Supplemental material

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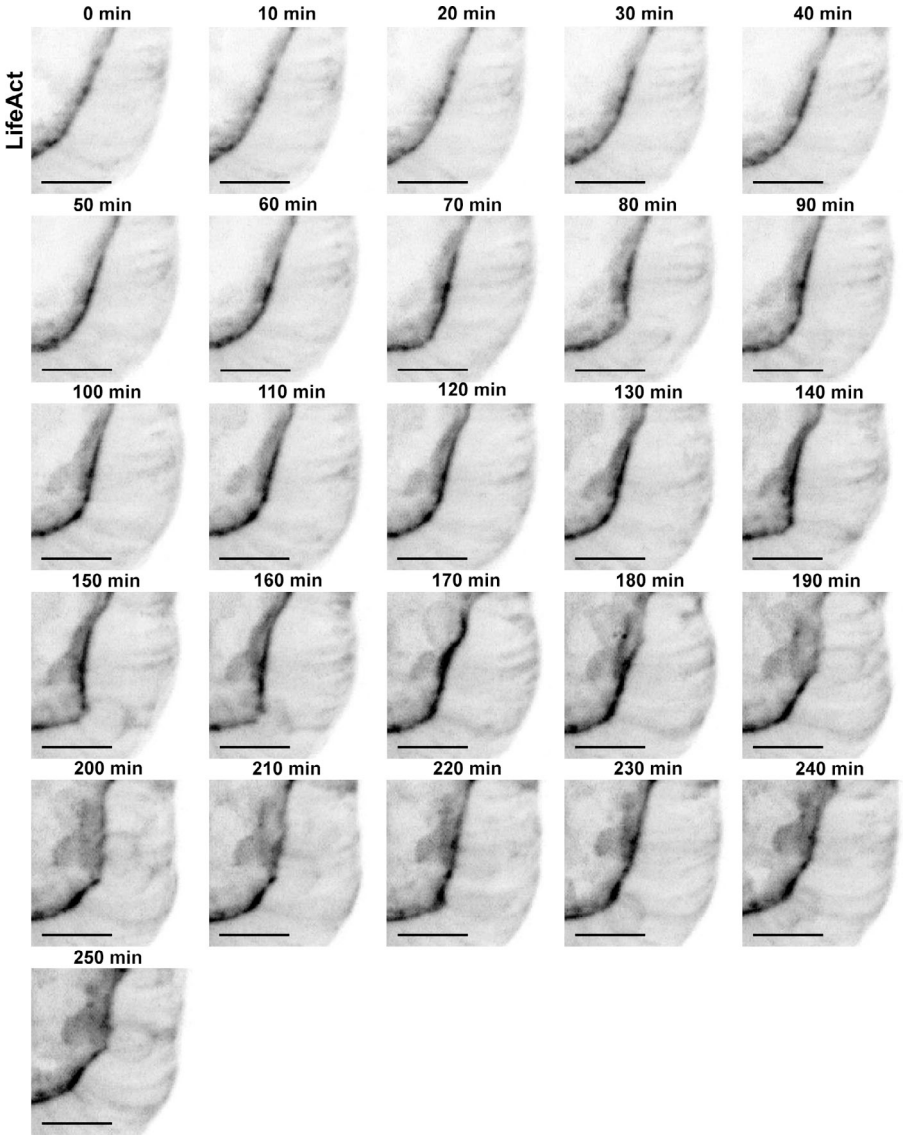


Figure S1. **Live imaging of control LifeAct-derived enteroids shows no internalization of F-actin.** Enteroids generated from the proximal small intestine (duodenum) of neonatal control LifeAct mice were imaged overnight at 37°C with 5% CO_2 to determine whether endocytosis of the apical membrane could be visualized. In control-derived enteroids, no LifeAct (F-actin)-positive inclusions were observed. Scale bars = 25 µm.

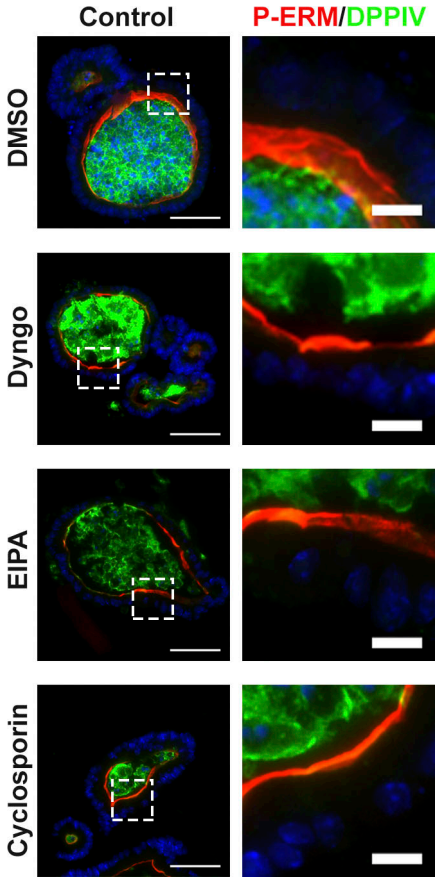


Figure S2. **Control enteroids treated with DMSO, Dyngo, EIPA, and cyclosporin show no alterations in apical membrane.** Enteroids generated from the proximal small intestine (duodenum) of neonatal control mice did not change in response to administration of DMSO, Dyngo, EIPA, or cyclosporin. The apical membrane was not altered (P-ERM, red), and DPPIV (green) was not observed below the apical brush border as is seen in Myo5B KO–derived enteroids. Enteroids generated from five mice, 15 enteroids analyzed per treatment. Scale bars = 50 µm for left panel and 10 µm for right panel.

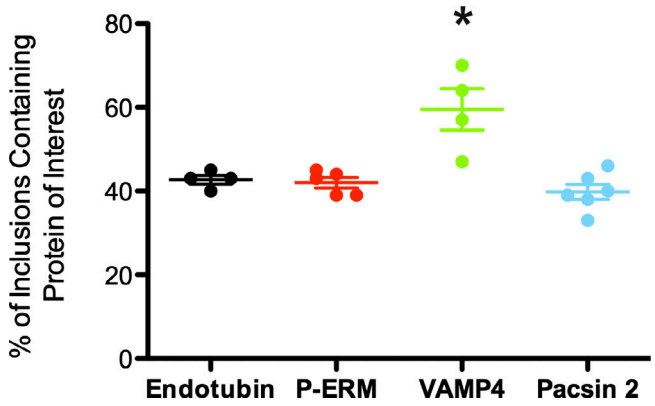


Figure S3. **Quantification of inclusions containing endotubin, P-ERM, VAMP4, or Pacsin 2.** The duodenum and jejunum of Myo5b KO mice were analyzed to determine the quantity of inclusions that were DPPIV positive and positive for endotubin, P-ERM, VAMP4, or Pacsin 2. Total inclusions were counted based on DPPIV immunostaining. Three 20× fields were analyzed per mouse to determine the percentage of inclusions that were positive for the protein of interest. Inclusions that were endotubin, P-ERM, or Pacsin 2 positive and DPPIV positive were ∼40% of the total inclusions. VAMP4- and DPPIV-positive inclusions accounted for ∼60% of the total DPPIV-positive inclusions. *n* = 4–6 Myo5b KO mice per group. *, P < 0.05. One-way ANOVA was performed with the Bonferroni post hoc test; error bars are SEM.

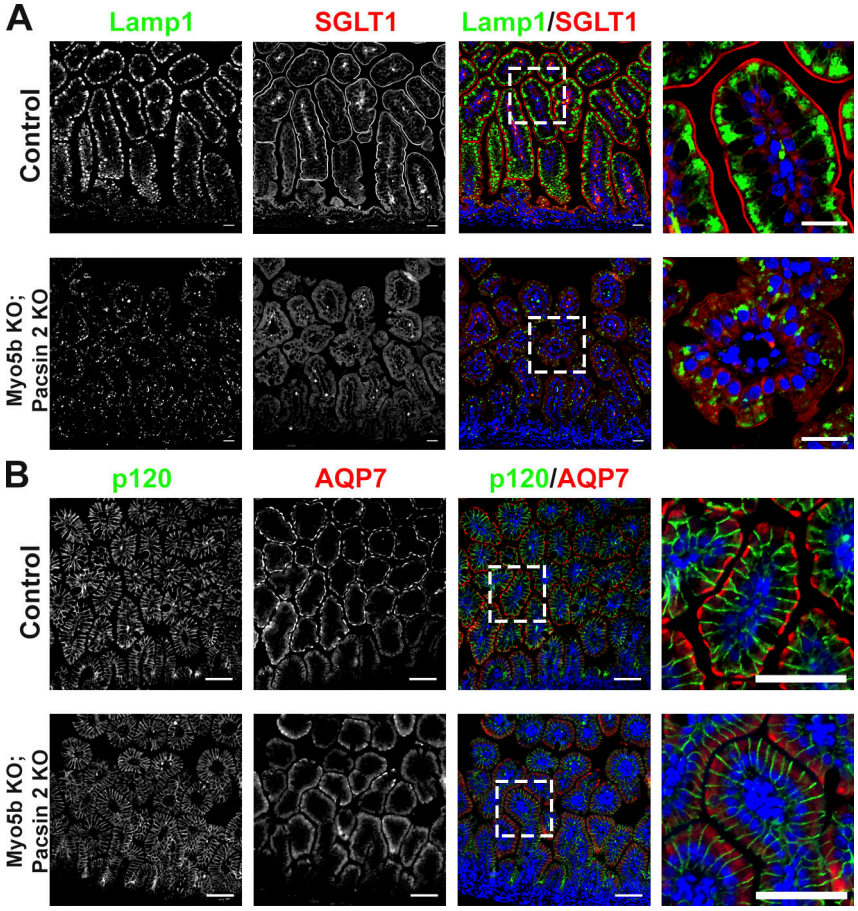
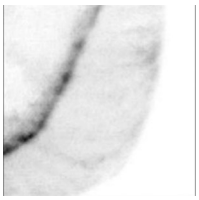
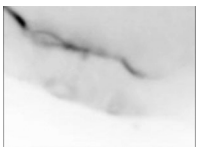


Figure S4. **Decreased inclusion formation does not correlate with improved trafficking of apical membrane proteins. (A)** Myo5b KO;Pacsin 2 KO (double KO) mice exhibited decreased apical expression of key regulators of water absorption. The sodium glucose cotransporter, SGLT1 (red), showed decreased apical expression in Myo5b KO;Pacsin 2 KO (double KO) mice, similar to Myo5b KO mice. Lamp1-positive lysosomes (green) are normally aligned below the apical membrane of neonatal enterocytes; however, in Myo5b KO;Pacsin 2 KO mice (double KO), Lamp1 is dispersed from its normal position. **(B)** The aquaporin water channel AQP7 was observed in its proper location on the apical membrane in control enterocytes. In Myo5b KO;Pacsin 2 KO mice (double KO), AQP7 expression was significantly decreased from the apical membrane. These results closely correspond to the mislocalization of apical proteins reported in Myo5b KO mice. These data suggest that a decrease in inclusion formation does not improve trafficking defects that result from loss of Myo5B. *n* = 3 mice per group. Scale bars = 50 µm.



Video 1. **Live imaging confocal microscopy of enteroids expressing LifeAct derived from the proximal small intestine of control mice.** Enteroids generated from neonatal control LifeAct mice show no inclusion formation. The apical brush border appeared stable, with no F-actin internalization. Images were acquired every 2 min; 12 frames/s.



Video 2. **Inclusion formation in LifeAct;Myo5b KO enteroids using confocal microscopy.** Confocal live imaging of LifeAct; Myo5b KO–derived enteroids show endocytosis of the apical brush border as defined by LifeAct (F-actin). Images were acquired every 2 min; 16 frames/s.