



Figures and figure supplements

Ezrin activation by LOK phosphorylation involves a PIP₂-dependent wedge mechanism

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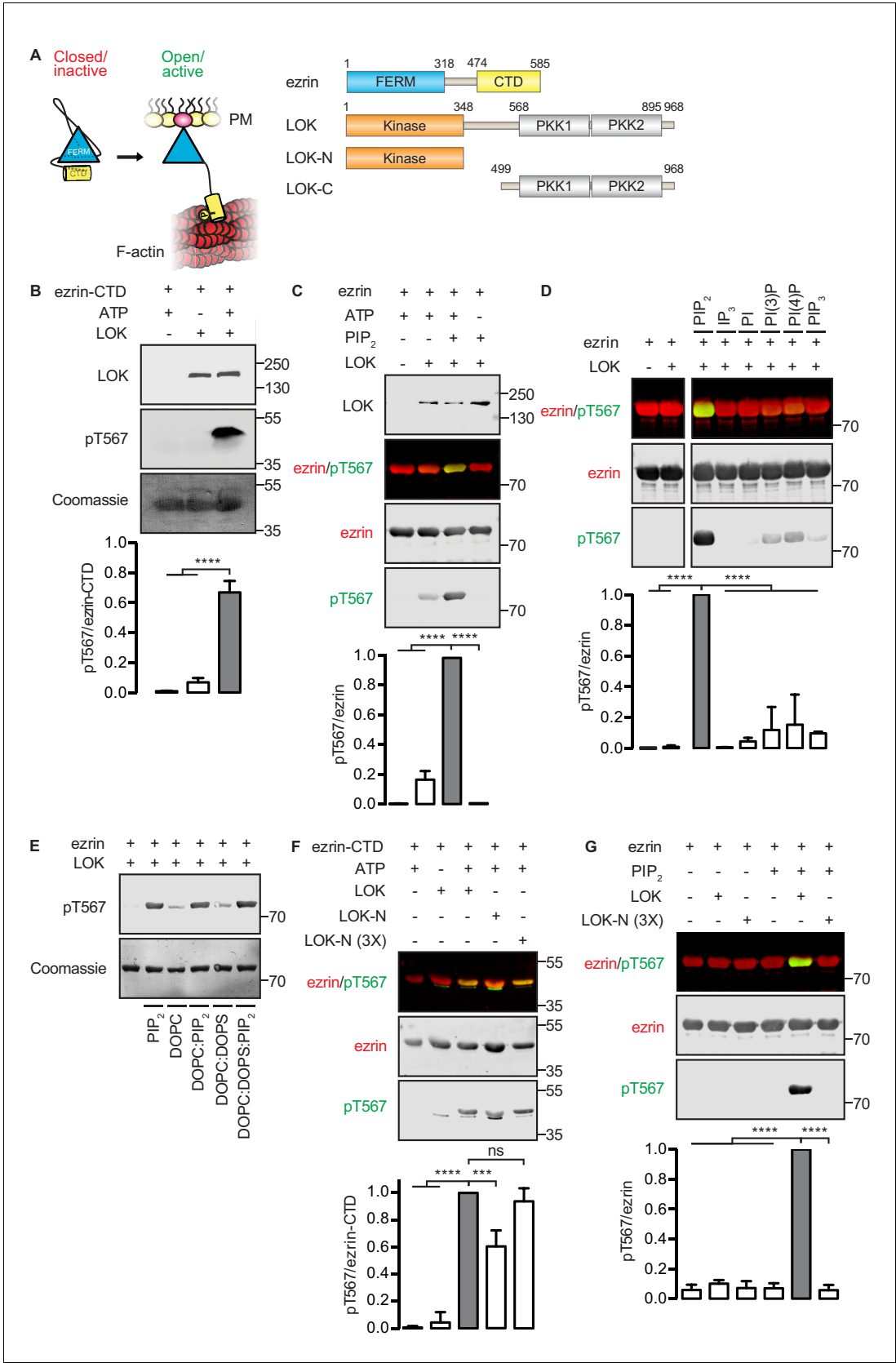


Figure 1. *In vitro* phosphorylation of full-length ezrin requires PIP₂ and LOK C-terminal domain. (A) Left panel: A cartoon illustration of cytoplasmic closed/inactive ezrin versus membrane-tethered open/active ezrin acting as crosslinker between the plasma membrane (PM) and the cytoskeletal F-actin. Right panel: Schematic of ezrin (FERM, CTD), LOK (Kinase, PKK1, PKK2), LOK-N (Kinase), and LOK-C (PKK1, PKK2). Figure 1 continued on next page

Figure 1 continued

actin. Right panel: The domain structure of ezrin and LOK constructs used in this study. The numbers indicate amino acids residues at protein domain boundaries. (B) *In vitro* kinase assay showing that 10 nM LOK phosphorylates 18 μ M ezrin-CTD. Data are presented as mean \pm SE, n = 3, two-way ANOVA (See also **Figure 1—source data 1**), ****p<0.0001. (C) *In vitro* kinase assay showing LOK-mediated phosphorylation of full-length ezrin in presence of 90 μ M of PIP₂ micelles. Data are presented as mean \pm SE, n = 3, two-way ANOVA (See also **Figure 1—figure supplement 1** and **Figure 1—source data 1**), ****p<0.0001. (D) *In vitro* kinase assay showing that 18 μ M ezrin is specifically primed by 90 μ M of PIP₂ micelles and not by IP₃ or other phospholipids at 90 μ M concentrations. Blots are derived from same membrane. Data are presented as mean \pm SE, n = 3, two-way ANOVA (See also **Figure 1—source data 1**), ****p<0.0001. (E) *In vitro* kinase assay showing that unilamellar liposomes DOPC:PIP₂ (90 mol% DOPC, 10% PIP₂) or DOPC:DOPS:PIP₂ (80 mol% DOPC, 10 mol% DOPS, 10 mol% PIP₂) promote phosphorylation of 18 μ M ezrin by 10 nM LOK, whereas DOPC (100 mol % DOPC) or DOPC:DOPS (70 mol% DOPC, 30 mol% DOPS) fail to promote LOK-mediated ezrin phosphorylation. (F–G) 10 nM LOK-N phosphorylates 18 μ M ezrin-CTD but not full-length ezrin in presence of 90 μ M PIP₂. Data are presented as mean \pm SE, n = 3, two-way ANOVA (See also **Figure 1—source data 1**), ***p<0.0002, ****p<0.0001. Total ezrin is shown in red and phosphorylation of T567 in green in dual color Western blots.

DOI: [10.7554/eLife.22759.002](https://doi.org/10.7554/eLife.22759.002)

The following source data is available for figure 1:

Source data 1. Experimental replicates for **Figure 1B,C, D, F and G**.

DOI: [10.7554/eLife.22759.003](https://doi.org/10.7554/eLife.22759.003)

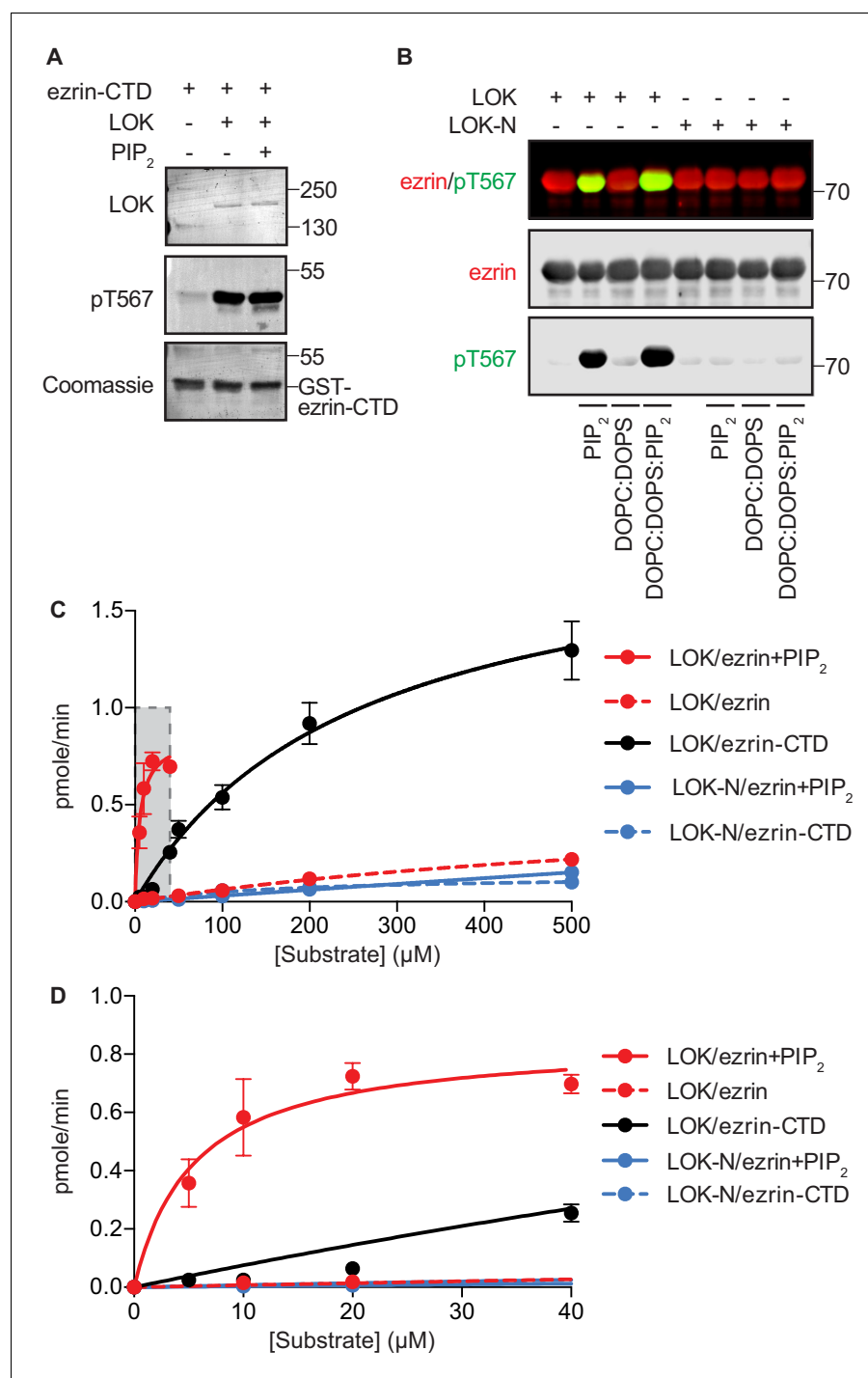


Figure 1—figure supplement 1. Phosphorylation of full-length ezrin by full-length LOK requires PIP₂ micelles or PIP₂-containing liposomes. (A) 10 nM LOK phosphorylates 18 μM ezrin-CTD regardless of the presence of 90 μM PIP₂. (B) 10 nM LOK requires 90 μM PIP₂ micelles or 1 mM DOPC:DOPS:PIP₂ unilamellar liposomes containing 10 mol% PIP₂ (DOPC:DOPS:PIP₂) for phosphorylation of 18 μM ezrin. 10 nM LOK-N cannot phosphorylate 18 μM ezrin in presence of PIP₂ micelles or DOPC:DOPS:PIP₂. Total ezrin is shown in red and phosphorylation of T567 in green in dual color western blots. (C) Michaelis-Menten curves of kinase-substrate pairs showing that PIP₂ and LOK-C render full-length ezrin an optimal substrate for LOK kinetics. The shaded region (0–40 μM substrate) is enlarged in (D). Data are represented as mean ± SE, n = 3 (See also **Table 1** and **Table 1—source data 1**).

DOI: [10.7554/eLife.22759.004](https://doi.org/10.7554/eLife.22759.004)

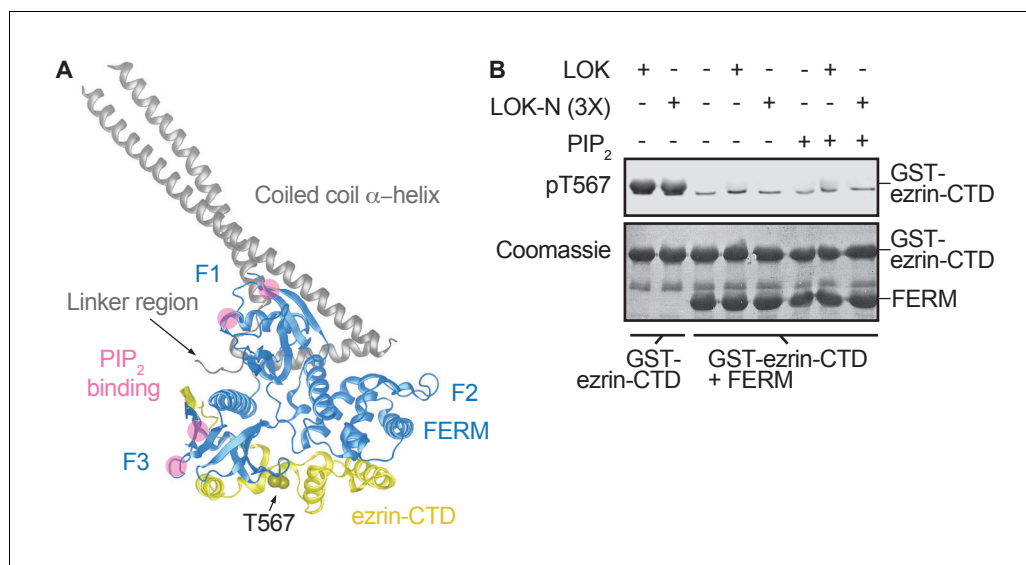


Figure 2. The central α -helical coiled coil hairpin region of ezrin is required for PIP₂-dependent phosphorylation. (A) Crystal structure of the ERM protein *Sfmoesin* (PDB: 211K) in a closed/inactive state. Lobes F1-3 represent the lobes in FERM domain (blue). The α -helical coiled coil hairpin is shown in gray. An acidic linker region (gray) of the hairpin occupies a PIP₂-binding site (pink), embedded between F1 and F3 lobes. T567 represents the position of ezrin T567 superimposed on *Sfmoesin*-CTD (yellow). This residue is masked in this closed state. (B) 10 nM LOK and 10 nM LOK-N phosphorylate 18 μ M GST-ezrin-CTD, but both LOK and LOK-N fail to phosphorylate 18 μ M of a GST-ezrin-CTD+FERM complex in absence or presence of 90 μ M PIP₂. Phosphorylation of GST-ezrin-CTD was detected with pT567 antibody. Total protein was visualized using Coomassie.

DOI: [10.7554/eLife.22759.005](https://doi.org/10.7554/eLife.22759.005)

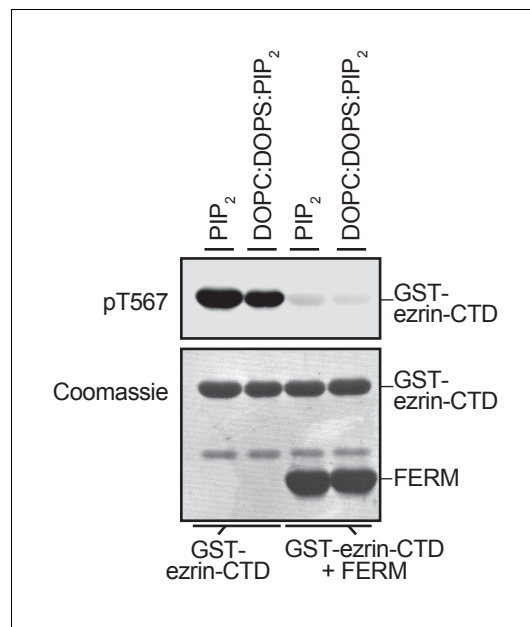


Figure 2—figure supplement 1. GST-ezrin-CTD +FERM complex, lacking the central α -helix region, is not phosphorylated in presence of PIP₂ micelles or PIP₂-containing liposomes. 10 nM LOK phosphorylates 18 μ M GST-ezrin-CTD independent of PIP₂ micelles, DOPC:DOPS or DOPC:DOPS:PIP₂ unilamellar liposomes, but 10 nM LOK fails to phosphorylate GST-ezrin-CTD+FERM primed with PIP₂ micelles or DOPC:DOPS:PIP₂ unilamellar liposomes. Phosphorylation of GST-ezrin-CTD was detected with pT567 antibody. Total protein was visualized using Coomassie.

DOI: [10.7554/eLife.22759.006](https://doi.org/10.7554/eLife.22759.006)

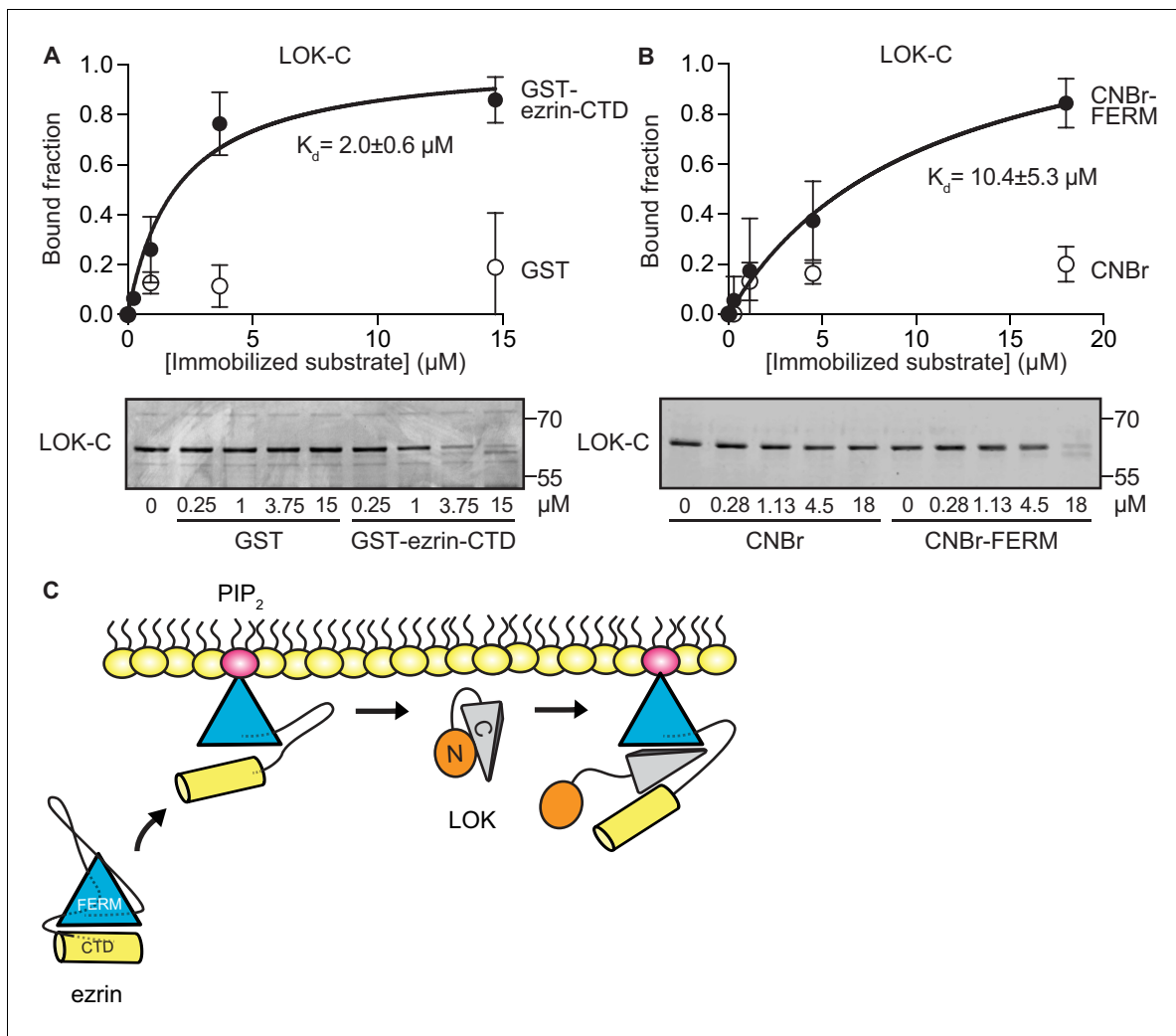


Figure 3. LOK C-terminal domain binds both ezrin FERM and C-terminal domains. **(A)** A binding curve for binding of 0–15 μM immobilized GST-ezrin-CTD to 200 nM LOK-C at $K_d = 2.0 \pm 0.6 \mu\text{M}$. Bottom panel depicts representative experiment and Coomassie staining of unbound fraction of LOK-C. Data are represented as mean \pm SE, $n = 3$ (See also [Figure 3—source data 1](#)). **(B)** Binding curve for binding of 0–18 μM immobilized CNBr-FERM to 200 nM LOK-C at $K_d = 10.4 \pm 5.3 \mu\text{M}$. Bottom panel depicts representative experiment and Coomassie staining of unbound fraction of LOK-C. Data are represented as mean \pm SE, $n = 3$ (See also [Figure 3—source data 1](#)). **(C)** A model of LOK-C acting as a wedge that pries open FERM and ezrin-CTD in a PIP_2 -primed ezrin molecule.

DOI: [10.7554/eLife.22759.009](https://doi.org/10.7554/eLife.22759.009)

The following source data is available for figure 3:

Source data 1. Analysis for binding curves in [Figure 3A–B](#).

DOI: [10.7554/eLife.22759.010](https://doi.org/10.7554/eLife.22759.010)

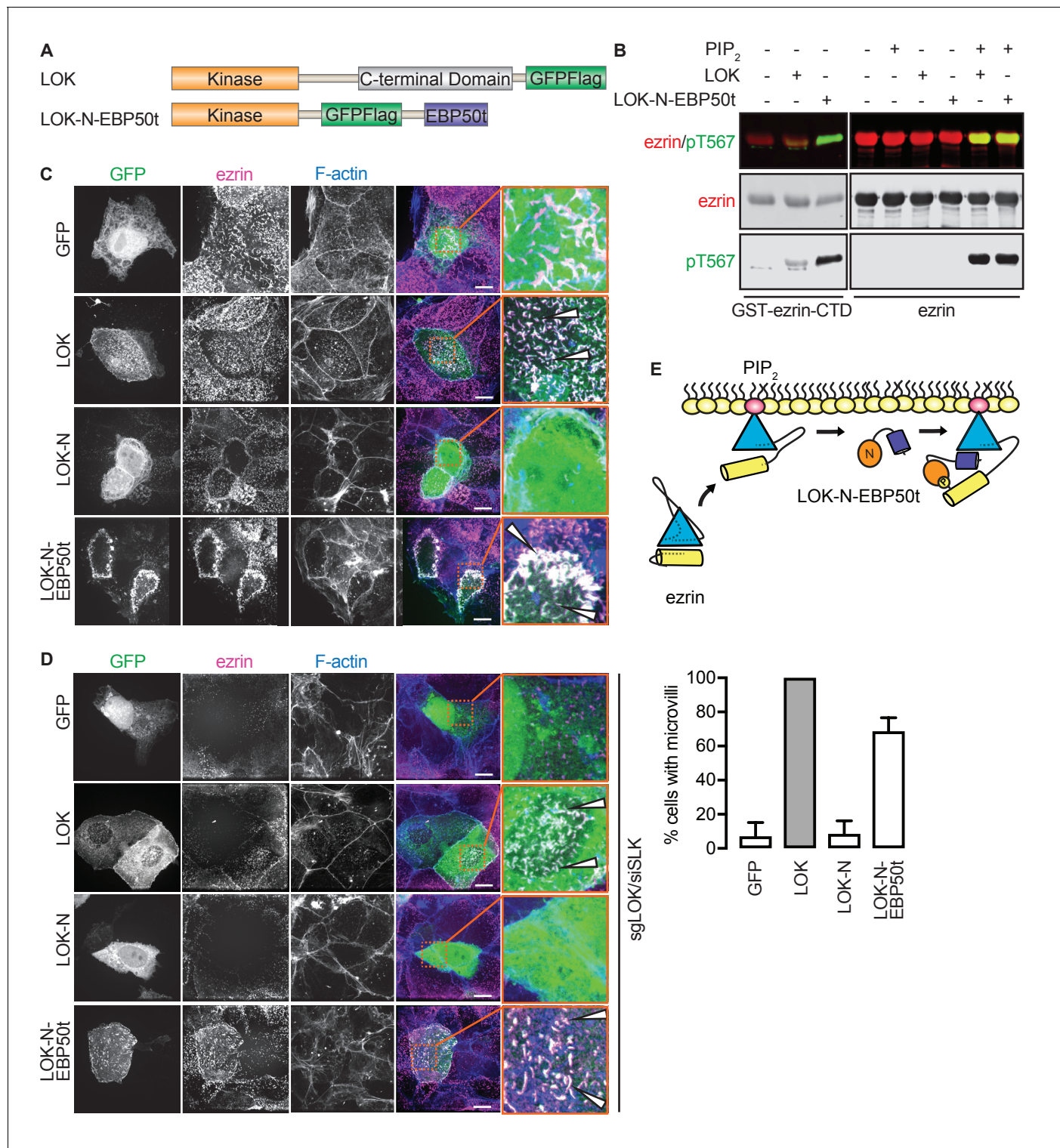


Figure 4. The tail of EBP50 compensates for lack of LOK C-terminus in PIP₂-dependent ezrin phosphorylation. **(A)** A schematic protein domain comparison of full-length LOK fused to GFP-Flag tag (green) and LOK-N fused to EBP50 tail (purple) via an internal GFP-Flag tag. **(B)** *In vitro* kinase assay showing that 10 nM LOK and 10 nM LOK-N-EBP50t both phosphorylate 18 μM ezrin-CTD, but only phosphorylate 18 μM ezrin in presence of 90 μM PIP₂. Total ezrin is shown in red and phosphorylation of T567 in green in dual color western blots. **(C)** Maximum projections of wild type JEG-3 cells overexpressing GFP, and GFP fusions of LOK, LOK-N or LOK-N-EBP50t (green) show that LOK and LOK-N-EBP50t are targeted to microvilli, while GFP and LOK-N are cytoplasmic. Ezrin in magenta. F-actin in blue. Insets represent area within dotted boxes. Arrows indicate microvilli. Scale bars, 10 μm. **(D)** Representative maximum projections of ERM kinase-suppressed sgLOK/siSLK JEG-3 cells overexpressing GFP, and GFP fusions of LOK, LOK-N or LOK-N-EBP50t (green) show that LOK and LOK-N-EBP50t are targeted to microvilli, while GFP and LOK-N are cytoplasmic. Ezrin in magenta. F-actin in blue. Insets represent area within dotted boxes. Arrows indicate microvilli. Scale bars, 10 μm. **(E)** Schematic diagram of the proposed mechanism. PIP₂ recruits ezrin to the membrane. LOK-N-EBP50t then phosphorylates ezrin at T567, leading to its activation and recruitment to microvilli.

Figure 4 continued on next page

Figure 4 continued

LOK-N-EBP50t (green) show that LOK and LOK-N-EBP50t rescue microvilli, while GFP and LOK-N failed to rescue microvilli. Ezrin in magenta. F-actin in blue. Insets represent area within dotted boxes. Arrows indicate microvilli. Scale bars, 10 μ m. Bar graph shows quantification of confocal images. Data are represented as mean \pm SE, n = 3. (See also **Figure 4—source data 1**) (E) A model depicting how LOK-N-EBP50t binding to FERM in PIP₂-primed ezrin resembles the wedging of ezrin by LOK-C prior to phosphorylation of T567.

DOI: [10.7554/eLife.22759.011](https://doi.org/10.7554/eLife.22759.011)

The following source data is available for figure 4:

Source data 1. Source data for quantification of microvilli in **Figure 4D** and **Figure 4—figure supplement 1B**.

DOI: [10.7554/eLife.22759.012](https://doi.org/10.7554/eLife.22759.012)

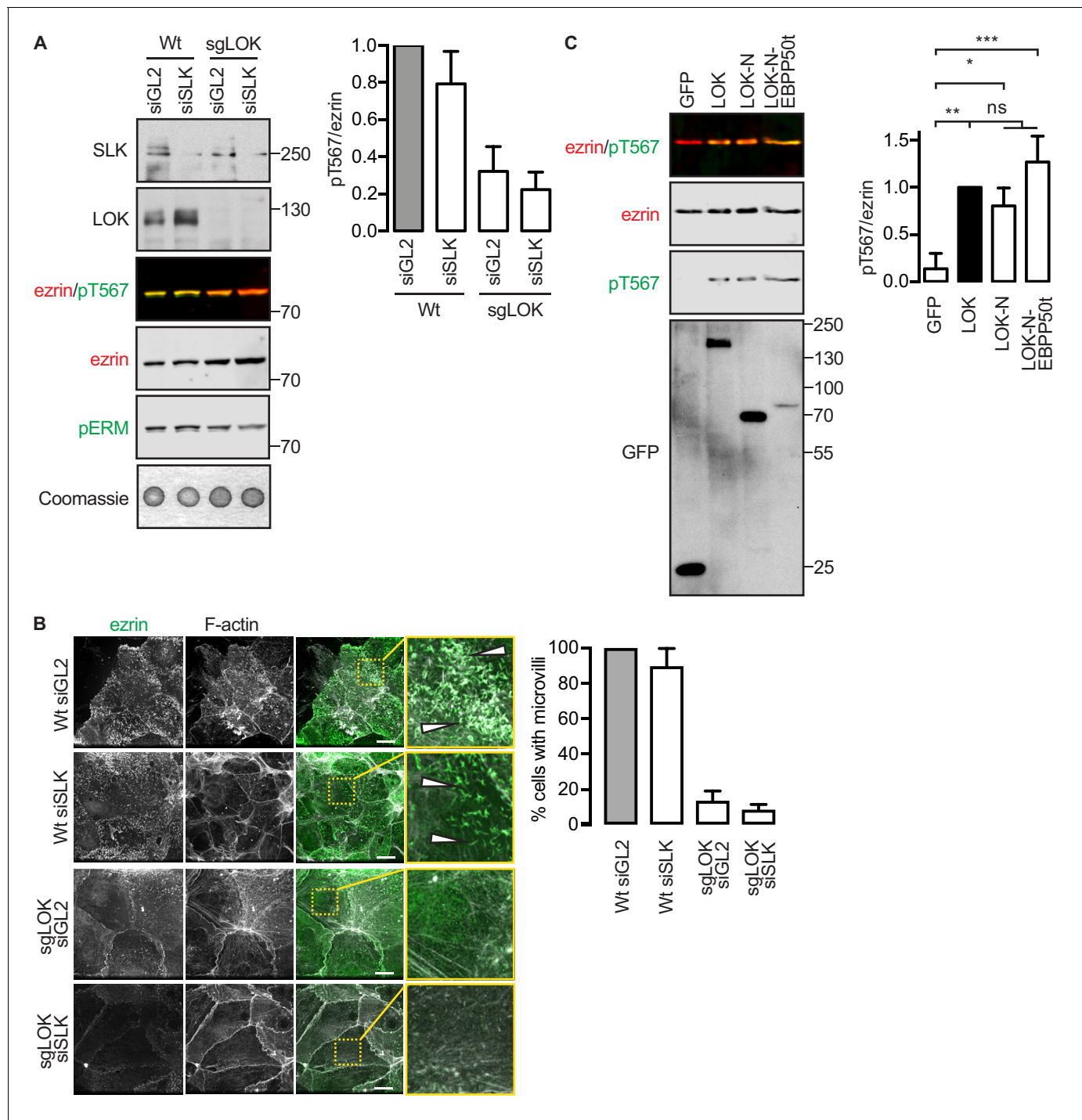


Figure 4—figure supplement 1. LOK-N-EBP50t rescues ezrin phosphorylation in kinase-suppressed JEG-3 cells. (A) A western blot showing that ezrin phosphorylation is suppressed in sgLOK/siSLK JEG-3 cells. Data are represented as mean \pm SE, $n = 3$. (B) Representative maximum projections showing that microvilli formation is suppressed in sgLOK/siSLK JEG-3 cells. Ezrin in green. F-actin in gray. Insets represent area within dotted boxes. Scale bars, 10 μ m. Bar graph shows quantification of confocal images. Data are represented as mean \pm SE, $n = 3$. (See also **Figure 4—source data 1**). (C) Western blot showing that LOK-N-EBP50t restores ezrin phosphorylation in sgLOK/siSLK JEG-3 cells. Total ezrin is shown in red and phosphorylation of T567 in green in dual color western blots. Data are represented as mean \pm SE, $n = 3$, two-way ANOVA, $*p=0.0332$, $**p=0.0021$, $***p=0.0002$. DOI: 10.7554/eLife.22759.013

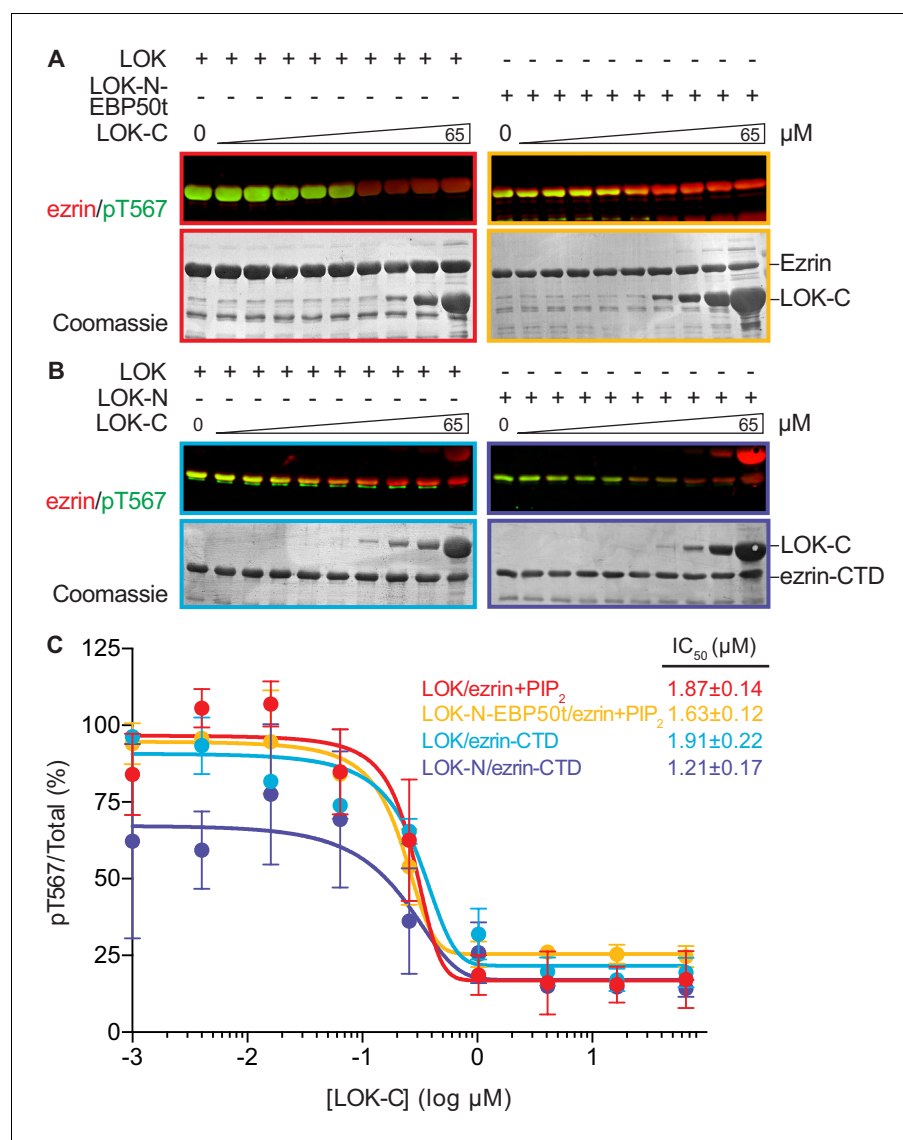


Figure 5. LOK C-terminus is a regulatory domain that inhibits the LOK kinase domain. (A) *In vitro* kinase assay showing that LOK-C (0–65 μM) inhibits phosphorylation of 18 μM ezrin phosphorylation by 10 nM LOK (red panel) and 10 nM LOK-N-EBP50t (yellow panel) in presence of 90 μM PIP₂. (B) *In vitro* kinase assay showing that LOK-C (0–65 μM) inhibits LOK-mediated phosphorylation of 18 μM ezrin-CTD (blue panel), and LOK-N-mediated phosphorylation of 18 μM ezrin-CTD (purple panel). Total ezrin is shown in red and phosphorylation of T567 in green in dual color western blots. (C) Inhibition curves of LOK/ezrin+PIP₂ (red), LOK-N-EBP50t/ezrin+PIP₂ (yellow), LOK/ezrin-CTD (blue), and LOK-N/ezrin-CTD (purple), with their corresponding IC₅₀-values. Data are represented as mean ± SE, n = 2 (see also **Figure 5—source data 1**).

DOI: [10.7554/eLife.22759.014](https://doi.org/10.7554/eLife.22759.014)

The following source data is available for figure 5:

Source data 1. Data summary and analysis for **Figure 5C**, based on experimental replicates represented in **Figure 5A–B**.

DOI: [10.7554/eLife.22759.015](https://doi.org/10.7554/eLife.22759.015)

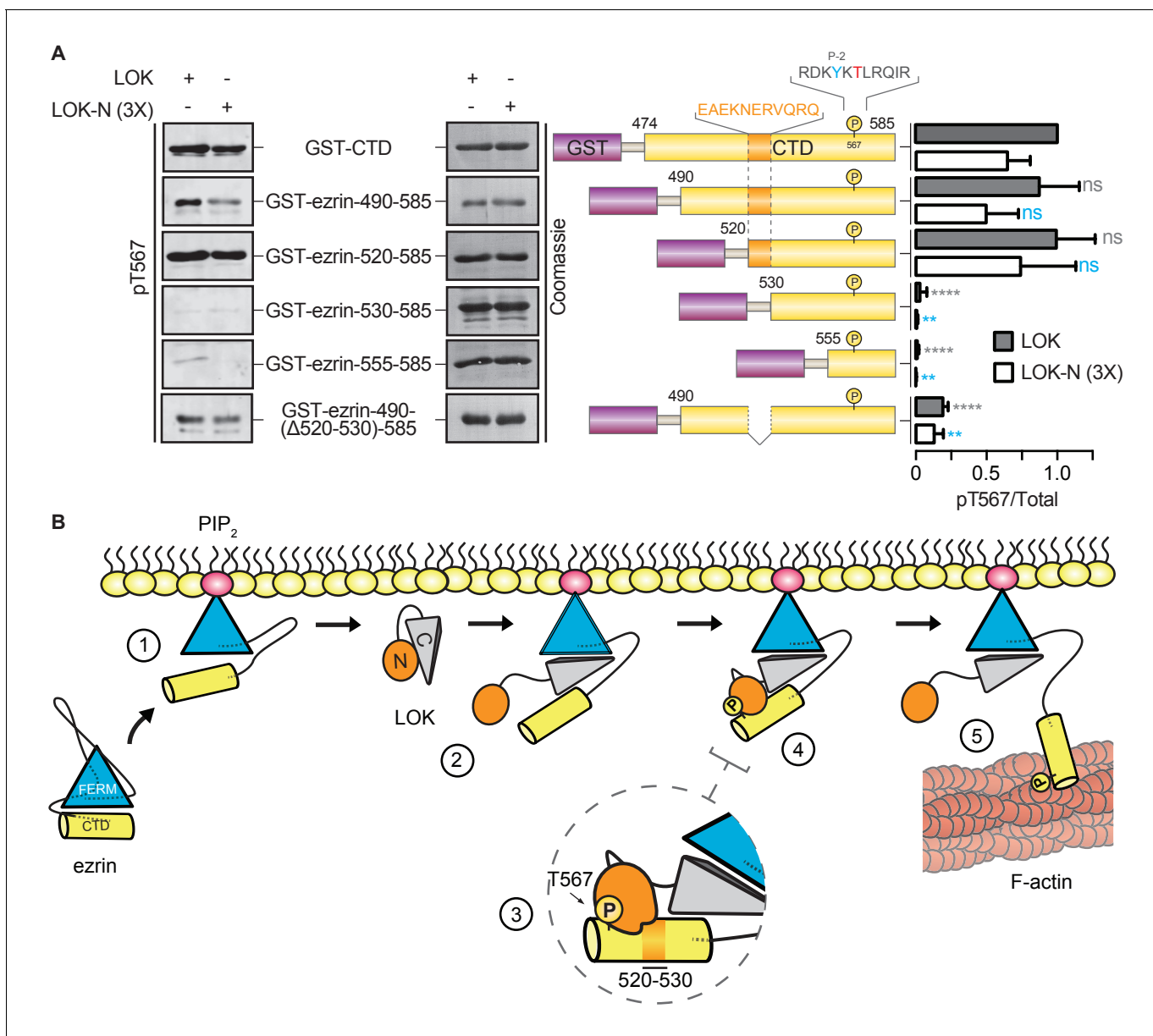


Figure 6. LOK kinase domain recognizes a docking site distal to T567 phosphorylation site. **(A)** *In vitro* kinase assay showing that 10 nM LOK and 30 nM LOK-N phosphorylate GST-ezrin-CTD, and the truncations GST-ezrin-490–585 and GST-ezrin-520–585. Phosphorylation decreases in the truncations GST-ezrin-530–585, GST-ezrin-555–585 and GST-ezrin-490(Δ520–530)–585. All substrate concentrations were 18 μM. Schematic illustration of GST-ezrin-CTD and truncations shows the distal docking site in orange letters and box. LOK kinase consensus sequence is shown in black, in which the T567 phosphorylation site is in red letters and the conserved Y565 is in blue letters. Data are represented as mean ± SE, n = 3, two-way ANOVA (See also **Figure 6—source data 1**), **p<0.0021, ****p<0.0001. **(B)** A working model depicting the proposed coincidence detection mechanism for ezrin phosphorylation. (1) Ezrin is recruited to PIP₂ and primed for phosphorylation. (2) Autoinhibition of LOK is relieved as LOK-C wedges in between FERM and ezrin-CTD. (3) LOK-N binds to distal docking site on ezrin-CTD and, (4) phosphorylates T567. (5) LOK remains tethered to activated ezrin, allowing phosphorylation of microvillar ezrin in a positive feedback loop.

DOI: [10.7554/eLife.22759.016](https://doi.org/10.7554/eLife.22759.016)

The following source data is available for figure 6:

Source data 1. Experimental replicates for **Figure 6A**.

DOI: [10.7554/eLife.22759.017](https://doi.org/10.7554/eLife.22759.017)