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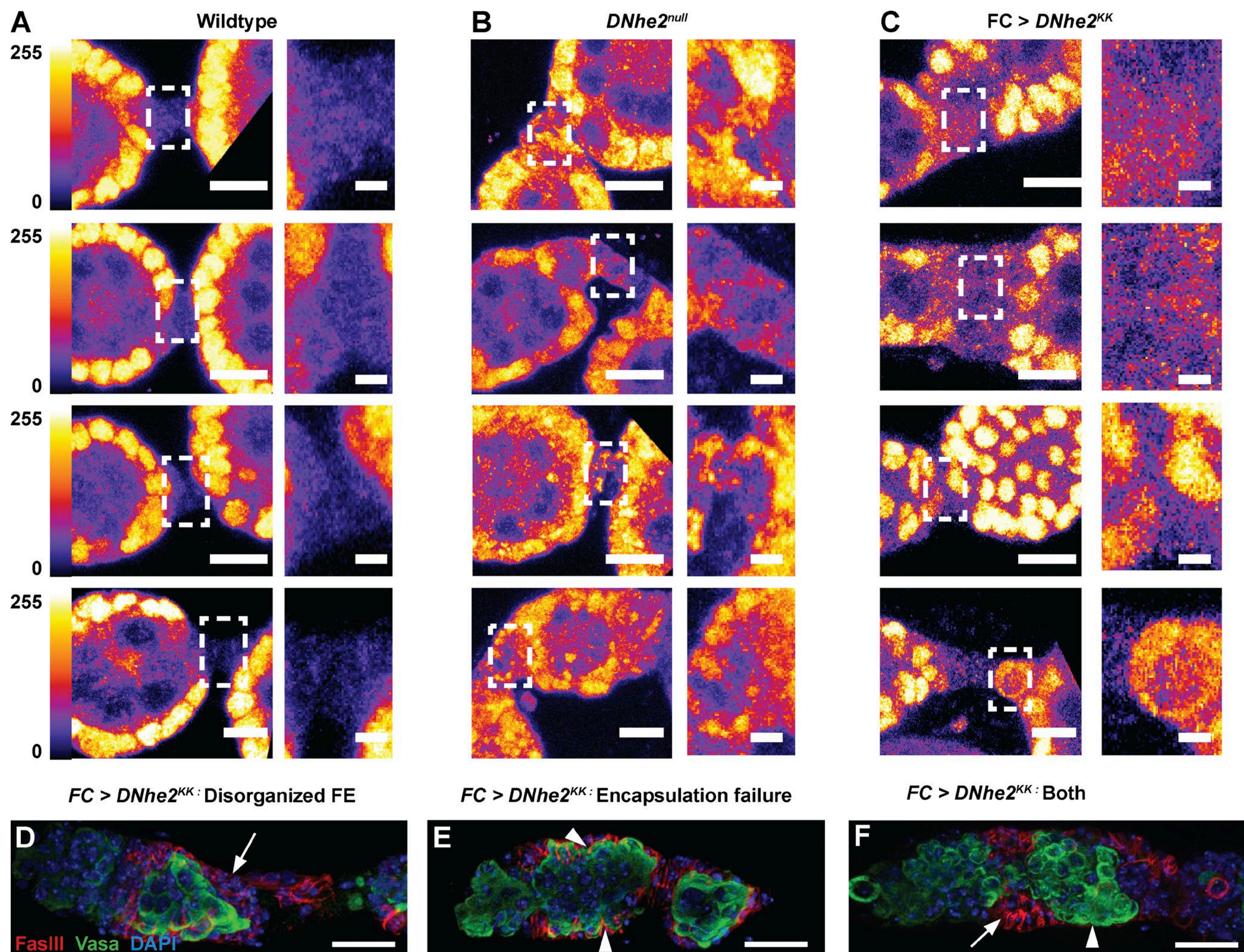

Figure S1. **Additional images of the Eya channel for images stained with Cas and Eya.** (A–C) Additional examples of Eya staining in the stalk cells of ovarioles from wild-type (A), *DNhe2*^{null} (B), and *FC-Gal4>UAS-DNhe2*$^{KK\ RNAi}$ (C) flies. The Eya channel has been pseudocolored to reflect intensity values. Eya is misexpressed and enriched in the cytoplasm of stalk cells *DNhe2*^{null} and *DNhe2*^{KKRNAi} expressing ovarioles. Boxed regions are enlarged to the right of each image. Bars: (main images)10 µm; (insets) 2 µm. (D–F) Expression of *DNhe2*$^{KK\ RNAi}$ with FC-Gal4 causes morphological defects in the germarium that are similar to the phenotypes observed in *DNhe2*^{null} germaria, including a disorganized follicle epithelium (arrow; D), encapsulation defects (arrowheads; E), and both a disorganized follicle epithelium (arrow) and encapsulation defects (arrowhead; F). Bars, 20 µm.

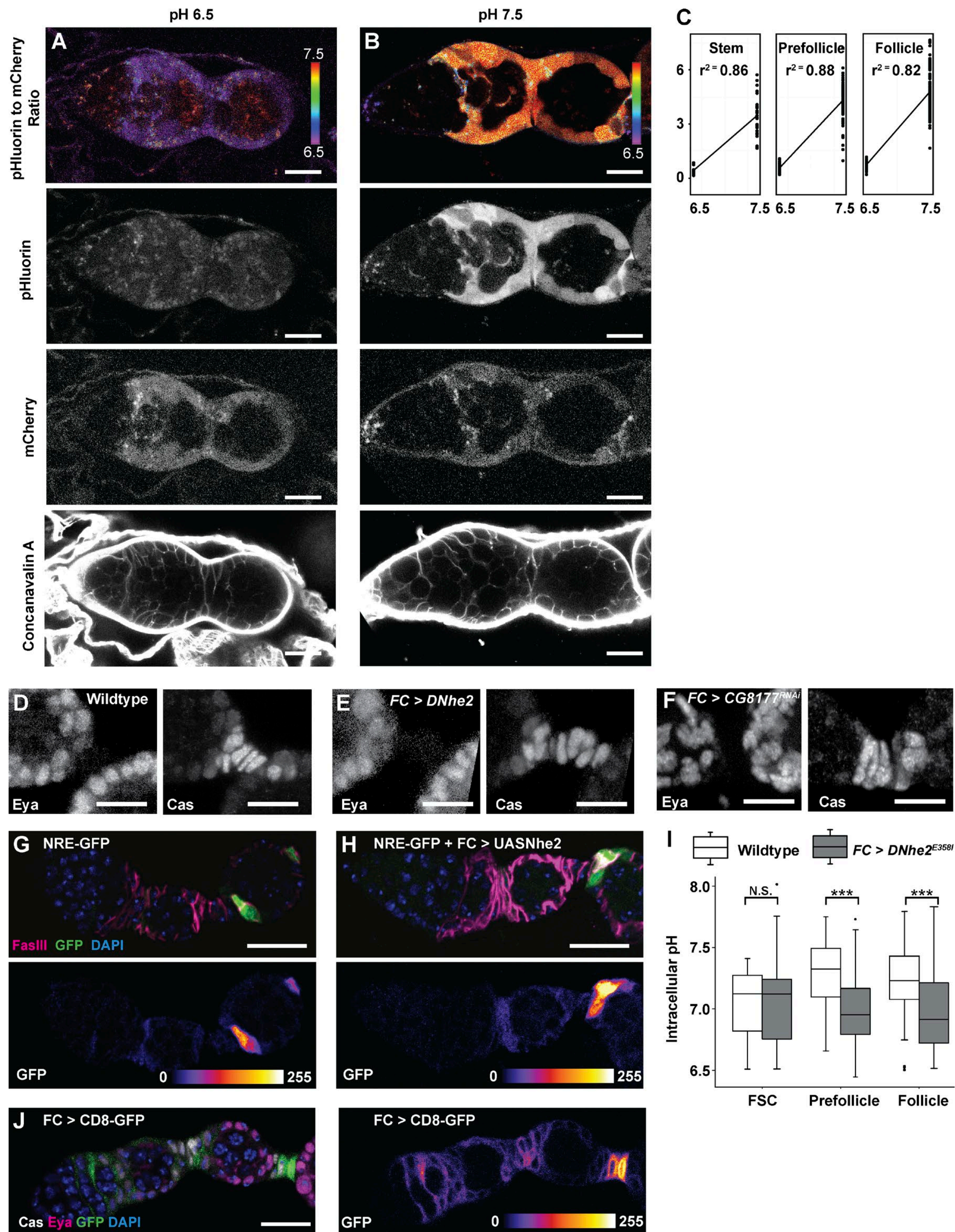

Figure S2. **Example images of calibration curves, example calibrations for quantitative pH imaging, and images of the Notch reporter, NRE-GFP, as well as quantitative pH measurements for *FC > DNhe2*^{E358I}.** (A and B) Germaria in nigericin buffer with pHi equilibrated to pH 6.5 (A) or 7.5 (B) units. In these conditions, the ratio of pHluorin fluorescence to mCherry fluorescence became uniform throughout the germaria, with low pHluorin fluorescence intensity at a pH of 6.5 and high pHluorin fluorescence intensity at a pH of 7.5. The pseudocoloring indicates the ratio of the fluorescence intensities of pHluorin to mCherry, which correlates with pHi. Colored bars indicate pHi. For pHi measurements, germaria were stained with concanavalin A conjugated to Alexa Fluor 647 (bottom panels in A and B) to identify cell boundaries so each cell could be accurately measured independently. (C) Representative calibration curves for a typical experiment, in this case wild-type FSC clones with mCherry::pHluorin. r^2 values are indicated. (D–F) Single Eya (D–F, left) and Cas (D–F, right) channels corresponding to the insets in Fig. 3 (C–E, right). (G and H) Germaria stained for FasIII and GFP with the Notch pathway reporter, NRE-GFP, alone (G) or in combination with *DNhe2* overexpression (H). Pseudocolored image reflecting the intensity of the GFP channel is shown in G and H (bottom). (I) pHi does not increase when a mutationally inactive *DNhe2*^{E358I} is overexpressed. n = 19–25 germaria; n = 4 independent replicates. ***, $P < 0.001$. (J) A germarium stained with Cas (gray), GFP (green), Eya (magenta), and DAPI (blue) with FC-Gal4 driving expression of CD8::GFP. Pseudocoloring in J (right) shows uniform intensity of the GFP channel in pFCs and FCs. Bars: (A, B, G, H, and J) 20 µm; (D–F) 10 µm.

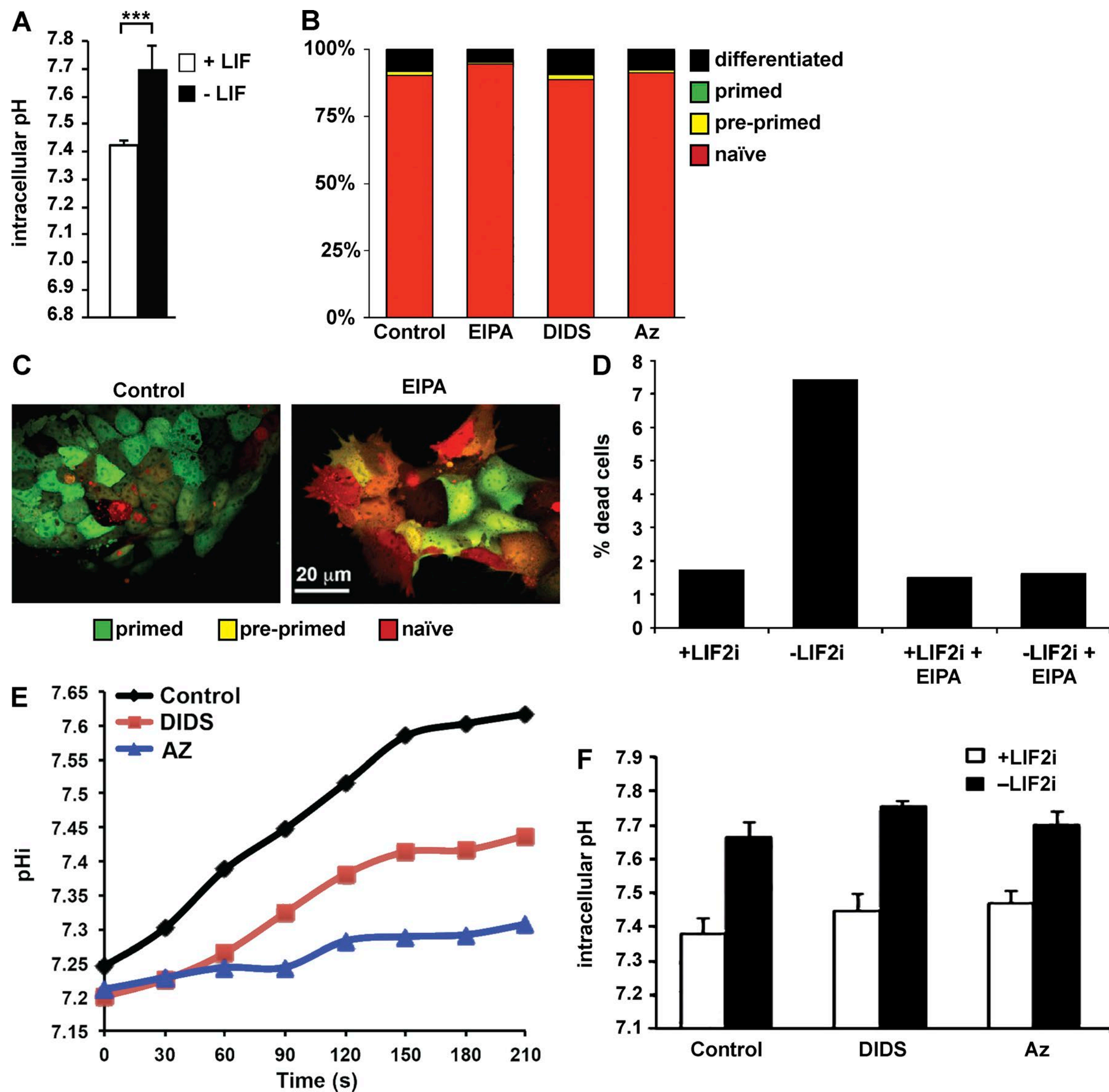

Figure S3. **Additional aspects of the interaction between changes in pHi and mESC differentiation, including lack of an effect of EIPA on cell survival, dual-reporter expression indicating EIPA maintains more cells in a naive state, and increased pHi in differentiating cells being independent of relieving MEK inhibition.** (A) Cells maintained for 2 wk in LIF (without 2i) have increased pHi 48 h after LIF removal. ***, P < 0.001. (B) The proportion of naive (*mir-290-mCherry*$^+$, *mir-302-eGFP*$^-$) dual-reporter cells cultured with LIF2i was not different in the presence of EIPA, DIDS, or Az compared with controls. (C) Images of dual-reporter cells maintained without LIF2i for 5 d show that more cells express *mir-290-mCherry*$^+$ in the presence versus absence of EIPA. (D) Cell death was measured by flow cytometry as increased DAPI intensity. The percentage of dead cells increased with the removal of LIF2i (1.7 to 7.4%) but did not change with EIPA treatment (1.5% with LIF2i, 1.6% without LIF2i). *n* = 2 independent cell preparations. (E) DIDS and Az attenuate pHi recovery after rapidly switching cells from a nominally HCO_3-free Hepes buffer at 0% CO_2 to a Hepes-free buffer containing 25 mM $NaHCO_3$ superfused with 5% CO_2. (F) The pHi of cells maintained for 72 h with and without LIF2i was not different in the presence of DIDS or Az compared with controls.

Table S1. **Differences in the slope and intercept of the linear regression model using two or three calibration points**

Cell type	Intercept	Slope	r^2
Two-point calibration			
Follicle	−6.222	1.035	0.917
Prefollicle	−5.741	0.954	0.903
Stem	−5.785	0.954	0.852
Three-point calibration			
Follicle	−6.247	1.038	0.922
Prefollicle	−5.776	0.958	0.909
Stem	−5.685	−0.945	0.867
Three point versus two point			
Follicle	0.025	−0.003	−0.005
Prefollicle	0.035	−0.004	−0.006
Stem	−0.100	0.009	−0.015

Table S2. **Summary of the pHi estimates for the wild-type controls in all experiments**

Experiment	*DNhe2*overexpression	*DNhe2 RNAi*	*DNhe2*^{E358I} overexpression	CG8177 RNAi	Flipout clones
Driver	FC-Gal4	FC-Gal4	FC-Gal4	FC-Gal4	Actin-Gal4
Stem pHi	7.07	6.92	7.04	7.05	6.79
pFC pHi	7.10	7.14	7.27	7.22	7.03
FC pHi	7.17	7.09	7.24	7.25	7.27
Δ *pHi* (stem versus pFC)	0.03	0.21	0.23	0.17	0.25
P-value for stem versus pFC	0.58	3.83×10^{-3}	0.01	0.04	4.85×10^{-5}
Δ *pHi* (stem versus FC)	0.11	0.17	0.20	0.21	0.48
P-value for stem versus pFC	0.06	1.06×10^{-3}	0.03	0.01	3.03×10^{-13}
n (germaria)	20	16	19	17	36