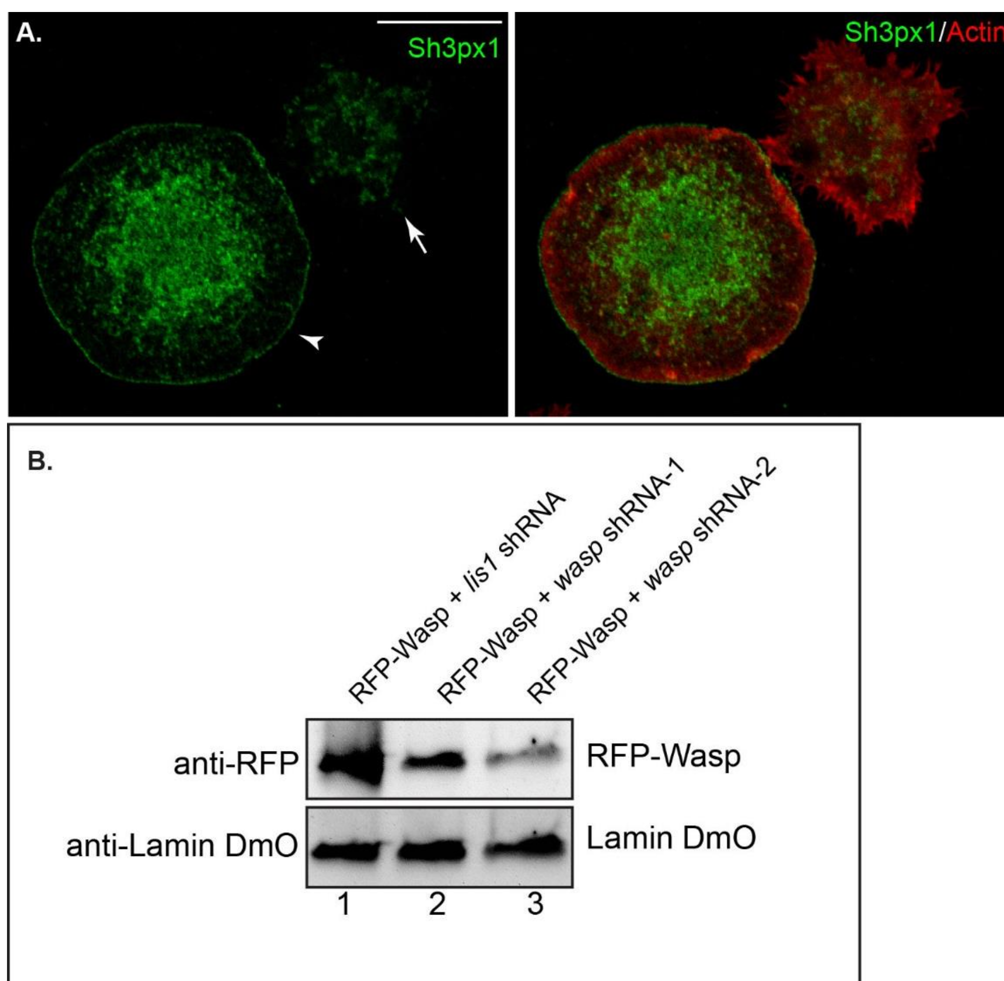

Fig. S1. Depletion phenotypes. (A) S2 cells were transfected with a plasmid expressing an shRNA targeting *sh3px1*. Three days after transfection, the cells were spotted onto con A coverslips and allowed to adhere for 2 hours. The cells were then fixed and processed for immunofluorescence using an antibody against Sh3px1 (green). The cells were also counterstained to reveal F-actin (red). The arrow indicates a cell that has been depleted of Sh3px1, whereas the arrowhead represents an un-transfected cell containing normal levels of Sh3px1. (B) S2 cells were transfected with a plasmid expressing RFP-Wasp and *lis1* shRNA (lane 1), *wasp* shRNA-1 (lane 2) or *wasp* shRNA-2 (lane 3). Three days after transfection, the cells were harvested and lysates were prepared. The lysates were run on an SDS gel and analyzed by western blotting using the indicated antibodies. Lamin DmO serves as the loading control.

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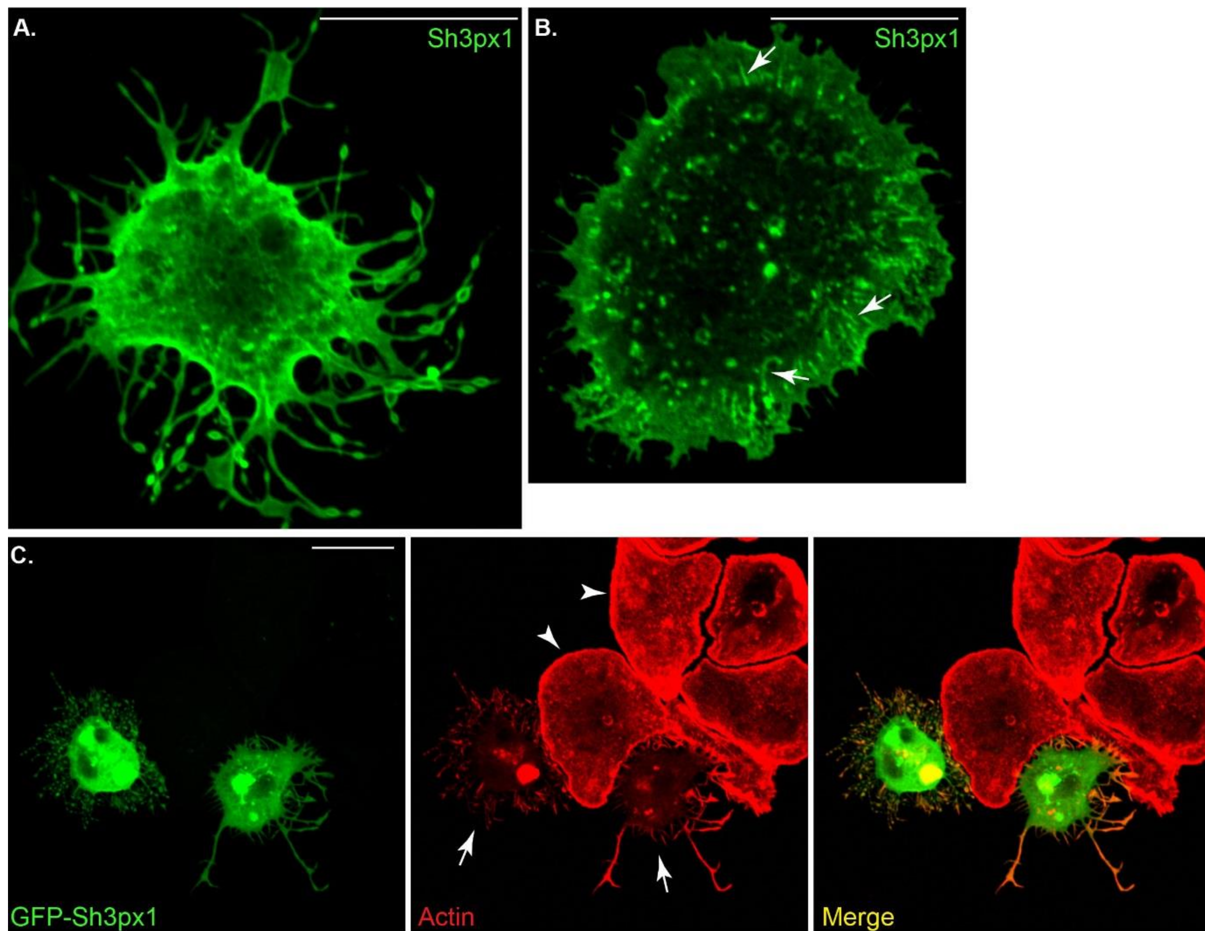

Fig. S2. Sh3px1 over-expression phenotypes. (A-B) S2 cells were transfected with a plasmid encoding untagged Sh3px1. Two days post transfection, the cells were spotted onto con A coated coverslips and allowed to adhere. Next, the cells were fixed and processed using an antibody against Sh3px1. Panel A is an example of a cell over-expressing untagged Sh3px1 that has formed long protrusions. Panel B is an example of a cell over-expressing untagged Sh3px1 that has formed tubules (arrows). (C) S2 cells were transfected with a plasmid encoding GFP-Sh3px1. Two days after transfection, the cells were spotted onto con A coated coverslips and allowed to adhere. Next, the cells were fixed, counterstained with Phalloidin-TRITC, and imaged. The cortical band of actin that is present in untransfected cells (arrowheads) is not seen in cells over-expressing GFP-Sh3px1. Instead, F-actin is present within the long cell protrusions (arrows).

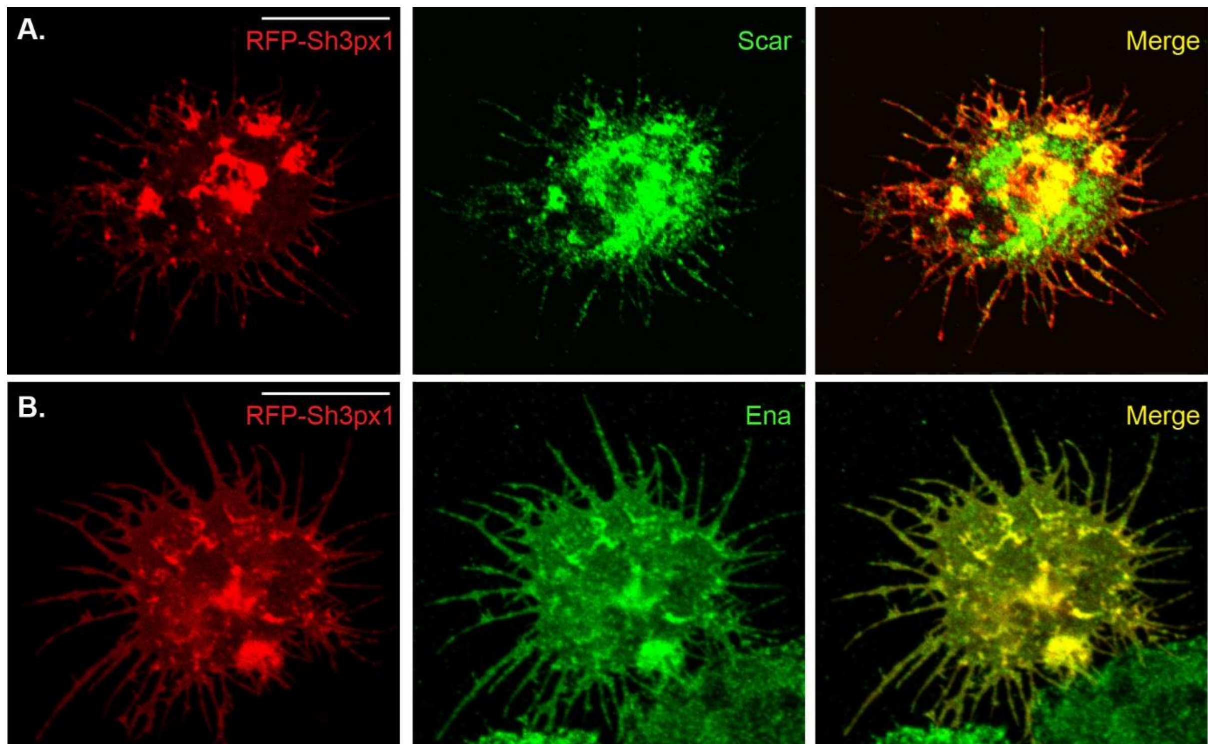

Fig. S3. Localization of Scar and Ena in Sh3px1 over-expression cells. S2 cells were transfected with a plasmid encoding TagRFP-t-Sh3px1. Two days post transfection, the cells were spotted onto con A coated coverslips and allowed to adhere. Next, the cells were fixed and processed using an antibody against Scar (A) or Ena (B).

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	Nwk	Mim
Snx9	<0.0001	<0.0001
Snx18	0.0139	0.0024
Snx33	<0.0001	<0.0001

Fig. S4. Statistic for co-localization studies.

The P-value for comparing the co-localization scores of Snx9, Snx18 and Snx33 with endogenous Sh3px1 with the co-localization scores of Mim and Nwk with endogenous Sh3px1 is indicated.

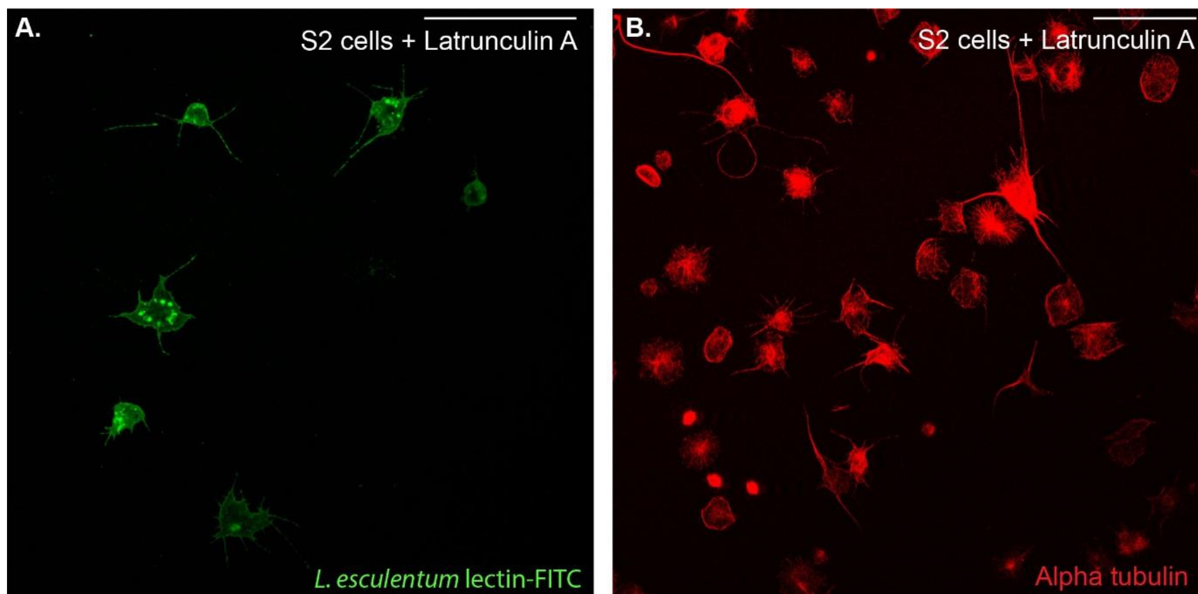

Fig. S5. Latrunculin treatment causes protrusion formation. Untransfected S2 cells were treated with LatrunculinA for 2 hours. The cells were then fixed and stained with FITC conjugated *Lycopersicon esculentum* lectin (tomato lectin) to visualize the plasma membrane (A). The cells were also labeled using an antibody against Alpha-tubulin (B). Treatment of S2 cells with this actin destabilizing drug results in the formation of cell protrusions. Ling and colleagues have shown that this process results from unrestrained microtubule growth (Ling et al., 2004).

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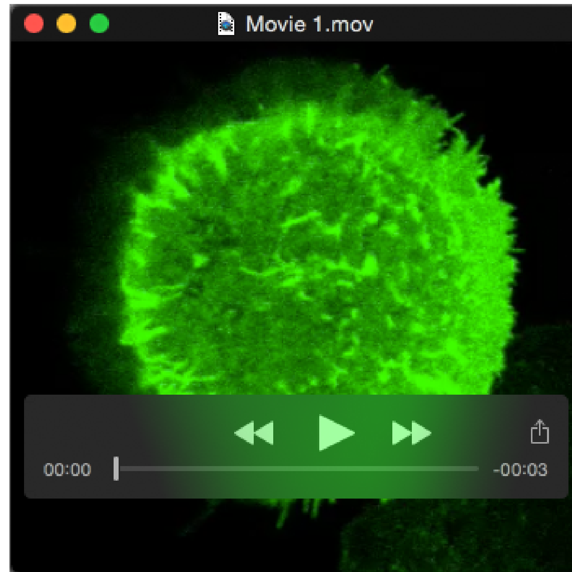
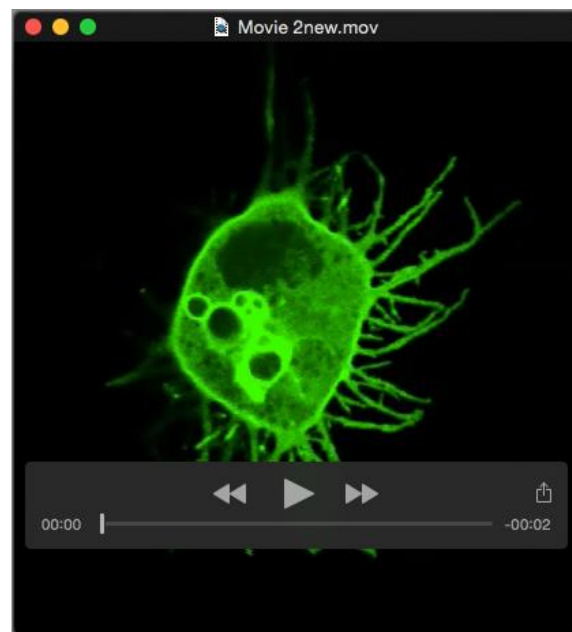
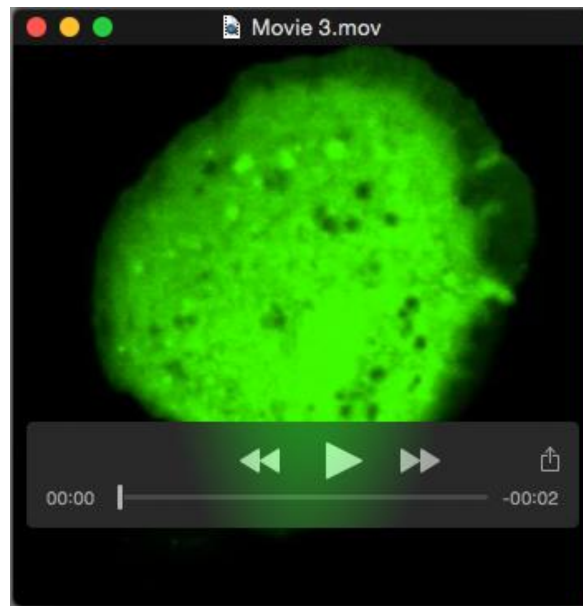


Movie 1. Example of a cell over-expressing GFP-Sh3px1. The experiment was performed by plating cells on glass bottom coverslip dishes and imaging using an inverted Zeiss 780 confocal microscope. This cell displays internal tubules and short protrusions.

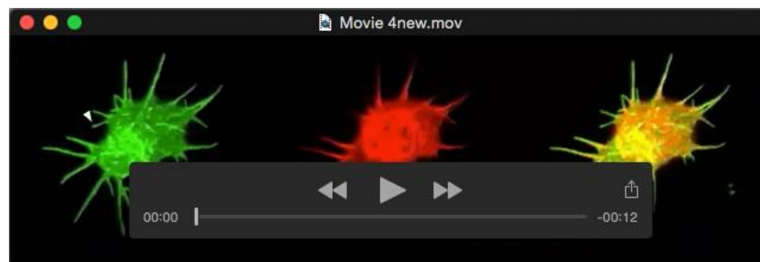


Movie 2. Example of a cell over-expressing GFP-Sh3px1. The experiment was performed by plating cells on glass bottom coverslip dishes and imaging using an inverted

Zeiss 780 confocal microscope. This cell displays long dynamic protrusions.



Movie 3. Example of a cell over-expressing GFP. The experiment was performed by plating cells on glass bottom coverslip dishes and imaging using an inverted Zeiss 780 confocal microscope. Tubules and protrusions were not observed in GFP expressing cells.



Movie 4. Example of a cell over-expressing GFP-Sh3px1 and mRuby2-Lifeact. The experiment was performed by plating cells on glass bottom coverslip dishes and imaging using an inverted Zeiss 780 confocal microscope. This cell displays long dynamic protrusions. Although GFP-Sh3px1 was present in all protrusions, mRuby2-Lifeact was not detected in some of the newly formed protrusions (arrow).

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Movie 5. Additional example of a cell over-expressing GFP-Sh3px1 and mRuby2-Lifeact. The experiment was performed by plating cells on glass bottom coverslip dishes and imaging using an inverted Zeiss 780 confocal microscope. As with the previous example, GFP-Sh3px1 was present in all protrusions. However, mRuby2-Lifeact was not detected in some of the newly formed protrusions (arrow).