Vesicle Trafficking Regulator Genes	RNAi lines	c306-Gal4 driven RNAi		*upd*-Gal4 driven RNAi	
		No border cell cluster	Incomplete BC cluster migration	No border cell cluster	Incomplete BC cluster migration
α-Snap (soluble NSF attachment protein)	KK107910	lethal	-		
	JF03266	**46.4%** (n=97)	ns	**88.3%** (n=120)	ns
	HMS00872	0% (n=38)	0%		
	GD11986 *	**22.7%** (n=88)	31.8%	**80.4%** (n=46)	ns
Rab5	JF03335	0% (n=22)	91%	0% (n=104)	ns
	HMS00147	0% (n=64)	ns		
Syntaxin 1A	JF01829 *	**50%** (n=40)	35%	**50.6%** (n=89)	14.6%
Syntaxin 4	JF01714 *	0% (n=136)	ns		
	JF01460	0% (n=93)	ns		
	HMS02771	0% (n=86)	ns		
Syntaxin 5	JF03330 *	ns (n=67)	20.9%	0% (n=72)	12.5%
Syntaxin 6	JF03125	0% (n=148)	ns		
Syntaxin 7	JF02436 *	0% (n=65)	40%	0% (n=48)	0%
Syntaxin 8	JF02038	0% (n=122)	ns		
Syntaxin 13	HMS01723	0% (n=48)	ns		
	JF01920	0% (n=98)	ns		
Syntaxin 16	JF01924	0% (n=174)	ns	0% (n=132)	ns
	HMC03430	0% (n=104)	ns		
Syntaxin 17	JF01937 *	0% (n=52)	0%		
Syntaxin 18	JF02263 *	0% (n=87)	ns		
NSF 1 (*comatose*)	HMS01261	0% (n=56)	ns		
	JF01459	0% (n=89)	0%		
	JF01233	0% (n=65)	ns	0% (n=27)	0%
NSF2	JF02765	0% (n=48)	ns	lethal	
	HMS01262	lethal	-	lethal	
γ-Snap	JF03124	0% (n=90)	ns		

Table S1. Cell-type specific depletion of *α-Snap* or *Syx1A* leads to a failure of border cell specification, while depletion of other vesicle trafficking regulators leads to defects in border cell migration or normal, migrating border cells. The percent penetrance of

the phenotype was scored in stage 10 egg chambers. Penetrance of the phenotype caused by c306- and *upd*-Gal4 driven JF03266 *α-Snap* RNAi is the average of four and two independent experiments, respectively. Penetrance of the phenotype caused by *Syx1A* RNAi is the average of two independent experiments. While some *α-Snap* RNAi lines and *Syx1A* RNAi occasionally led to cell migration defects, the no-border-cells phenotype was more unusual; thus, this was our focus for further characterization. If the border cell migration defect was observed in less than 10% of stage 10 egg chambers, it was deemed not significant (ns) since control (c306-Gal4) egg chambers sometimes displayed defects to this extent. Rab5 disruption was used as a positive control, which resulted in incomplete border cell migration (Assaker et al., 2010); however, border cell fate was not affected and *Rab5* depletion in polar cells yielded a wild-type phenotype. In all experiments flies were incubated overnight at 29^{o}C. RNAi lines with an asterisk have been shown to cause mutant phenotypes in other tissues, which indicates they are functional (Mummery-Widmer et al., 2009, Schnorrer et al., 2010, Khuong et al., 2013, Meehan et al., 2015, Peng et al., 2015, Harris et al., 2016, Mauvezin et al., 2016).

Assaker, G., D. Ramel, S. K. Wculek, M. Gonzalez-Gaitan and G. Emery (2010). Spatial restriction of receptor tyrosine kinase activity through a polarized endocytic cycle controls border cell migration. *Proc Natl Acad Sci U S A* **107**(52): 22558-22563.

Harris, K. P., Y. V. Zhang, Z. D. Piccioli, N. Perrimon and J. T. Littleton (2016). The postsynaptic t-SNARE Syntaxin 4 controls traffic of Neuroligin 1 and Synaptotagmin 4 to regulate retrograde signaling. *Elife* **5**.

Khuong, T. M., R. L. Habets, S. Kuenen, A. Witkowska, J. Kasprowicz, J. Swerts, R. Jahn, G. van den Bogaart and P. Verstreken (2013). Synaptic PI(3,4,5)P3 is required for Syntaxin1A clustering and neurotransmitter release. *Neuron* **77**(6): 1097-1108.

Mauvezin, C., A. L. Neisch, C. I. Ayala, J. Kim, A. Beltrame, C. R. Braden, M. K. Gardner, T. S. Hays and T. P. Neufeld (2016). Coordination of autophagosome-lysosome fusion and transport by a Klp98A-Rab14 complex in Drosophila. *J Cell Sci* **129**(5): 971-982.

Meehan, T. L., S. E. Kleinsorge, A. K. Timmons, J. D. Taylor and K. McCall (2015). Polarization of the epithelial layer and apical localization of integrins are required for engulfment of apoptotic cells in the Drosophila ovary. *Dis Model Mech* **8**(12): 1603-1614.

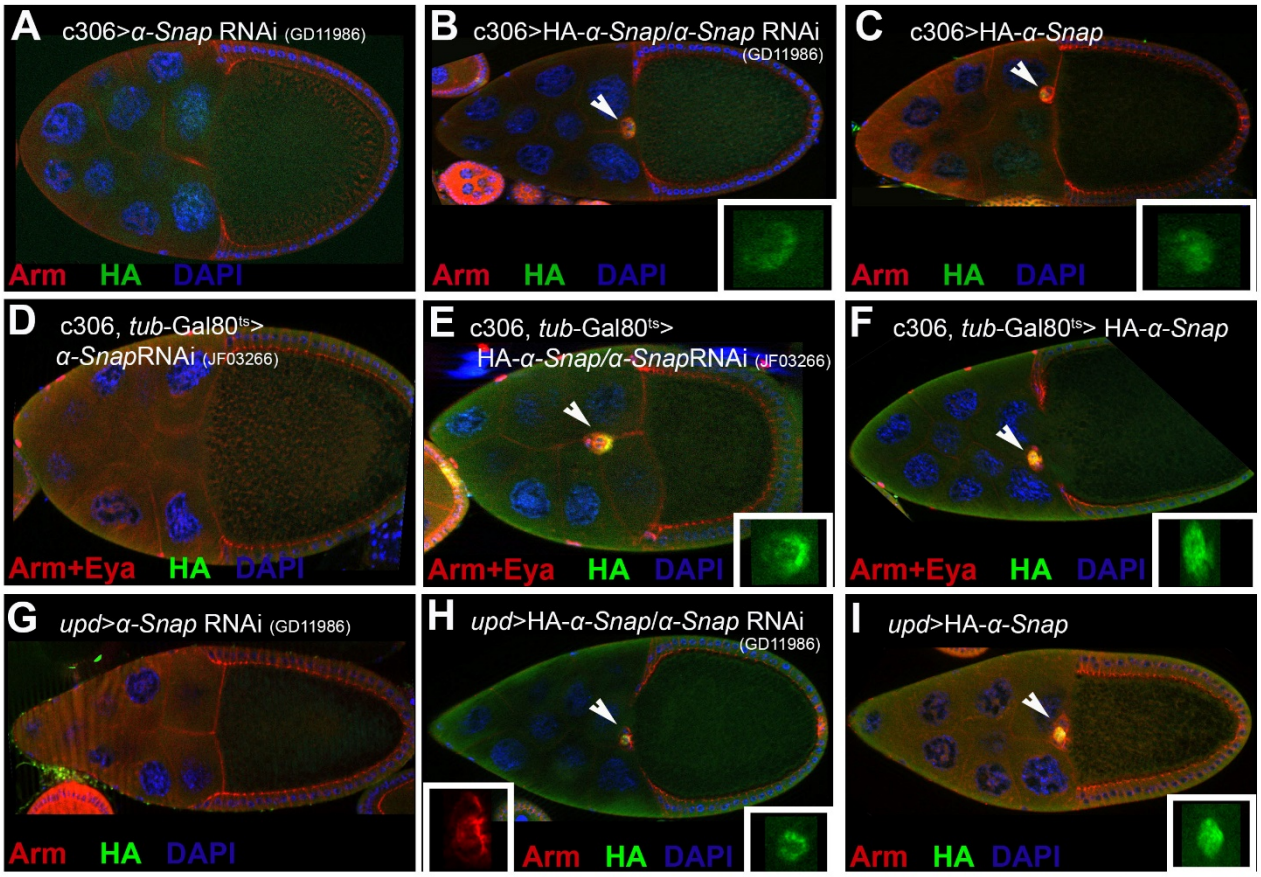
Mummery-Widmer, J. L., M. Yamazaki, T. Stoeger, M. Novatchkova, S. Bhalerao, D. Chen, G. Dietzl, B. J. Dickson and J. A. Knoblich (2009). Genome-wide analysis of Notch signalling in Drosophila by transgenic RNAi. *Nature* **458**(7241): 987-992.

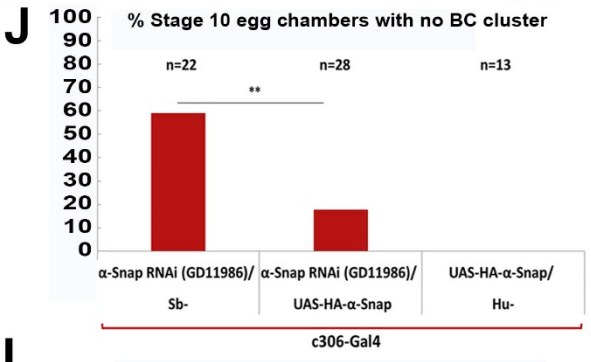
Peng, Y., J. Lee, K. Rowland, Y. Wen, H. Hua, N. Carlson, S. Lavania, J. Z. Parrish and M. D. Kim (2015). Regulation of dendrite growth and maintenance by exocytosis. *J Cell Sci* **128**(23): 4279-4292.

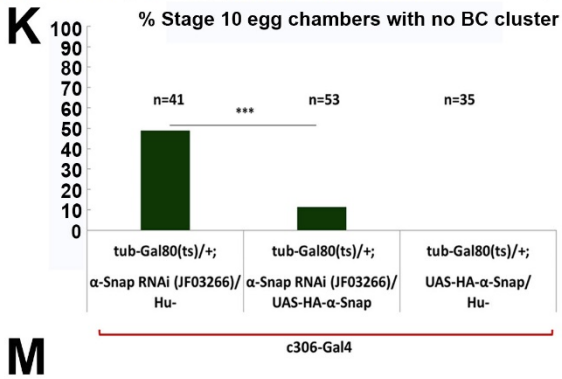
Schnorrer, F., C. Schonbauer, C. C. Langer, G. Dietzl, M. Novatchkova, K. Schernhuber, M. Fellner, A. Azaryan, M. Radolf, A. Stark, K. Keleman and B. J. Dickson (2010). Systematic genetic analysis of muscle morphogenesis and function in Drosophila. *Nature* **464**(7286): 287-291.

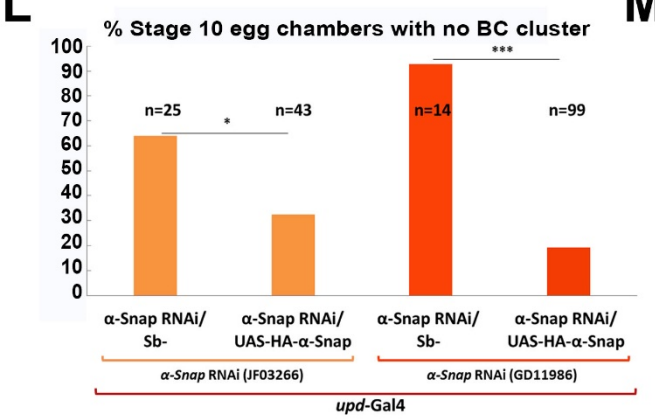
Gene	RNAi line	*tub*-Gal4 driven RNAi in embryos		c306-Gal4 driven RNAi	
		Fold change	Primers	Single RNAi	Interaction tests
α-Snap (soluble NSF attachment protein)	KK107910			lethal	
	JF03266	**0.19**	α-snap ex1-2; α-snap ex2-3; α-snap FRP		Lethal with JF01233 HMS01261, (NSF RNAi), JF02765 (NSF2 RNAi)
	GD11986				Lethal with JF02765, HMS01262 (NSF2 RNAi)
NSF (*comatose*)	HMS01261	**0.31**	comt set2; comt ex4-6		Lethal with JF02765 (NSF2 RNAi)
	JF01459				
	JF01233				
NSF2	JF02765	**0.02**	NSF2 ex2-3 NSF2 ex3-4		Lethal with HMS01261 (NSF RNAi)
	HMS01262	**0.09**	NSF2 ex2-3 NSF2 ex3-4	lethal at 25°	

Table S2. Verification of functional RNA interference. To test knockdown efficiency, *tub*-Gal4 females were crossed to males from the indicated RNAi lines. Total RNA was collected from F1 embryos and assayed by qRT-PCR in triplicates. Fold changes are represented as averages of two or four experiments. Endogenous ovarian expression of *α-Snap, NSF,* and *NSF2* mRNAs was verified using the same primer sets. For interaction tests, RNAi lines were balanced and combined as indicated, then males carrying two RNAi insertions were crossed to c306-Gal4 females.









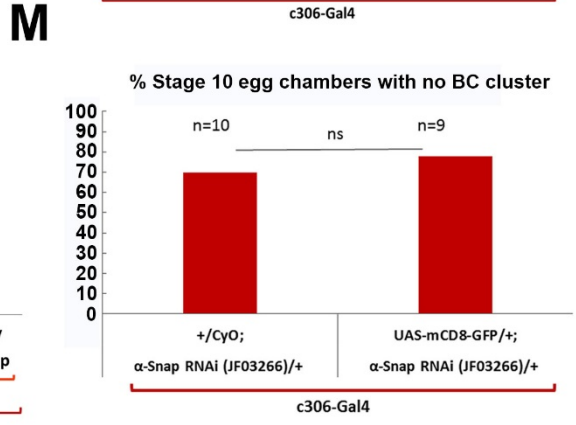


Figure S1. α-Snap is required in the anterior follicle cells to induce border cell fate.

Depletion of *α-Snap* in the anterior follicle cells (A&D) and in polar cells (G) leads to egg chambers that lack the border cell cluster. c306- and *upd*-Gal4-driven expression of HA-tagged *α-Snap* in the depleted egg chambers rescues the lack of a border cell cluster phenotype caused by the RNAi (B, E&H, arrowheads, insets; border cells are marked by HA expression (green) and Arm (red), and DAPI stain (blue). Over-expression of *α-Snap* alone in the control egg chambers (c306-Gal4, c306-Gal4; *tub*-Gal80 (ts) and *upd*-Gal4) does not affect border cell specification (C, F&I). (J-M) Comparison of the penetrance of the no border cell (BC) cluster phenotype caused by *α-Snap* RNAi to that caused by the RNAi plus expression of HA-tagged *α-Snap* in the anterior follicle cells (J&K) and polar cells (L). The presence of a second UAS in the genome (UAS-GFP), which might dilute out the concentration of Gal4, does not lead to suppression of the phenotype caused by *α-Snap* RNAi (M). *indicates $p< 0.05$, **$p<0.005$, *** $p< 0.0005$ significance value by two-tailed Fisher's exact test. Not significant change is shown as ns. Genotypes with no bar did not have any cases of the no border cell cluster phenotype. (A-C) and (J) flies were incubated at 29°C for 20 hours prior to dissection. (D-F) and (K) flies were incubated at 31°C (non-permissive temperature for $Gal80^{ts}$) for 54 hours prior to dissection. (G-I) and (L) flies were incubated at 29°C for 45 hours (for the JF03266 RNAi line (L)) and for 26 hours (for the GD11986 RNAi line (G-I & L)) prior to dissection. (M) flies were incubated at 29°C for 48 hours prior to dissection.

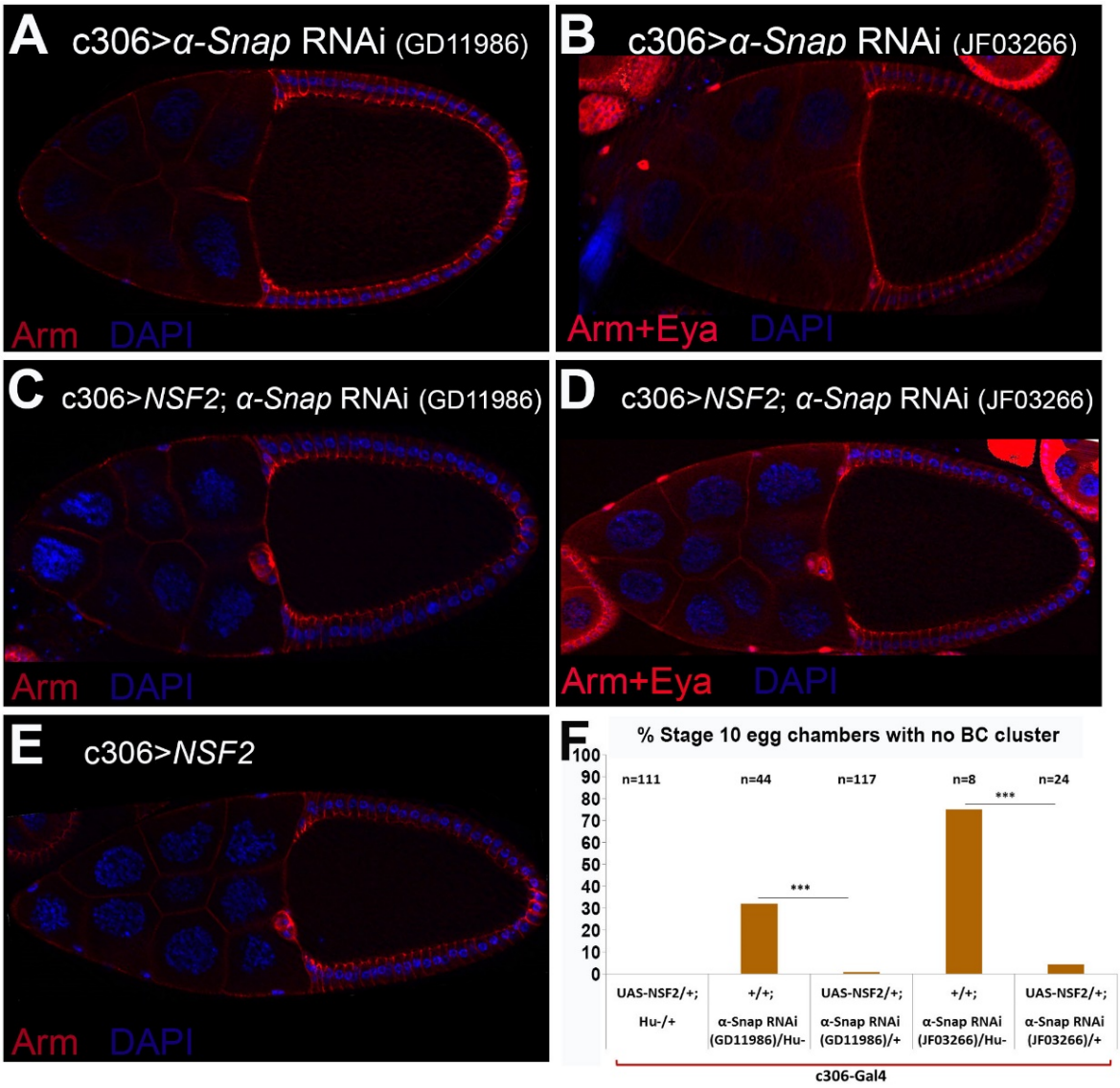


Figure S2. **NSF2 and α-Snap function together to regulate border cell specification.** (A-B) c306-Gal4-driven depletion of *α-Snap* using either the GD11986 (A) or JF03266 (B) RNAi line results in egg chambers that lack a border cell cluster. (C-E) Over-expression of *NSF2* using c306-Gal4 rescues the phenotype caused by *α-Snap* RNAi (C-D), while overexpression of *NSF2* alone yields a wild-type phenotype (E). (F) The penetrance of the phenotype caused by *α-Snap* RNAi is significantly reduced upon over-expression of *NSF2*. ***indicates p< 0.0005 significance value by two-tailed Fisher's exact test. Flies bearing JF03266 RNAi and the ones additionally expressing *NSF2* (c306-Gal4; UAS-*NSF2*; *α-Snap* RNAi (JF03266) flies) were incubated at 29°C for 24-48 hours prior to dissection.

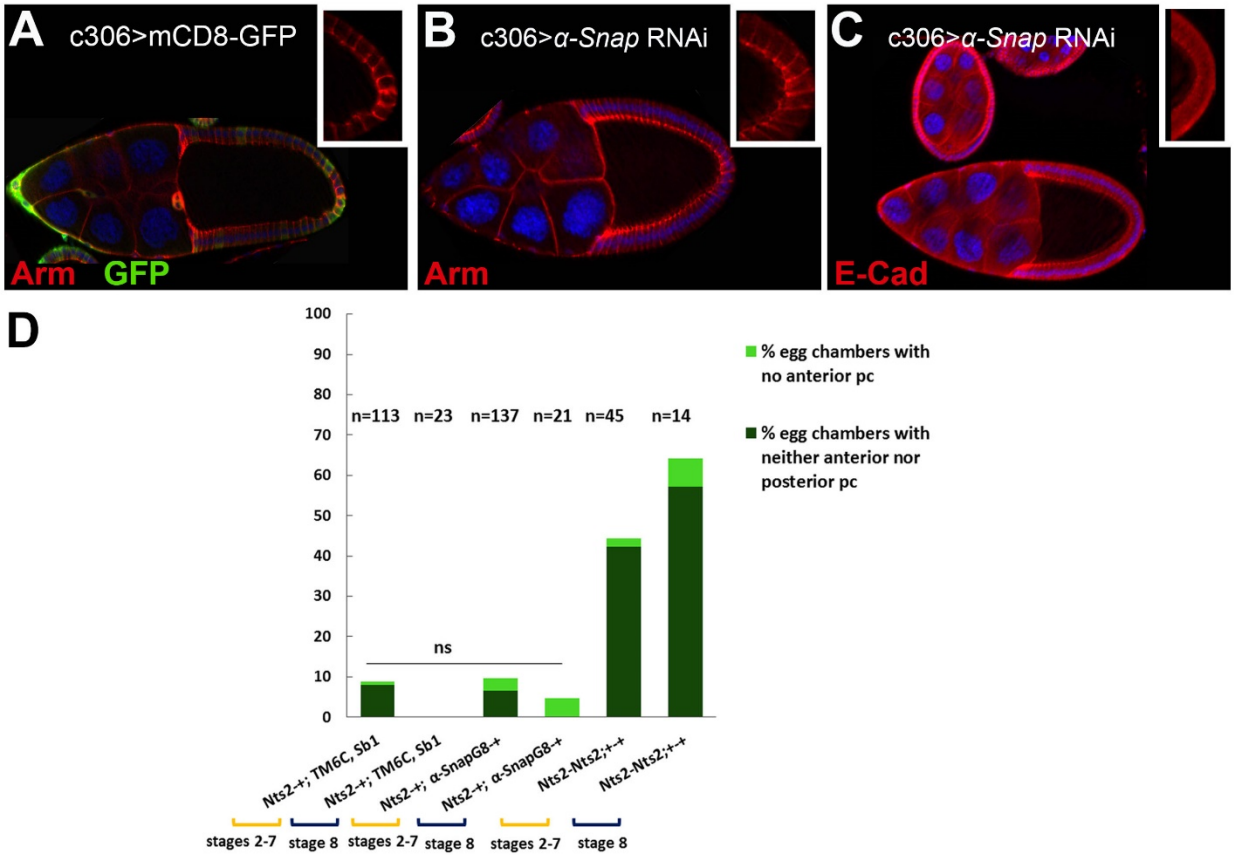
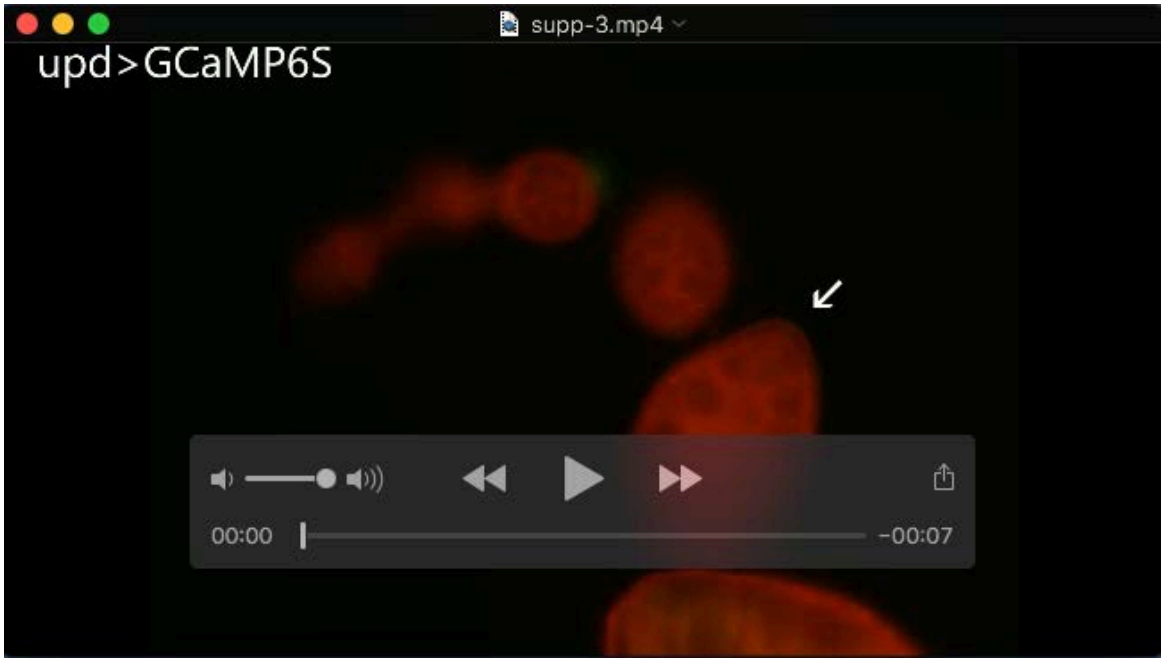


Figure S3. α-Snap does not affect trafficking of all cell surface molecules. (A-C) In egg chambers with *α-Snap* depletion, cell-surface adhesion molecule complexes are properly localized. c306-Gal4 drives membrane-tethered GFP expression (green) in the anterior and posterior follicle cells (A). Arm (A,B, red) and E-Cad (C, red) localization patterns in the posterior follicle cells appear normal in a c306-Gal4-driven *α-Snap* RNAi egg chamber. Insets in (A-C) are magnified views of posterior follicle in the control and in the *α-Snap* depleted egg chambers. (D) *α-Snap* and *Notch* (*N*) do not genetically interact during polar cell specification. Penetrance of stages 2-8 egg chambers that lack either only the anterior or both the anterior and the posterior polar cells (pc) in the N^{ts2}/+ heterozygous, N^{ts2}/+; α-Snap^{G8}/+ double heterozygous or N^{ts2}/N^{ts2} homozygous flies. Two-tailed Fisher's exact test was run to calculate the statistical significance. All flies were incubated at 29°C (non-permissive temperature for *ts2* allele of *N*) for 116 hours.

Movie 1: Transient GFP fluorescence in a polar cell prior to border cell specification in a *tub*-Gal4>UAS-GCaMP6m egg chamber. A 7:20 (seven minute, twenty second) portion of a 40 minute-long time lapse movie. Images were captured every 10 seconds with a 1 second exposure time. Pulses are seen in the stage 6/7 egg chamber.



Movie 2: Transient GFP fluorescence in polar cells in a stage seven *upd*-Gal4>UAS-GCaMP6s egg chamber. An 11 minute portion of an hour-long time lapse movie. Images were capture every 20 seconds, with a 1.24second exposure time; red shows FM4-64 marking the cell membranes. A pulse is seen in the stage 8 egg chamber.

Movie 3: Periodic GFP fluorescence in polar cells prior to border cell specification in a c306-Gal4-UAS-GCaMP6m egg chamber. A 1 h, 37 min, 20 s (01:37:20) portion of a two hour-long time lapse movie. Images were captured every 20 second with a 1 second exposure time in the green channel. Pulses are seen in the anterior of the stage 7/8 egg chamber and the posterior of the younger egg chamber.