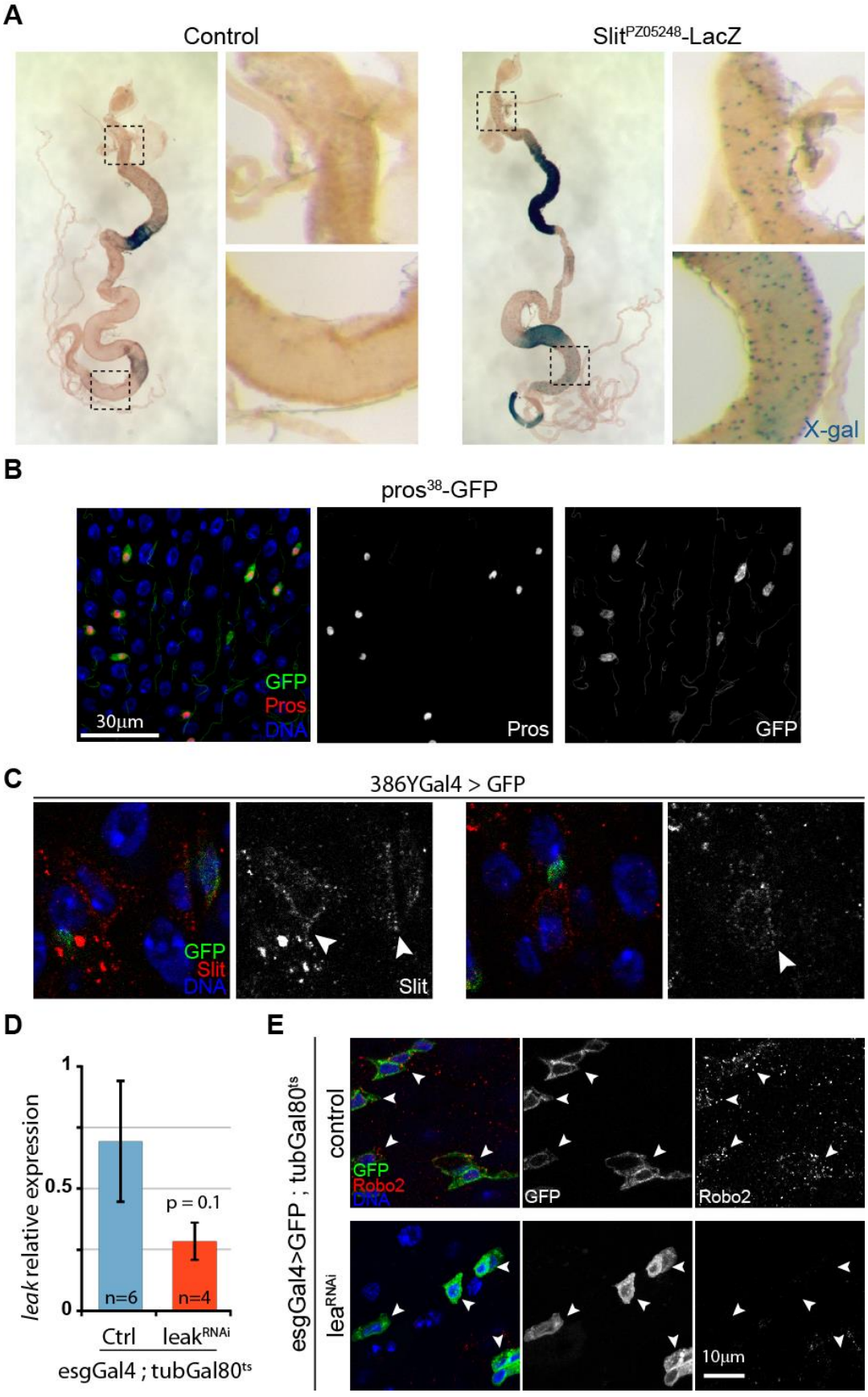
A
Control
Slit^PZ05248-LacZ
X-gal
B
pros^38-GFP
GFP
Pros
DNA
30µm
Pros
GFP
C
386YGal4 > GFP
GFP
Slit
DNA
Slit
D
leak relative expression
1
0.5
0
p = 0.1
n=6
n=4
Ctrl
leak^RNAi
esgGal4 ; tubGal80^ts
E
esgGal4>GFP ; tubGal80^ts
control
lea^RNAi
GFP
Robo2
DNA
GFP
Robo2
10µm

**Figure S1 (relates to Figure 1). Characterization of Slit and Prospero reporter lines, Slit expression pattern and manipulation of the Robo2 expression in ISCs.**

(A) Whole mount of Drosophila intestines labeled by X-gal staining, from wild-type flies or flies carrying a P-element inserted in the *slit* locus ($Slit^{PZ05248}$). Control gut shows the endogenous β-galactosidase activity.

(B) The GFP-containing P-element inserted in the *prospero* locus in the fly line $pros^{38}$ allows the expression of GFP in prospero-positive EEs.

(C) Images from single confocal sections showing the accumulation of the Slit protein at the periphery of ISC/EBs cells. GFP expression marks EEs in this genotype.

(D,E) Expression of a dsRNA directed against Robo2 ($leak^{RNAi}$) using the $esgGal4^{ts}$ driver, for 10 days at 29°C, is sufficient to decrease the expression of this receptor in the intestine and in esg-positive, as shown by qRTPCR and immunocytochemistry.

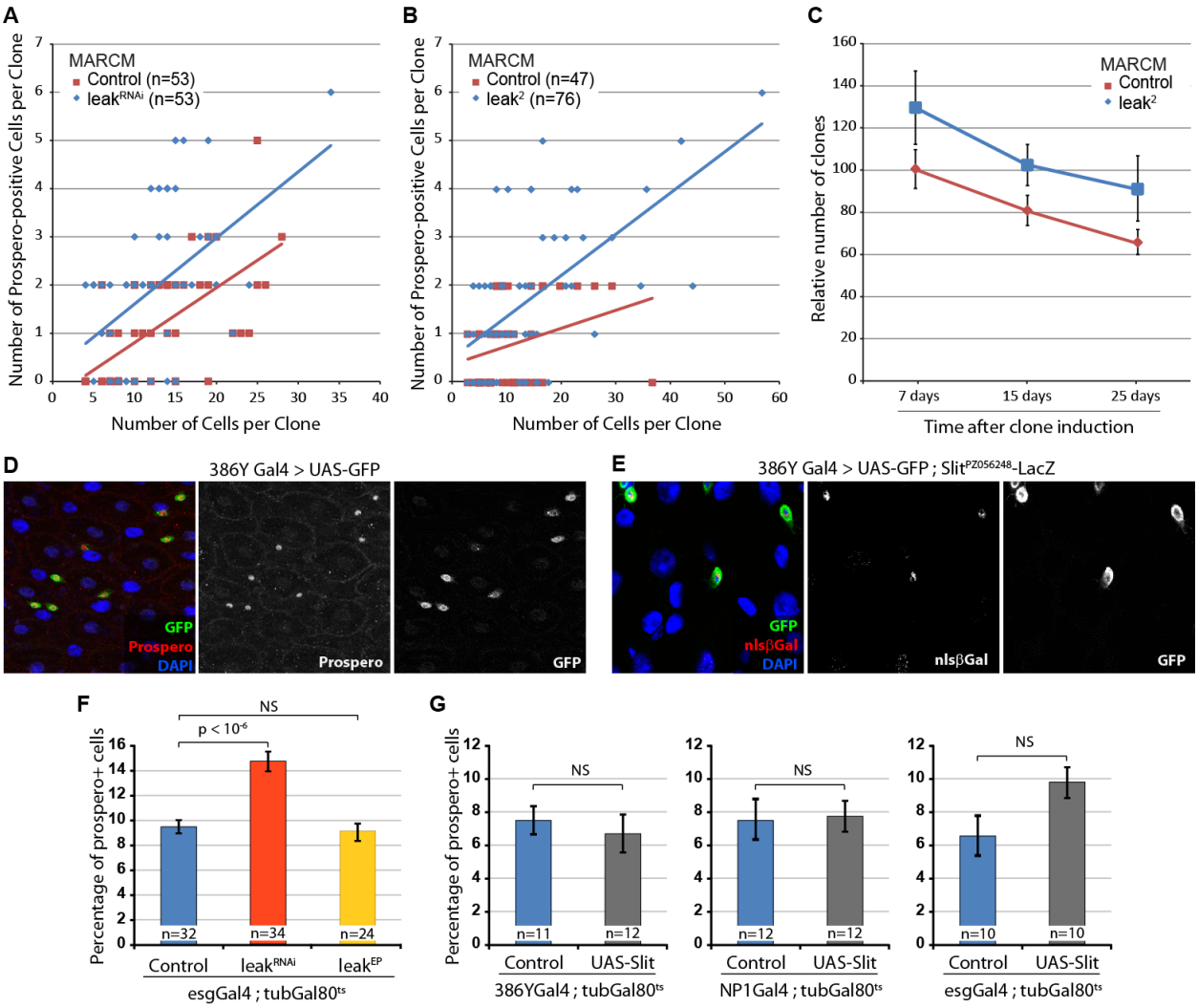


**Figure S2 (relates to Figure 2). Knock-down or mutation of the Robo2 receptor increases the proportion of EEs in ISC clones, while over-expression of Slit or Robo2 does not affect the composition of the intestinal epithelium.**

(A,B) Quantification of the total number of cells and the number of Prospero-positive cells in lea$^{RNAi}$ expressing clones (A) and *lea*$^{2}$ homozygous clones (B), compared to their respective controls. Solid lines represent linear regressions of the data sets.

(C) Time course analysis of the maintenance of wild-type and *lea*$^{2}$ homozygous MARCM clones in the fly posterior midgut. The number of GFP-labeled clones was normalized to the number of

clones in wild-type flies 7 days after heat-shock. Mutant clones are maintained at the same rate as control clones, demonstrating that Robo2 mutation does not affect ISC self-renewal.

(D,E) The 386YGal4 driver is active in enteroendocrine cells. The Gal4-containing P-element P{GawB}386Y inserted in the *amontillado* locus is sufficient to drive the expression of UAS-GFP in Prospero-positive (D) and Slit-expressing (E) EEs.

(F) Knocking-down the expression of Robo2/leak ($leak^{RNAi}$) in ISCs, for 10 days, leads to an increase of EE proportion in the posterior midgut, while over-expression of Robo2/leak ($leak^{EP}$) does not affect the composition of the epithelium.

(G) Over-expressing Slit in EEs (386YGal4$^{ts}$ driver), ECs (NP1Gal4$^{ts}$) and ISC/EBs (esgGal4$^{ts}$) for 10 days does not affect the proportion of EEs in the posterior midgut.

n represents the number of clones in A and B and the number of intestines analyzed in F and G. p-value from two-tailed Student's t-test. NS, Not Significant.

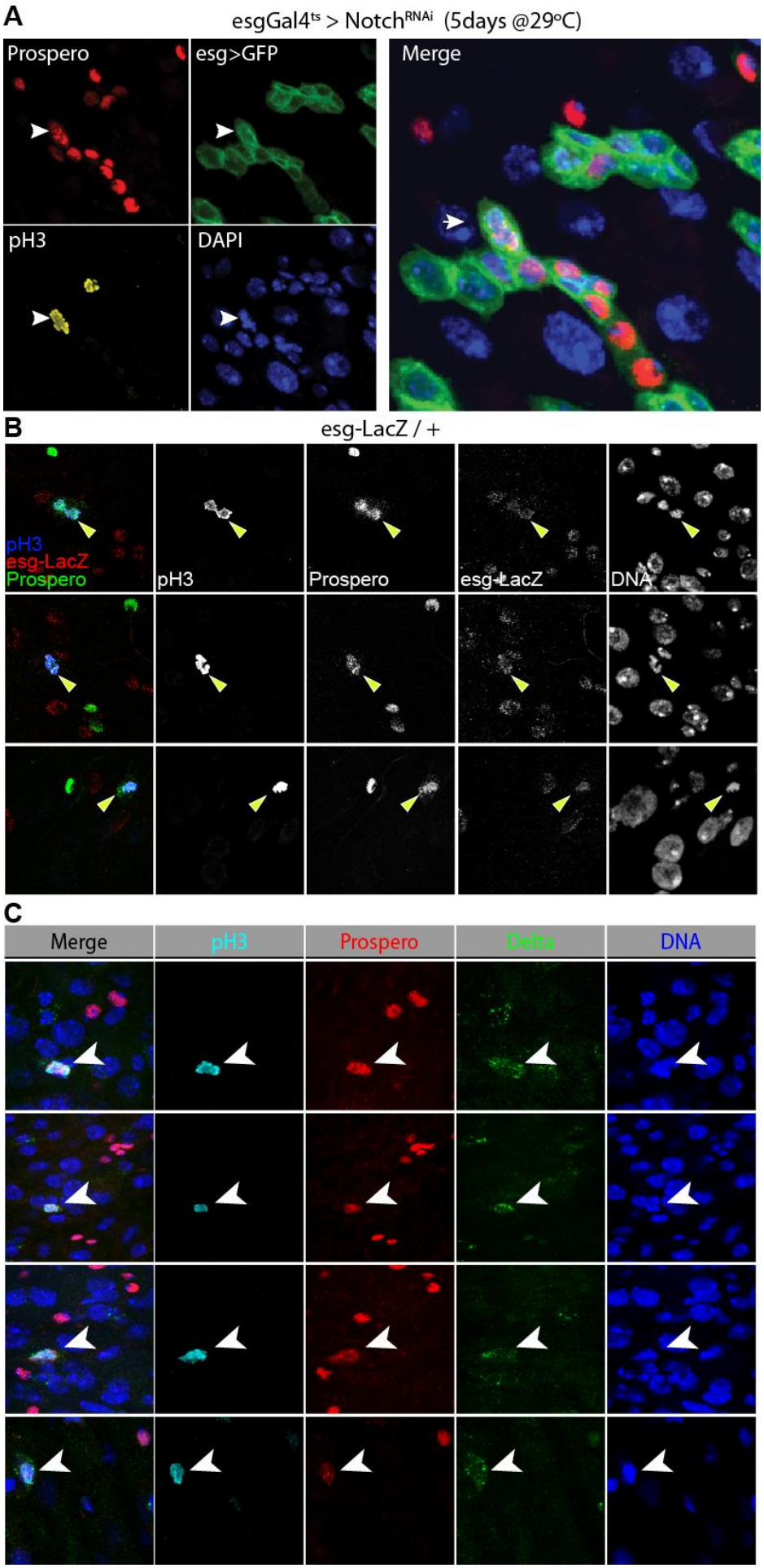
A
esgGal4ts > NotchRNAi (5days @29°C)
Prospero
esg>GFP
Merge
pH3
DAPI
B
esg-LacZ / +
pH3
esg-LacZ
Prospero
pH3
Prospero
esg-LacZ
DNA
C
Merge
pH3
Prospero
Delta
DNA

**Figure S3 (relates to Figure 4). Prospero is expressed in a subset of mitotic stem cells.**

(A) Confocal images of ISCs and EEs cell clusters in the intestine of UAS-Notch$^{RNAi}$;esgGal4,UAS-GFP;tubGal80$^{ts}$ females, 5 days after transgenes induction. The Prospero protein can be detected by immunocytochemistry in a subset of phosphoHistoneH3- and esg-positive cells.

(B) Representative images of esg-LacZ expressing pH3+ pros+ cells.

(C) Additional images demonstrating the co-expression of the Delta and Prospero proteins in a sub-population of mitotic stem cells in the posterior midgut.

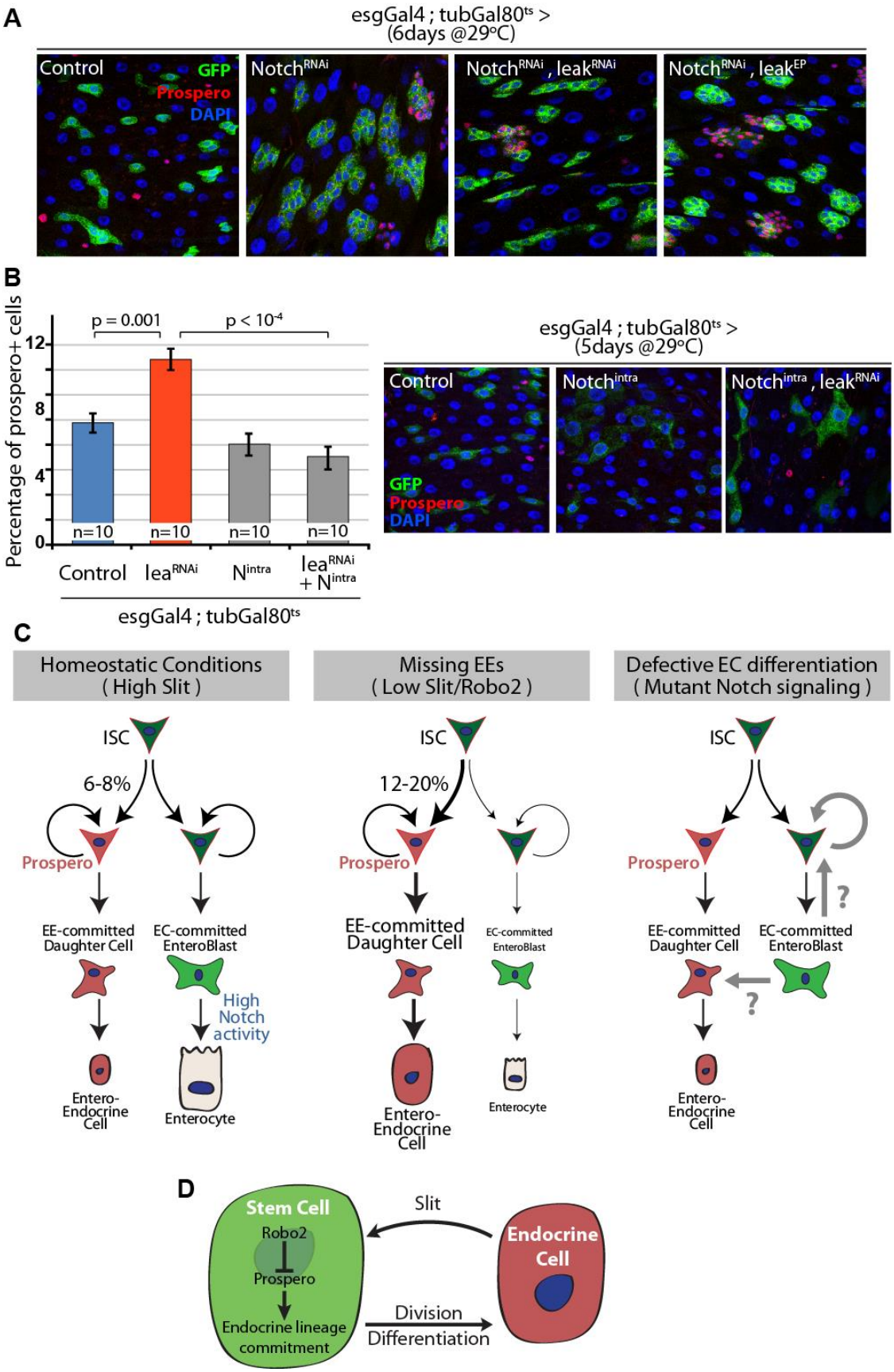
A
esgGal4 ; tubGal80ts >
(6days @29°C)
Control
GFP
Prospero
DAPI
NotchRNAi
NotchRNAi , leakRNAi
NotchRNAi , leakEP
B
Percentage of prospero+ cells
p = 0.001
p < 10-4
12
8
4
0
n=10
n=10
n=10
n=10
Control
leaRNAi
Nintra
leaRNAi + Nintra
esgGal4 ; tubGal80ts
esgGal4 ; tubGal80ts >
(5days @29°C)
Control
Notchintra
Notchintra , leakRNAi
GFP
Prospero
DAPI
C
Homeostatic Conditions
( High Slit )
Missing EEs
( Low Slit/Robo2 )
Defective EC differentiation
( Mutant Notch signaling )
ISC
6-8%
12-20%
Prospero
EE-committed Daughter Cell
EC-committed EnteroBlast
High Notch activity
Entero-Endocrine Cell
Enterocyte
?
D
Stem Cell
Robo2
Prospero
Endocrine lineage commitment
Slit
Endocrine Cell
Division
Differentiation

**Figure S4 (relates to Figure 4). Genetic interaction between leak/Robo2 and the Notch signaling pathway in ISCs.**

(A) Genetic interaction between Robo2/leak and Notch. Expression of $N^{RNAi}$ in ISCs and EBS causes an accumulation of ISCs and EEs in the posterior midgut. Knocking-down ($lea^{RNAi}$) or over-expressing ($lea^{EP}$) Robo2 in this genetic background does not affect the $N^{RNAi}$-mediated phenotype.

(B) Ectopic Notch activity in esg-positive cells (expression of $Notch^{intra}$ construct) is sufficient to drive endoreplication in these cells, as shown by accumulation of the large GFP-expressing cells in the posterior midgut, and abolishes the increased proportion of EEs observed when lea/robo2 is knocked-down. n represents the number of intestines analyzed. p-value from two-tailed Student's t-test.

(C) Updated model of the intestinal stem cell lineage in the *Drosophila* posterior midgut. Our data strongly suggests that in response to low level of Slit signal, the proportion of ISC expressing the prospero transcription factor is increased, promoting the commitment of ISCs and their daughter cells to the endocrine lineage. In the absence of Notch signaling, EC differentiation is impaired and ISC daughter cells are committed to the EEs lineage by default or retain stem cell identity.

(D) Model representing the negative feedback loop controlling the proportion of EEs in the fly intestine. These cells express the Slit ligand, which negatively regulates the commitment of ISC daughter cells to the endocrine lineage.

## Supplemental Experimental Procedures

### Analysis of gene expression

Total RNA was extracted from 8 guts using Trizol (Invitrogen), and cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen). Quantitative Real-Time PCR was performed on a BioRad MyiQ Single Color Real Time PCR Detection System using SYBR green. Leak/Robo2 expression was normalized to the expression of Actin5C.