jedi-FLAG CHC siRNA phosphomutant CHC-GFP transfected cells by Student's *t* test).

through its ITAM domains (Scheib *et al.*, 2012). Syk recruitment and activity were required for Jedi-1-mediated phagocytosis. Therefore it is possible that binding of apoptotic bodies to Jedi-1 results in Syk activation and recruitment to the ITAM domain of Jedi-1, which then associates with CHC and phosphorylates it. Phosphorylation of CHC promotes its association with actin, which facilitated internalization (Bonazzi *et al.*, 2011), and we demonstrated that phosphorylation of CHC was required for internalization of microspheres (Figure 11). Of note, we also observed actin accumulation around phagocytosed microspheres, particularly at early stages of internalization (Figure 9). Because Syk can also regulate actin polymerization through activation of the Rac exchange factor Vav (Deckert *et al.*, 1996; Cougoule *et al.*, 2006), Jedi-1 may use both GULP-mediated CHC recruitment and Syk activation of Rac to form actin structures that facilitate engulfment.

Together with our previous findings (Wu *et al.*, 2009; Scheib *et al.*, 2012), the present findings suggest a remarkable conservation of engulfment mechanisms from *C. elegans* to *D. melanogaster* to mammals. Like its homologues, Draper and CED-1, Jedi-1 is expressed by "amateur" phagocytes and signals engulfment of apoptotic cells through recruitment of the adaptor protein GULP/CED-6. It is notable that a recent report demonstrated that in *C. elegans*, CED-6 forms a complex with CHC and the adaptor AP2 during cell corpse engulfment (Chen *et al.*, 2013).

MATERIALS AND METHODS

DNA constructs

Jedi-1 was cloned into the pEGFP or pFlag plasmid as described previously (Wu *et al.*, 2009). Jedi-1-GFP mutants were obtained by site-directed mutagenesis with PfuUltra HF (Stratagene) PCR and Dpn1 (New England Biolabs, Ipswich, MA) digestion. The GULP deletion mutant was created by mutation of residue R324 to create a new stop codon using the same reagents used to create the Jedi-1 mutants, and GULP was expressed in the pEBB triple-HA vector. To make the GULP shRNA targeting both mouse and human GULP mRNA, the synthetic oligonucleotide 5'-GATCCG-GAACAGAAGTTGTGAGAGATGTTCAAGAGACATCTCTCA-

CAACTTCTGTTCCCTTTTACGCGTG-3' and the reverse complement were annealed and ligated into pSIREN-RetroQ-ZsGreen (Clontech, Mountain View, CA). The scrambled shRNA was in the same vector. siRNA targeting the CHC17 target sequence, 5'-AAG CAA TGA GCT GTT TGA AGA, and control nontargeting siRNA were purchased from Qiagen (Valencia, CA). Wild-type or phosphomutant CHC-GFP rescue constructs resistant to CHC17 siRNA (pcDNA3.1/Zeo-siRNA(AS)resCHC17WT-GFP and pcDNA3.1/Zeo-siRNA(AS)resCHC17&Y1477F+Y1487F-GFP) were previously described (Bonazzi *et al.*, 2011). The CHC shRNA (pBrain-GFP-CHC4), which targets both mouse and human CHC, and control shRNA, which targets rat CHC (pBrain-GFP-CHC1), were kindly provided by Stephen Royle (University of Liverpool, Liverpool, United Kingdom).

Cell culture and transfection

HeLa cells and MEFs were grown in DMEM with 10% fetal bovine serum in 5% CO₂. Transfections were carried out using Lipofectamine 2000 (Invitrogen, Grand Island, NY) per the manufacturer's instructions. MEF cell lines were described previously (Ma *et al.*, 2007). Briefly, an siRNA against GULP was designed to target nucleotides 125–145 (from the starting codon) with staggered ends to form *Bam*HI and *Hind*III restriction sites that would allow subsequent subcloning into the pSilencer2.1-U6 puro plasmid (Ambion, Austin, TX). Mouse embryonic fibroblasts (MEF-1) were transfected with this plasmid and selected with 6 μg/ml puromycin for stable GULP-knockdown clones. The control clone was transfected with a control plasmid (pApuro) and selected under the same conditions. For siRNA transfections, HeLa cells were transfected with HiPerfect (Qiagen) according to the manufacturer's instructions. Cells were assessed for siRNA efficacy 72 h after transfection by Western blotting. For CHC rescue experiments, cells were transfected with CHC rescue constructs 48 h after siRNA transfection using Lipofectamine 2000 according to the manufacturer's instructions.

Immunoprecipitation and Western blot analysis

Transfected cells were harvested in 500 μl of NP-40 lysis buffer (25 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1% NP-40, 10% glycerol, 1 mM Na₃VO₄, and Complete Mini EDTA-free Protease Inhibitor Cocktail tablet [Roche, Indianapolis, IN]). Jedi-1-GFP and mutants were immunoprecipitated with anti-GFP (Roche) and Protein A Sepharose (Invitrogen). GST-GULP was pulled down using glutathione Sepharose beads (Amersham Biosciences, Piscataway, NJ). Clathrin heavy chain was immunoprecipitated using the X-22 antibody (Abcam, Cambridge, MA). HA-GULP was immunoprecipitated with a monoclonal HA antibody (Covance, Princeton, NJ). Endogenous Jedi-1 was immunoprecipitated using a previously described polyclonal Jedi-1 antibody (Scheib *et al.*, 2012). After separation of the proteins by SDS-PAGE, Western blot analysis was performed using primary antibodies to the HA tag (1:3000; Covance), GFP (1:1000; Roche), GULP (1:1000; Abcam), clathrin heavy chain (1:1000; Santa Cruz Biotechnology, Dallas, TX), anti-FLAG (1:1000; Covance), anti-phospho-CHC (1:1000; developed by Frances Brodsky; Bonazzi *et al.*, 2011),

endogenous Jedi (1:1000), or α -tubulin (1:1000; Calbiochem, Billerica, MA).

Biotinylation

HeLa cells or MEFs were transfected with wild-type or APXA mutant Jedi-GFP. After 24–48 h, the cells were placed on ice, and surface proteins were biotinylated with EZ-Link Sulfo-NHS-SS-Biotin (Pierce, Rockford, IL). The biotinylation of surface proteins was reversed using 50 mM DTT at various time points after stimulation with 2- μ m carboxylate-modified latex beads (Invitrogen). Internalized biotinylated Jedi-1-GFP or mutant Jedi-1-GFP was pulled down with avidin agarose beads (Pierce) and detected by immunoblotting with a GFP antibody (Roche).

Engulfment assays

DRG cocultures and engulfment of apoptotic neuron assays were performed as described by Scheib *et al.* (2012). In brief, DRG from E13.5 CD1 mouse embryos of either sex were dissociated, and 50,000 cells were plated onto a collagen-coated glass coverslip in 1:1 UltraCULTURE (BioWhittaker, Radnor, PA):Neuralbasal medium (Invitrogen) with 3% fetal bovine serum and N2 and B27 supplements plus 50 ng/ml NGF. The glial cells were transfected using Effectene (Qiagen), and after 2 d, the NGF was removed to induce apoptosis. The cocultures were then fixed in 4% paraformaldehyde, and the transfected cells were detected using anti-GFP (1:500; Abcam) and anti-mouse labeled with Alexa Fluor 488 (1:400). The nuclei were detected using TO-PRO-3 (Life Technologies, Grand Island, NY). Photomicrographs of z-stacks were taken using a Zeiss LSM 510 inverted confocal microscope (Cell Imaging Shared Resource at Vanderbilt University Medical Center, Nashville, TN), and at least 50 cells were analyzed for each experiment. Any cell with an internalized TO-PRO–positive signal, other than its own nucleus, was counted as having phagocytosed an apoptotic body.

For the microsphere engulfment assay, 300,000 HeLa cells or MEFs were plated on 35-mm tissue culture plates. The next day, cells were transfected with the indicated plasmids using Lipofectamine 2000. After 24 h for expression or 48 h for shRNA knock-down, 50,000 of these cells were plated per well on collagen-coated right-well chamber slides. The next day, 2- μ m carboxylate-modified fluorescent polystyrene microspheres (Invitrogen) or nonfluorescent carboxylate-modified latex microspheres (Invitrogen) in phosphate-buffered saline (PBS) with 1 mg/ml bovine serum albumin were incubated with the cells at 37°C for 2 h, and then the unbound microspheres were removed by PBS rinses and the cells were fixed in 10% Formalin. The cells were then immunostained and imaged with a Zeiss LSM 510 inverted confocal microscope. The percentage of transfected cells that had engulfed one or more microspheres was determined for each condition. Microspheres were scored as being positive for actin or phospho-CHC accumulation if the staining was within 0.5 μ m of the microsphere and surrounded at least 50% of it, using ImageJ. Actin- or phospho-CHC–positive microspheres were placed into subcategories based on whether they were distal (>10 μ m) from the closest edge of the nucleus (based on 4',6-diamidino-2-phenylindole staining) or proximal (<10 μ m) to the nucleus using ImageJ.

Immunostaining

MEFs or MEFs with GULP knocked down were transfected with Jedi-1-GFP and plated using the same protocol as the microsphere engulfment assay. After microsphere incubation and fixation in 10% Formalin, cells were stained for Jedi-GFP (as in the engulfment assay) and phospho-CHC (primary, anti-phospho-CHC, 1:1000;

secondary, anti-mouse Alexa 647, 1:500; Invitrogen). For actin localization studies, MEFs were transfected with Jedi-1-FLAG (primary, anti-FLAG 1:500 [Sigma-Aldrich, St. Louis, MO]; secondary, anti-mouse Alexa 647, 1:500 [Invitrogen]) and incubated with Alexa 488–phalloidin (1:500; Invitrogen). For actin and total clathrin localization studies, Jedi-1–transfected MEF cells were stained for total clathrin using the X-22 antibody (1:500; Abcam) and incubated with secondary anti-mouse Alexa 647 (Invitrogen) along with phalloidin (1:500; Invitrogen).

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