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Transcriptional control of stem cell maintenance in the *Drosophila* intestine

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SUMMARY

Adult stem cells maintain tissue homeostasis by controlling the proper balance of stem cell self-renewal and differentiation. The adult midgut of *Drosophila* contains multipotent intestinal stem cells (ISCs) that self-renew and produce differentiated progeny. Control of ISC identity and maintenance is poorly understood. Here we find that transcriptional repression of Notch target genes by a Hairless-Suppressor of Hairless complex is required for ISC maintenance, and identify genes of the *Enhancer of split* complex [*E(spl)-C*] as the major targets of this repression. In addition, we find that the bHLH transcription factor Daughterless is essential to maintain ISC identity and that bHLH binding sites promote ISC-specific enhancer activity. We propose that Daughterless-dependent bHLH activity is important for the ISC fate and that E(spl)-C factors inhibit this activity to promote differentiation.

KEY WORDS: *Drosophila*, Notch signaling, Adult stem cells

INTRODUCTION

The homeostasis of adult tissues is controlled through the renewal of differentiated cells by adult stem cells or committed progenitors. A detailed understanding of how stem cells are controlled in their in vivo environment has proved challenging owing to the requirement for precise genetic manipulation and in vivo lineage-labeling techniques. The posterior midgut of the adult *Drosophila* intestine is a simple model system in which to understand how adult stem cells are maintained. The posterior midgut epithelium contains intestinal stem cells (ISCs) distributed throughout the tissue that self-renew and produce differentiated cells during the adult lifetime (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Upon cell division, each ISC produces one daughter cell that retains the ISC fate and one enteroblast (EB) that differentiates into either an absorptive enterocyte (EC) or a secretory enteroendocrine cell (ee) (Ohlstein and Spradling, 2006) (see Fig. 1N). This simple cell lineage greatly facilitates the analysis of fate decisions using precise lineage labeling and genetic manipulation of the ISC and its progeny.

Recent studies have begun to address the control mechanisms of stem cell self-renewal and proliferation. Self-renewal of ISCs is influenced by Wingless (Wg) (Lee et al., 2009; Lin et al., 2008). Wg, however, is not strictly required to maintain ISC identity, as ISCs are still detected in the absence of Wg signaling (Lin et al., 2008). Additionally, ISC proliferation is modulated by Insulin and Jak/Stat signaling, at least in response to intestinal damage (Amcheslavsky et al., 2009; Buchon et al., 2009; Jiang et al., 2009; Lin et al., 2010). Despite these significant recent advances, the signals and transcriptional programs that control ISC identity and maintenance are unknown. One important clue, however, has come from studies on the role of Notch in this lineage that showed that differentiation of ISC progeny requires Notch signaling and that forced expression of activated Notch results in the differentiation of ISCs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007).

In this study, we find that ISC maintenance requires transcriptional repression of Notch target genes by Hairless. We identify the *Enhancer of split* complex [*E(spl)-C*] genes as the key Notch targets that need to be repressed by Hairless to ensure ISC maintenance, while being upregulated in EBs to promote differentiation. Additionally, Daughterless (Da), a basic helix-loop-helix (bHLH) transcriptional activator, is also essential for ISC maintenance and bHLH E-box binding sites are required for expression of an ISC-specific enhancer. We propose a model in which Da and E(spl)-C factors act antagonistically to regulate maintenance of the ISC fate versus EB differentiation.

MATERIALS AND METHODS

Drosophila stocks and clone analysis

Drosophila stocks and crosses were kept at 18-25°C. Adults were aged at 25°C unless stated otherwise. Clones were induced in 3- to 6-day-old flies and analyzed in well-fed females. MARCM clones (Lee and Luo, 2001) were generated using the X chromosome *y w P[hs-FLP] P[pTub-GAL4] P[UAS-nlsGFP]* combined with FRT *P[pTub-GAL80]* chromosomes on the second and third chromosomes. MARCM clones on the X chromosome were induced with *hsflp122 P[pTub-GAL80] FRT19A; P[pAct-GAL4] P[UASGFP]* (Lin et al., 2008). The following mutant alleles were used to generate recombinant lines and for experiments: H^{E31}, H^{1}, H^{2}, $neur^{IF65}$, *P[gro+] Df(3R)* $gro^{32.2}$, *P[gro+]* on II, *P[l(2)35Bg+] Su(H)*$^{\Delta 47}$ [a small deletion of *Su(H)* with a genomic rescue construct of the neighboring gene (Morel and Schweisguth, 2000)], $numb^{2}$, $numb^{15}$, da^{10}, da^{3}, *Df(1)*sc^{B57}, $amos^{1}$, and ato^{1}. Control wild-type MARCM clones were generated using *FRT82B P[w+]90B*, *FRT40A* and *y w FRT19A* chromosomes. We generated the *E(spl)-C*$^{\Delta m\delta\text{-}m6}$ deletion by FLP-mediated recombination using lines XPd08311 and RBe00084 (Exelixis). This deficiency removes a genomic region containing the coding sequences (CDSs) of the *E(spl)-C* genes *mδ*, *mγ*, *mβ*, *mα*, *m1*, *m2*, *m3*, *m4*, *m5* and *m6*. It also removes promoter sequence from *m7*, but leaves the coding sequence intact. The *E(spl)-C*$^{\Delta m\delta\text{-}m6}$ deficiency does not remove *m8* or *groucho*. The entire *E(spl)-C* and *groucho* sequences are removed by *Df(3R)*$gro^{32.2}$ and *groucho* is rescued by *P[gro+]*.

We have adapted the MARCM technique to induce positively marked *Su(H) H* double-mutant clones in *y w P[hs-FLP] P[pTub-GAL4] P[UAS-nlsGFP]; FRT40A P[l(2)35Bg+] Su(H)*$^{\Delta 47}$ / *FRT40A P[pTub-GAL80];*

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FRT82B P[w+]90B H^{E31} / FRT82B P[pTub-GAL80] flies, in which GFP is expressed only upon recombination at both *FRT40A* and *FRT82B* sites. A two heat-shock protocol was used to alleviate residual Su(H) protein. The first heat shock creates three types of clones: unmarked $Su(H)^{\Delta 47}$ mutant clones that grow into large clones, unmarked H^{E31} mutant clones that do not proliferate, and marked double-mutant clones that do not proliferate; these double-mutant clones behave as *H* single-mutant clones (data not shown), probably owing to residual levels of Su(H) protein. A second heat shock, applied 5 days later, creates the three types of clones described above as well as a fourth type: marked double-mutant clones produced by a single recombination event at the *FRT82B* occurring in $Su(H)^{\Delta 47}$ mutant cells.

The *esg-GAL4 UAS-GFP; pTub-GAL80^{ts}* driver (Jiang et al., 2009) was used to ectopically express *UAS-scute* and *UAS-asense* in adults raised at 18°C and shifted to 29° C at 3 days of adulthood. The MARCM system was used to overexpress *UAS-Hairless* and *UAS-m7-VP16* (Jimenez and Ish-Horowicz, 1997). *E(spl)-mβ1.5-lacZ* (Cooper et al., 2000), *EE4-lacZ* (Culi and Modolell, 1998; Giagtzoglou et al., 2005) and *Su(H)-GBE-lacZ* (Furriols and Bray, 2001) flies were used.

The wild-type *mira-promoter-GFP* transgene consists of PCR-amplified genomic sequence flanking the *mira* CDS up to adjacent genes (for details, see Fig. S6 in the supplementary material), with the *mira* CDS replaced by nuclear-targeted *mGFP6* sequence (Haseloff et al., 1999). The resulting product was cloned with *Not*I/*Spe*I into the *Not*I/*Xba*I sites of the pattB vector (Bischof et al., 2007). The E-box-mutated version was generated by PCR amplifying seven individual regions (the E-box sequence CAGCTG was mutated to CAAATG within the primer sequences). All PCR was performed using Phusion polymerase (Finnzymes). The *mira-promoter-GFP* and *miraΔEbox-promoter-GFP* constructs were sequenced and injected into ϕX-22A flies (Bischof et al., 2007) to allow site-specific integration of the promoter constructs.

Immunostaining and in situ hybridization

Adult female intestines were dissected in PBS and fixed for 2 hours at room temperature (RT) in 4% paraformaldehyde. Intestines were rinsed in PBT (PBS containing 0.1% Triton X-100), trimmed and incubated for at least 30 minutes in PBS containing 50% glycerol, then equilibrated in PBT to osmotically clean the lumen. For Fig. S1 in the supplementary material and Fig. 6A, intestines were fixed for 15 minutes in 4% formaldehyde/heptane, dehydrated in methanol and rehydrated in PBT as described (Lin et al., 2008). Primary antibody incubations were either overnight at 4°C or 3-5 hours at RT. Secondary antibodies were incubated 3-5 hours at RT. DAPI (1 μg/ml) was added to the final wash. Intestines were mounted in 4% N-propyl-galate, 80% glycerol and imaged on Leica SP2 and SPE confocal microscopes. Images are selected layers of confocal stacks. For Fig. 4B,C, intestines were processed as previously described for embryos (Bardin and Schweisguth, 2006). The following antibodies were used: mouse anti-Delta [C594-9B, developed by the laboratory of S. Artavanis-Tsakonas (Harvard Medical School, Boston, MA, USA); 1:2000, DSHB], guinea pig anti-Hairless A (A. Preiss, Institute of Genetik, University of Hohenheim, Stuttgart, Germany; 1:500), mouse anti-Pros [MR1A, developed by the lab of C. Doe (University of Oregon, Eugene, OR, USA); 1:10, DSHB], rabbit anti-Pros (Y.-N. Jan, University of California, San Francisco, CA, USA; 1:2000), rabbit anti-Daughterless (Y.-N. Jan; 1:1000), rabbit anti-Asense (Y.-N. Jan; 1:5000) and goat anti-β-gal (1:1000, Biogenesis).

For the *scute* in situ hybridization, the protocol developed by the Bier laboratory was used (O'Neill and Bier, 1994), followed by anti-DIG-POD (1:2000, Roche, 11207733910). In situs were developed using the TS Plus Cyanine 3 System following the supplier's instructions (Perkin-Elmer, NEL741001KT).

Quantification and statistical analysis

Only clones located in the posterior midgut were considered for analysis and numerous clones of representative midguts were analyzed. In Fig. 1M and Fig. 5E, the number of cells per clone was counted. In Fig. 3E and Fig. 4H, $Delta^{-}$ GFP^{+}, $Delta^{+}$ GFP^{+}, as well as $Pros^{-}$ GFP^{+} and $Pros^{+}$ GFP^{+} cells were counted on images taken using a CoolSNAP Camera (Ropers Scientific) on a Leica DMRXE epifluorescence microscope. Two planes of focus, apical and basal, were analyzed for each field. Differences in total cell number (Fig. 1M, Fig. 5E) or in the proportion of $Delta^{+}$ and $Pros^{+}$ cells relative to GFP^{+} cells (Fig. 3E, Fig. 4H) between genotypes were tested for statistical significance using a standard normal distribution where $\varepsilon \geq 1.96$ indicates significance with 95% confidence. In Fig. 1M: wild type, n=215 clones; H^{E31}, n=334. Differences in the proportions of wild-type versus H^{E31} clones are significant for: 1-cell clones, ε=3.1; 2- to 5-cell clones, ε=3.2; ≥6-cell clones, ε=8.7. In Fig. 5E: wild type, n=139 clones; da^{10}, n=62. Differences in the proportions of wild-type versus da^{10} clones are significant for: 1-cell clones, ε=5.78; 2- to 5-cell clones, ε=5.62. Since there were no da^{10} clones with greater than 5 cells, the difference in proportions could not be tested.

For counting $Delta^{+}$ cells in *Hairless* mutants and in Fig. 3E at the 10 day time point: wild type, n=422 GFP^{+} cells; H^{E31}, n=202 GFP^{+} cells; $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$, n=983 GFP^{+} cells; $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$ H^{E31}, n=331 GFP^{+} cells. The following differences in proportions were statistically significant: wild type versus H^{E31}, ε=3.7; H^{E31} versus $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$ H^{E31}, ε=17.1. The difference in proportions between $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$ and $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$ H^{E31} is not significant (ε=1.7). For counting $Pros^{+}$ cells in Fig. 4H, we used a 6 day time point: wild type, n=294 GFP^{+} cells; H^{E31}, n=197 GFP^{+} cells; $E(spl)\text{-}C^{\Delta m\delta\text{-}m6}$, n=622 GFP^{+} cells; $neur^{IF65}$, n=327 GFP^{+} cells. Wild type is significantly different from $neur^{IF65}$ (ε=17.5). This statistical test was not used for cases in which the number of incidents was less than five.

EC density in Fig. 5D was determined on confocal stacks in which nuclear Pdm1 expression and nuclear size were measured and divided by the total area of the mutant or wild-type tissue. We found that $Delta^{+}$ cells and $Pros^{+}$ cells have nuclei ranging between 3 and 5 μm in diameter; we therefore use a cut-off of 7 μm to define the EC. Several representative stacks were analyzed. Error bars represent s.d. from the mean. In Fig. 6J, the number of clones at 10 days AHS that contained at least one $Pros^{+}$ cell was assessed in wild-type versus *Df(1)scB57* clones from Leica SPE confocal stacks. Twelve out of 32 wild-type clones contained at least one $Pros^{+}$ cell, compared with none out of 37 *Df(1)scB57* clones. This difference is statistically significant (P=0.0000256, Fisher's exact test).

For Fig. S2 in the supplementary material, confocal sections were used to count the total number of cells and the $Delta^{+}$, $Pros^{+}$ and phosphorylated Histone H3 $(PH3)^{+}$ cells per clone. Error bars represent s.d. from the mean. In Fig. S2A,B in the supplementary material: for the overexpression of Hairless, n=20 clones; wild type, n=25 clones. In Fig. S2D in the supplementary material: for the overexpression of Hairless, n=25 clones; wild-type, n=28 clones. Thirteen out of 25 clones overexpressing *Hairless* have at least one $PH3^{+}$ cell, compared with 3/28 in wild type (P=0.0008, two-tailed Fisher's exact test). Additionally, 4/25 clones overexpressing *Hairless* contained more than one dividing cell, compared with 0/28 for wild type (P=0.043, two-tailed Fisher's exact test).

RESULTS

Hairless is required for self-renewal of ISCs

Upon division of the ISC, Delta is inherited by both daughter cells and Notch is similarly present in both daughter cells (Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007). This raises the possibility that Notch receptors are activated in both daughter cells. Since Notch activity in ISCs can cause terminal differentiation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007), we hypothesized that expression of Notch target genes might be repressed in ISCs. The Hairless-Su(H) co-repressor complex acts in a limited number of developmental contexts to repress Notch target gene expression (Furriols and Bray, 2000; Koelzer and Klein, 2003; Koelzer and Klein, 2006; Morel and Schweisguth, 2000). The adaptor protein Hairless binds to Su(H) and is required for the repressor activity of Su(H) by recruiting the co-repressors CtBP and Groucho, as well as the Histone H3/H4 chaperone Asf1, thereby promoting efficient silencing through repressive chromatin (Bang and Posakony, 1992; Barolo et al., 2002; Brou et al., 1994; Castro et al., 2005; Goodfellow et al., 2007; Morel et al., 2001; Nagel et al., 2005). Upon Notch receptor activation, Notch is cleaved and the active form of Notch, NICD (Notch intracellular domain),

translocates to the nucleus where it interacts with Su(H) and replaces Hairless (reviewed by Kopan and Ilagan, 2009; Bray, 2006; Schweisguth, 2004). First, we examined the expression of Hairless in the intestine of adult flies. Hairless was detected in the nuclei of ISCs and EBs, as well as in a small number of differentiated progeny (see Fig. S1 in the supplementary material). By contrast, lower or undetectable levels of Hairless were present in more mature ECs (see Fig. S1 in the supplementary material). This expression is consistent with a role of Hairless in ISCs and/or ISC daughter cells.

Next, we examined the role of *Hairless* in ISC maintenance. Growth of clones that were homozygous for the null allele *Hairless*^{E31} (H^{E31}) was examined using the mosaic analysis with repressible cell marker (MARCM) technique (Lee and Luo, 2001), in which GFP was heritably expressed in either the ISC and its progeny cells, forming a growing stem cell clone, or in the EB, forming a transient single-cell clone with a ~1-week turnover rate (Ohlstein and Spradling, 2006). Whereas wild-type clones proliferated over time and formed large clones by 14 days after heat shock (AHS) (Fig. 1A-D), H^{E31} mutant clones failed to grow, forming either very small groups of cells or remaining as single cells at 14 days AHS (Fig. 1E-H). To quantify the self-renewal potential of *Hairless* mutant clones, we counted the number of large stem cell clones (6 cells or more), small stem cell clones (2-5 cells), and single-cell clones (transient clones and stem cell clones that are non-proliferating) at 6 days AHS (Fig. 1M). This analysis revealed that only 4% of the H^{E31} mutant clones contained 6 cells or more, compared with 31% for wild-type clones. Furthermore, 55% of the H^{E31} mutant clones were single-cell clones (n=215), compared with only 42% of wild-type clones (n=334). These differences are statistically significant (see Materials and methods). H^{1} and H^{2} clones similarly lost self-renewing capacity over time and failed to produce large clones at 14 days AHS (data not shown), indicating that this phenotype is unlikely to result from unrelated mutations on this chromosomal arm. These results clearly show that *Hairless* mutant clones have a considerably diminished capacity to grow.

We next examined the fate of ISCs in *Hairless* mutants using Delta as a marker. At 10 days AHS, only 7% of cells in H^{E31} mutant clones were Delta^{+} (n=202; Fig. 1I-J‴ and Fig. 3E), whereas 18% of cells in wild-type clones were Delta^{+} (n=422), indicating that the ISC fate is not properly maintained upon loss of *Hairless* activity. We further examined differentiation in *Hairless* mutant clones. At 6 days AHS, 40% (n=158) of the H^{E31} mutant cells expressed the EC marker Pdm1 (Nubbin – FlyBase) (Lee et al., 2009) and 59% (n=675) had a large polyploid nucleus, a characteristic of ECs (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), whereas only 0.5% (n=202) of the H^{E31} mutant cells expressed the ee marker Prospero (Pros) (Fig. 3E). This indicates that *Hairless* mutant cells can differentiate and, when they do so, they become ECs and not ees. This fate choice is consistent with the known role of Hairless in antagonizing Notch, as strong Notch signaling favors adoption of the EC fate (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). Nevertheless, 27% (n=675) of the H^{E31} mutant cells had a small diploid nucleus and 14% had an intermediate size nucleus. Thus, many *Hairless* mutant cells appear to have lost ISC characteristics (i.e. the expression of Delta and the ability to form large clones over time) without properly differentiating into ECs.

The effect of *Hairless* overexpression on clone growth and cell fate was tested. *Hairless* overexpression for 6 days caused a large increase in average clone size (85±57 cells, n=20 clones; see Fig. S2A in the supplementary material), as compared with wild-type clones (14±10 cells, n=25 clones). Additionally, *Hairless* overexpression resulted in many clones containing at least one

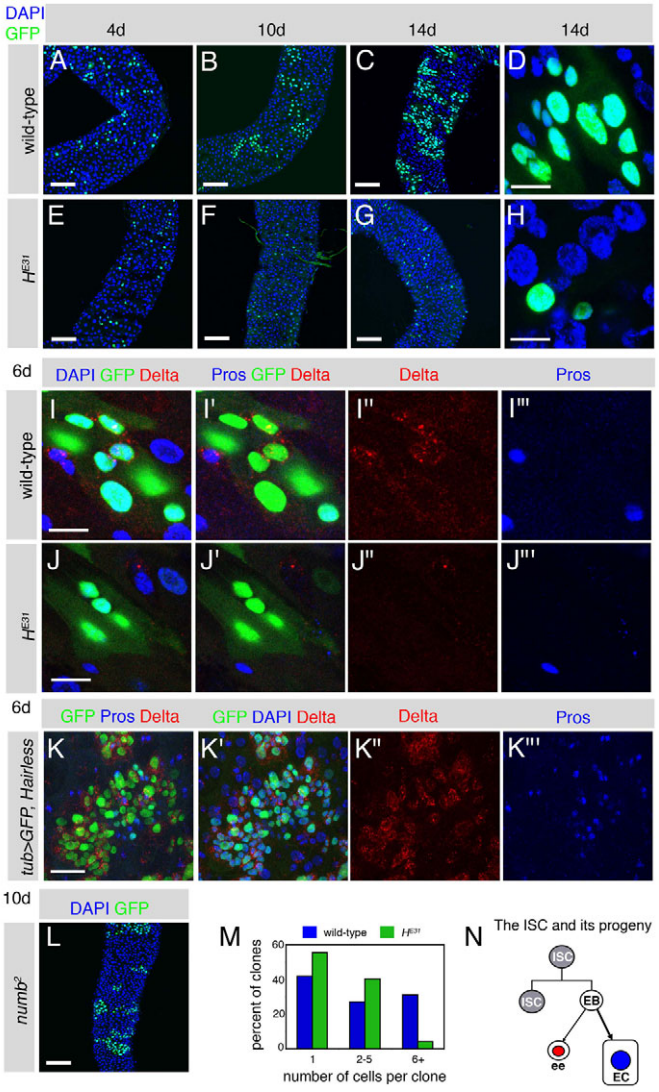

Fig. 1. *Hairless* is required for ISC self-renewal. The growth of positively marked clones (GFP, green) as well as the expression of Delta (red) and Pros (blue) were monitored over time in the posterior midgut (nuclear DAPI, blue). (**A-H**) The growth of positively marked wild-type (A-D) and H^{E31} mutant (E-H) clones was monitored over a 2-week period at 4, 10 and 14 days (d) after heat shock (AHS). (D,H) High-magnification views at 14 days. (**I-J‴**) Whereas wild-type control clones (I-I‴) usually contained 1-2 Delta^{+} small cells and 0-2 Pros^{+} cells, H^{E31} clones (J-J‴) lacked Delta^{+} small cells and Pros^{+} cells. Note that Delta expression was detected in some Pros^{+} cells in wild-type midguts (see I′). (**K-K‴**) Overexpression of *Hairless* in MARCM clones under the control of *tub-GAL4* over a 6-day period resulted in ectopic Delta^{+} ISC-like cells and ectopic Pros^{+} cells. (**L**) *numb*^{2} mutant ISCs proliferated into large clones by 10 days AHS. (**M**) Analysis of the size of wild-type and H^{E31} clones at 6 days AHS. For each category of clone size, the number of clones is given as the percentage of the total number of clones (wild-type, n=215 clones; H^{E31}, n=334 clones). The frequency of single-cell clones, corresponding to transient clones and non-proliferative ISC clones, was significantly increased upon loss of *Hairless* activity, whereas large H^{E31} mutant clones (6 cells or more) were rare compared with wild-type clones (see Materials and methods for statistics). (**N**) The ISC and its progeny. The ISC produces an ISC and an enteroblast (EB) upon division. The EB further differentiates into either an enteroendocrine cell (ee) expressing the transcription factor Pros or a large polyploid enterocyte (EC). Scale bars: 100 μm in A-C,E-G,L; 10 μm, in D,H,I-K.

mitotic cell (52%, *n*=25 clones; see Fig. S2C,D in the supplementary material), with one clone containing five cells undergoing division at the same time, including two that were directly adjacent, strongly suggesting that these two cells had acquired ISC fate. In addition, *Hairless* overexpression was sufficient to promote the formation of many ectopic small cells that expressed Delta and/or Pros (Fig. 1K-K″ and see Fig. S2 in the supplementary material). The increased number of both Delta$^+$ and mitotic cells indicates that the overexpression of *Hairless* induces ectopic ISCs. We conclude that Hairless is both necessary and sufficient to produce ISC-like cells, indicating that Hairless regulates the maintenance of ISCs.

By contrast, Numb, another negative regulator of Notch, plays no essential role in ISC self-renewal: both *numb*^{2} (Fig. 1L) and *numb*^{15} (data not shown) mutant cells produced large clones over time. We therefore conclude that *Hairless*, but not *numb*, is required for ISC self-renewal and maintenance.

Hairless acts in an *Su(H)*-dependent manner to maintain the ISC fate

Repression of Notch target genes by Hairless requires the sequence-specific DNA-binding protein Su(H), which recruits the repression complex onto DNA (Barolo et al., 2002; Furriols and Bray, 2001; Morel et al., 2001; Morel and Schweisguth, 2000). To test whether Hairless acts via Su(H) to regulate ISC maintenance, we generated clones lacking both *Hairless* and *Su(H)* activities (see Materials and methods). The loss of *Hairless* activity resulted in defective self-renewal (Fig. 2C-D′; see also Fig. 1), whereas loss of *Su(H)* activity led to an overspecification of small Delta$^+$ ISC-like cells (Fig. 2A-B′) (see Ohlstein and Spradling, 2007). Positively marked *Su(H)*$^{\Delta 47}$ *H*^{E31} double-mutant clones generated within *Su(H)*$^{\Delta 47}$ single-mutant unmarked clones proliferated and expressed the ISC marker Delta (Fig. 2E-H′). Therefore, the activity of *Su(H)* is required for the ISC loss seen in *Hairless* mutant clones and we conclude that *Su(H)* is epistatic to *Hairless* with respect to Delta expression and ISC self-renewal. This suggests that Hairless acts via Su(H) to repress Notch target genes in ISCs in order to promote stem cell maintenance. Interestingly, the loss of *Su(H)* led to an increased number of ISC-like cells, similar to Notch loss-of-function, whereas loss of *Hairless* activity led to a loss of ISC self-renewal, similar to the overexpression of an activated Notch receptor. This suggests that loss of Hairless/Su(H)-mediated repression, on its own, does not lead to ISC loss and that Su(H)-mediated target gene activation is also required for ISC loss in this context.

Deletion of the *E(spl)-C* suppresses the *Hairless* loss-of-ISC phenotype

We next sought to identify the Notch target genes that need to be repressed by Hairless in ISCs. The *E(spl)-C* genes are well characterized targets of the Notch pathway (Bailey and Posakony, 1995; Jennings et al., 1994; Lecourtois and Schweisguth, 1995). We found that an *E(spl)mβ-lacZ* construct (Cooper et al., 2000) was highly upregulated in EB cells, whereas it was expressed at only low levels in ISCs (Fig. 3F-F″). Moreover, loss of *E(spl)-C* genes, using either *E(spl)-C*$^{\Delta m\delta\text{-}m6}$ or the *Df(3R) gro*$^{32.2}$ combined with the *groucho* rescue construct, *P[gro+]*, resulted in an increased number of small Delta$^+$ ISC-like cells (Fig. 3A-A″,E; see Fig. S3A-C‴ in the supplementary material). This indicates that *E(spl)-C* genes are required in ISC daughter cells to promote EB differentiation and/or block self-renewal in presumptive EB cells. To test whether this phenotype was due to the loss of the *E(spl)-C* bHLH transcriptional repressors, we took advantage of an ‘activator’ version of m7 (HLHm7 – FlyBase), m7-VP16, in which fusion of the transcriptional activator VP16 to the bHLH m7 converts m7 from a transcriptional repressor into an activator (Jimenez and Ish-Horowicz, 1997). Expression of m7-VP16 similarly resulted in an increase in small Delta$^+$ ISC-like cells and a loss of Pros$^+$ cells (see Fig. S3F-F″ in the supplementary material). We therefore conclude that EB differentiation and/or inhibition of ISC self-renewal are likely to result from the loss of one or more *E(spl)-C* bHLH genes.

To test whether the loss of ISCs seen in the absence of *Hairless* activity was due to derepression of *E(spl)-C* genes, we examined the phenotype of *E(spl)-C Hairless* double-mutant clones. We found that deletion of the *E(spl)-C*, using either *E(spl)-C*$^{\Delta m\delta\text{-}m6}$ or the *Df(3R) gro*$^{32.2}$ *P[gro+]*, rescued the growth defect of *Hairless* mutant clones at 6 and 10 days AHS (Fig. 3B-B‴,D; see Fig. S3D-E‴ in the supplementary material; compare with *Hairless* single-mutant clones in Fig. 1E-H,J-J‴). The observation of large clones at 10 days AHS clearly indicated that *Hairless E(spl)-C* double-mutant ISCs retained their ability to self-renew over time (Fig. 3D and see Fig. S3D-D″ in the supplementary material). Consistent with this, ISC-like Delta$^+$ cells were seen in *Hairless E(spl)-C*

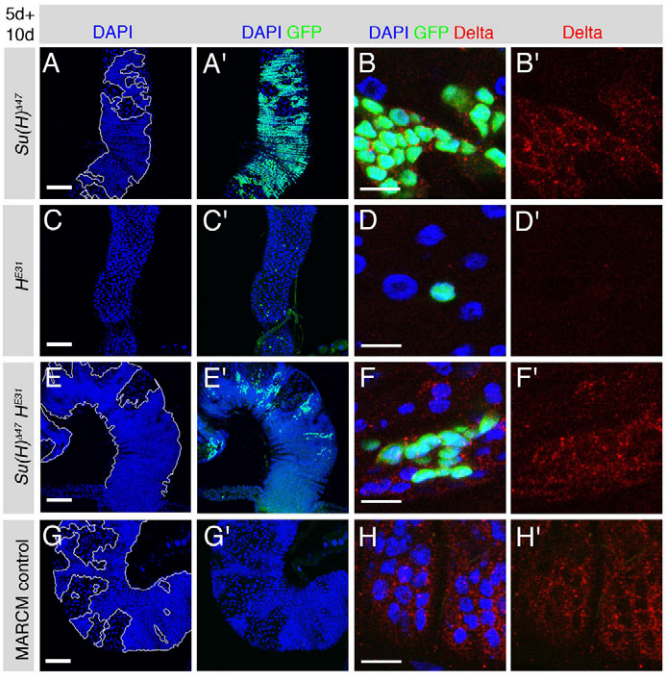

Fig. 2. *Su(H)* is required for the *Hairless* ISC maintenance defect. Clone growth (GFP, green; DAPI, blue) and Delta expression (red) were examined in clones produced using a two heat-shock protocol. Low (A,A′,C,C′,E,E′,G,G′) and high (B,B′,D,D′,F,F′,H,H′) magnification views of representative midguts are shown. (**A-B′**) *Su(H)*$^{\Delta 47}$ clones (outlined in A) contained ISC-like cells expressing Delta. (**C-D′**) *H*^{E31} clones failed to proliferate and did not express Delta. (**E-F′**) *H*^{E31} positively marked clones within *Su(H)*$^{\Delta 47}$ unmarked clones (outlined in E) proliferated and contained Delta-expressing ISC-like cells. (**G-H′**) Negative control clones. The potential loss of GAL80 expression independent of a recombination event was monitored in *Su(H)*$^{\Delta 47}$ single-mutant clones (outlined in G) in *y w P[hs-FLP] P[pTub-GAL4] P[UAS-nlsGFP]; FRT40A P[l(2)35Bg] Su(H)*$^{\Delta 47}$*/ FRT40A P[pTub-GAL80]; FRT82B P[pTub-GAL80] / MKRS* flies. This genotype produced virtually no GFP$^+$ cells, indicating that the spontaneous loss of GAL80 expression cannot account for the phenotype seen in E-F′. Scale bars: 100 μm in A,A′,C,C′,E,E′,G,G′; 10 μm in B,B′,D,D′,F,F′,H,H′.

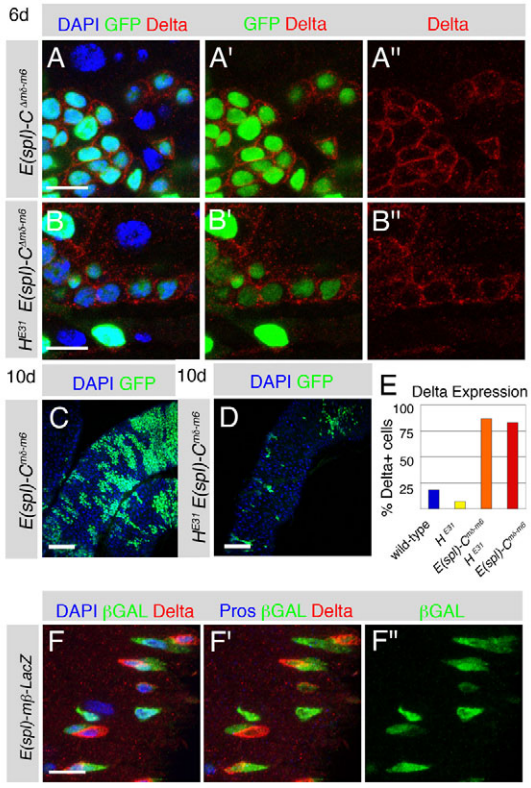

Fig. 3. Deletion of the *E(spl)-C* suppresses the *Hairless* ISC maintenance defect. (**A-B″**) Both *E(spl)-C*$^{mδ\text{-}m6}$ (A-A″) and *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} (B-B″) positively marked mutant clones (GFP, green; nuclear DAPI, blue) grew and contained many small Delta^{+} (red) ISC-like cells at 6 days AHS. (**C**,**D**) *E(spl)-C*$^{mδ\text{-}m6}$ clones (C) were larger than *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} clones (D) at 10 days AHS. (**E**) Analysis of the percentage of Delta^{+} cells in wild-type, *H*^{E31}, *E(spl)-C*$^{mδ\text{-}m6}$ and *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} clones at 10 days AHS. The difference between *H*^{E31} and either *E(spl)-C*$^{mδ\text{-}m6}$ or *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} was statistically significant, whereas the difference between *E(spl)-C*$^{mδ\text{-}m6}$ and *E(spl)-C*$^{mδ\text{-}m6}$ *H*^{E31} was not (see Materials and methods for statistics). (**F-F″**) *E(spl)-mβ1.5-lacZ* expression (β-galactosidase, green) was high in presumptive EBs adjacent to ISCs (Delta, red) that exhibited low *E(spl)-mβ1.5-lacZ* expression. Only basal nuclei are shown in this confocal section (Pros and DAPI, blue). Scale bars: 10 μm in A-B″, F-F″; 100 μm in C,D.

double-mutant clones (Fig. 3B-B″,E and see Fig. S3E-E‴ in the supplementary material). Quantification of Delta^{+} cells showed that *H*^{E31} *E(spl)-C*$^{Δmδ\text{-}m6}$ mutant clones contain a similar proportion of Delta^{+} cells (83%, *n*=331 cells) as *E(spl)-C*$^{Δmδ\text{-}m6}$ mutant clones (87%, *n*=983 cells) at 10 days AHS, a significant rescue of the 7% of Delta^{+} cells in *Hairless* mutants clones (*n*=202 cells; Fig. 3E). We note, however, that *E(spl)-C* single-mutant clones were larger than *Hairless E(spl)-C* double-mutant clones (Fig. 3C,D). We interpret this difference in clone growth to suggest the existence of additional Notch targets that act to limit self-renewal and are subject to derepression in the *Hairless E(spl)-C* mutant cells. Expression of these targets would therefore limit the growth of *Hairless E(spl)-C* double-mutant clones. Taken together, our data indicate that *E(spl)-C* genes promote EB differentiation and/or block ISC self-renewal in presumptive EB cells and that their inhibition in the ISC by Hairless-mediated repression prevents ISC loss.

E(spl)-C genes play a non-essential role in enteroendocrine differentiation

E(spl)-C mutant cells do not express the ee markers Pros [Fig. 4A-B‴,H and see Fig. S3 in the supplementary material; only 0.5% of mutant cells are Pros^{+} (*n*=622 cells) compared with 6.1% of cells in wild-type clones (*n*=294 cells)] and Allatostatin (data not shown). Expression of m7-VP16 produced similar phenotypes to those when *E(spl)-C* is lost (see Fig. S3F-F″ in the supplementary material). By contrast, EC cells were properly specified, as shown by both large nuclear size (>7 μm) and expression of the EC marker Pdm1 (Fig. 4B-D and see Fig. S3C in the supplementary material; note that Pdm1 appeared to also mark early ECs with a nucleus smaller than 7 μm). The density of large ECs in *E(spl)-C* mutant tissue was similar to that of wild-type tissue, whereas the density of Pdm1^{+} cells was slightly increased in the *E(spl)-C* mutant tissue (Fig. 4B-D). Thus, loss of *E(spl)-C* genes led to a large excess of Delta^{+} ISC-like cells, a normal density of differentiated EC-like cells and a loss of Pros^{+} ee-like cells. Despite the large increase in small Delta^{+} cells, no general hyperplasia of the tissue was observed (Fig. 4B-D). We conclude that genes of the *E(spl)-C* are important to limit ISC fate specification and to promote ee differentiation, but are dispensable for the production of ECs. Since Notch signaling is required for EC fate (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), this implies that Notch targets other than those encoded by the *E(spl)*-C act to promote EC differentiation. Furthermore, whereas the *E(spl)-C* genes are important for ee differentiation, this role could be bypassed by reducing Delta signaling activity using a *neuralized* (*neur*) mutation. Indeed, expression of Pros was restored in *neur E(spl)-C* double-mutant clones (Fig. 4E-G‴), showing that *E(spl)-C* genes are not strictly required for ee specification.

Daughterless is required for ISC maintenance

A major function of the E(spl) bHLH repressors in both *Drosophila* and vertebrate development is to inhibit the activity of the bHLH Daughterless (Da)/E47-based dimeric transcriptional activators (reviewed by Kageyama et al., 2007; Alifragis et al., 1997; Gigliani et al., 1996; Heitzler et al., 1996; Oellers et al., 1994). Consistent with this, we found that Da is expressed in cells of the ISC lineage (see Fig. S4 in the supplementary material). To test the possible role of *da* in this tissue, we studied the growth of *da* mutant clones. We found that ISCs are lost in *da*^{10} (Fig. 5C-E) and *da*^{3} (data not shown) clones, as seen by loss of clone growth and loss of Delta^{+} ISC cells. *da* mutant clones consisted of single or pairs of polyploid EC-like cells, showing that *da* is important for ISC maintenance.

Da-dependent bHLH transcriptional activity is mediated through E-box motifs. To test whether Da regulates ISC-specific gene expression, we examined the expression of a known Da/proneural target (Reeves and Posakony, 2005), *miranda* (*mira*), using a reporter gene in which the *mira* coding sequence is replaced with nuclear GFP (*mira-promoter-GFP*; Materials and methods and see Fig. S6 in the supplementary material). We found that *mira-promoter-GFP* was specifically expressed in ISCs (Fig. 5F-G″). Moreover, mutation of the seven E-box motifs present in this construct largely eliminated ISC-specific gene expression (Fig. 5H-I″), indicating that E-box sites are necessary to drive such expression. In addition, multimerized E-boxes in front of a minimal promoter were sufficient to direct expression in ISCs, albeit weakly (see Fig. S5 in the supplementary material). We conclude that Da-binding motifs can mediate ISC-specific gene expression.

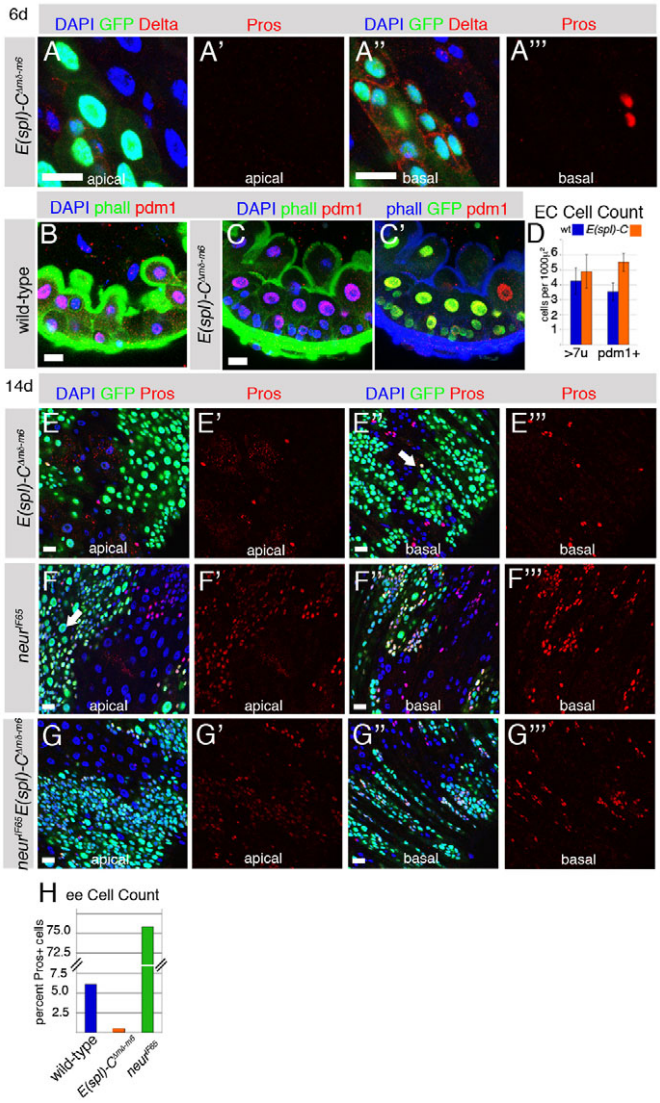

Fig. 4. *E(spl)-C* genes regulate enteroendocrine differentiation. (**A-A‴**) The expression of Pros as a marker for ee fate and polyploidy (DAPI, blue) as a marker for EC fate were assessed in *E(spl)-C* and *neur* mutant clones (GFP, green). EC nuclei were detected in apical planes (A). A schematic of cells in apical/basal planes is shown in Fig. S3B in the supplementary material. Apical (A,A′) and basal (A″,A‴) views are shown of a 6-day AHS *E(spl)-C*$^{mδ\text{-}m6}$ mutant clone. *E(spl)-C*$^{mδ\text{-}m6}$ mutant clones produced many ISC-like cells expressing Delta (red in A,A″) but not Pros (red in A′,A‴). (**B-C′**) At 6 days, *E(spl)-C*$^{mδ\text{-}m6}$ mutant clones (GFP, green in C′) had an increase in small nuclei (DAPI, blue) but overall tissue architecture (phalloidin, green in B,C and blue in C′; Pdm1, red) was similar to the wild type (B). (**D**) The density and specification of large (>7 μm) polyploid ECs was unaffected by the deletion of the *E(spl)-C*, although a slightly higher number of cells expressing Pdm1 was present. Error bars represent s.d. from the mean. (**E-E‴**) Apical and basal low-magnification views of a field of 14-day *E(spl)-C*$^{mδ\text{-}m6}$ mutant cells. Polyploid ECs were properly specified (E), whereas Pros was not expressed in *E(spl)-C*$^{mδ\text{-}m6}$ mutant cells (E-E‴; an exceptional Pros$^+$ cell is indicated in E″ by an arrow). (**F-F‴**) Apical and basal low-magnification views of a field with 14-day *neur*^{IF65} mutant cells. As previously noted (Ohlstein and Spradling, 2007), *neur*^{IF65} mutant clones contain few polyploid ECs (arrow in F) but many ectopic Pros$^+$ ee-like cells (F-F‴). (**G-G‴**) Apical and basal low-magnification views of a field with 14-day *neur*^{IF65} *E(spl)-C*$^{mδ\text{-}m6}$ mutant clones. Many Pros$^+$ ee-like cells were detected (G′,G‴). (**H**) Quantification of the number of Pros$^+$ ee cells. *E(spl)-C*$^{Δmδ\text{-}m6}$ mutant clones lack Pros$^+$ cells (0.5%, *n*=622 cells) compared with *neur*^{IF65} mutant (76%, *n*=327 cells) and wild-type (6%, *n*=294 cells) clones. See Materials and methods for statistics. Scale bars: 10 μm.

achaete-acute complex genes are required for ee, but not ISC, specification

Class I bHLH family members (Da/E47) can act as homodimers (Ellenberger et al., 1994; Murre et al., 1991; Oellers et al., 1994), as heterodimers with class II bHLH proteins (Powell and Jarman, 2008), and, at least in the *Drosophila* wing, evidence suggests that Da acts in concert with a Zn-finger transcription factor (Jafar-Nejad et al., 2006). Da/E47 members have a broad expression pattern, such that class II bHLH partners with a more restricted expression pattern often confer cell- or tissue-specificity to the heterodimeric complex. In the *Drosophila* embryo, Da is ubiquitous and acts with proneural bHLH factors of the *achaete-scute* complex (*AS-C*) that are expressed in adult midgut precursor cells to regulate their fate (Tepass and Hartenstein, 1995). Since Da expression was fairly general in the intestine, we similarly reasoned that a putative Da bHLH class II protein might be specifically expressed in the ISC to promote E-box-dependent ISC-specific expression, as seen for *mira*. To identify ISC-specific bHLH genes, we compared the transcriptional profile of wild-type posterior midguts with those mutant for *Su(H)* that contained a large excess of ISC-like cells and a mild increase in Pros$^+$ ee-like cells (data to be presented elsewhere). From this analysis, two class II bHLH family genes were highly upregulated: *scute* (60-fold) and *asense* (22-fold). The Asense protein was detected in a subset of Pros$^+$ ee cells (Fig. 6A-A″). Since Scute antibodies are no longer available, we used in situ hybridization to detect the *scute* RNA. *scute* transcripts were detected in *Su(H)* mutant clones that contain many ISC- and ee-like cells (Fig. 6B,B′), as well as in single basal cells with small nuclei in the heterozygous tissue outside of clones (Fig. 6C), suggesting that *scute* is expressed in the ISC, EB and/or ee.

We therefore investigated the role of *scute* and *asense* in ISC maintenance and ee differentiation using *Df(1)scB57*, which lacks all four *AS-C* genes (*achaete*, *scute*, *lethal of scute* and *asense*). No effect on clone growth or on Delta$^+$ expression was observed (Fig. 6D-I). We conclude that the *scute* and *asense* genes are not required for ISC maintenance. The bHLH genes *amos* and *atonal* were similarly not essential for ISC maintenance (data not shown). Whether proneural proteins act redundantly in the maintenance of ISCs or, alternatively, whether Da acts as a heterodimer with a non-proneural bHLH protein or as a homodimer, remains to be studied. We next studied ee differentiation and found that *Df(1)scB57* clones are devoid of Pros$^+$ cells (Fig. 6F,I,J). These expression and genetic data indicate that *asense* and/or *scute* are necessary for ee differentiation. Conversely, overexpression in ISCs of *asense* or *scute* using *esgGAL4 GAL80ts* increased the number of Pros$^+$ cells (Fig. 6J-O), indicating that *asense* and *scute* are sufficient to promote ee differentiation.

DISCUSSION

Adult stem cells self-renew and, at the same time, give rise to progeny that eventually differentiate. Our work provides evidence that one of the strategies used to maintain the identity of ISCs in *Drosophila* is to repress the expression of Notch target genes. Consistent with our finding, the loss of a general regulator of transcriptional repression, the Histone H2B ubiquitin protease

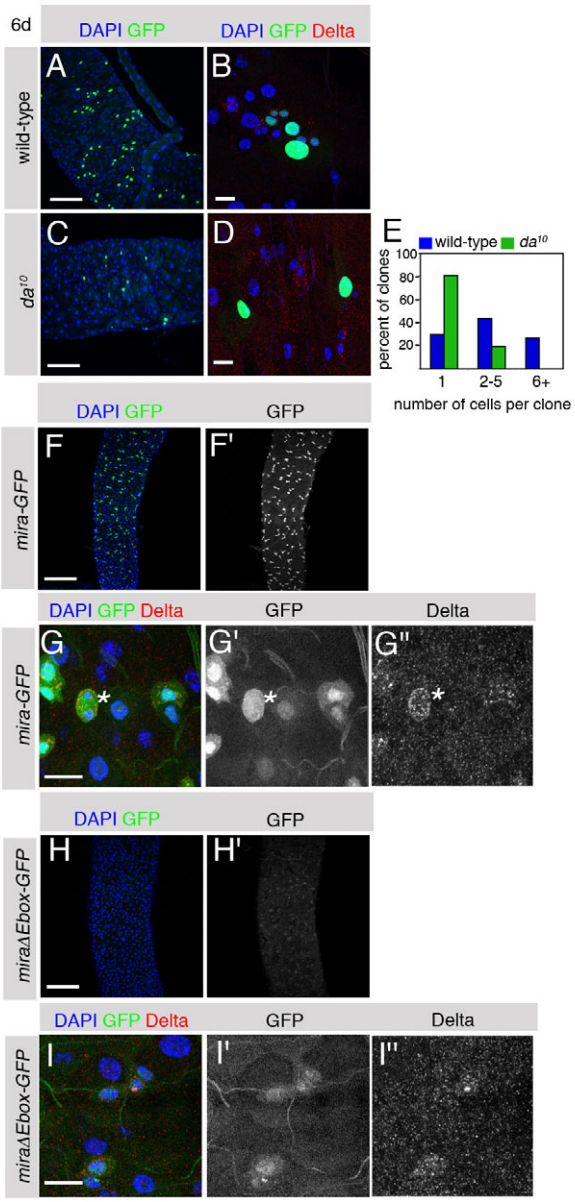

Fig. 5. *daughterless* is required for ISC identity. (**A-D**) Clone growth (nuclear GFP, green; DAPI, blue) and expression of Delta (red) were examined in wild-type (A,B) and da^{10} (C,D) clones at 6 days AHS. da^{10} mutant clones (C,D) did not contain Delta$^+$ cells, failed to grow and were composed of single or pairs of differentiated EC cells. (**E**) Quantification of clone size [number of cells in wild-type (blue, *n*=139) and da^{10} mutant (green, *n*=62) clones] at 6 days AHS. For each clone size category, the number of clones is given as the percentage of the total number of clones. The frequency of single-cell clones, corresponding to transient clones and non-proliferative ISC clones, was significantly increased upon loss of *da* activity, whereas large da^{10} mutant clones (6 cells or more) were not seen (see Materials and methods for statistics). (**F-G″**) The role of Da-binding motifs was assessed in the context of a *mira-promoter-GFP* transgene (*mira-prom-GFP*) that was specifically expressed in ISCs (nuclear GFP, green in G and white in G′; Delta, red in G and white in G″; DAPI, blue). A dividing ISC is marked by an asterisk in G. Pairs of GFP$^+$ cells were also seen, probably owing to inheritance of GFP by ISC progeny cells. (**H-I″**) Mutation of the seven E-box motifs in the *mira-promoter-GFP* transgene (*miraΔEbox-prom-GFP*) largely abolished nuclear GFP expression (compare nuclear signals in G′ and I′). Scale bars: 100 μm in A,C,F,F′,H,H′; 10 μm in B,D,G-G″,I-I″.

Scrawny, gives a similar phenotype to *Hairless* (Buszczak et al., 2009). Additionally, several recent studies indicate that transcriptional repression of differentiation genes may be a central hallmark of stem cells in general (Dejosez et al., 2008; Jepsen et al., 2007; Liang et al., 2008; Maines et al., 2007; Pietersen and van Lohuizen, 2008).

Two models have been proposed for Hairless activity. One proposes that Hairless competes with NICD for interaction with Su(H), thereby preventing transcriptional activation of Notch target genes by low-level Notch receptor activation (Bang et al., 1995; Morel et al., 2001). A second, non-exclusive, model proposes that Hairless antagonizes the transcriptional activation of Notch target genes by tissue-specific transcription factors other than Notch (Barolo et al., 2002; Barolo et al., 2000; Castro et al., 2005). Since the loss of Su(H) can suppress the phenotype of *Hairless* on ISC clone growth, we propose that Hairless promotes ISC maintenance by repressing the transcription of genes that would otherwise be activated by Notch signaling in ISCs (Fig. 7). Thus, Hairless appears to set a threshold level to buffer Notch signaling in ISCs. In the absence of this repression, the expression of *E(spl)-C* genes and other Notch targets would lead to loss of the ISC fate. Importantly, our findings suggest a mechanism for how the transcriptionally repressed state is turned off and activation of the differentiation program is initiated: high activation of Notch in EBs displaces Hairless from Su(H) and leads to expression of the *E(spl)-C* genes (Fig. 7).

E(spl)-C bHLH repressors act in part through their ability to inhibit bHLH activators (Kageyama et al., 2007). Our data demonstrate that Da is also essential to maintain ISC fate and that E-box Da-binding sites are required to promote ISC-specific enhancer activity. Thus, we propose that activation of *E(spl)-C* genes by Notch in EBs downregulates Da bHLH activity and thereby contributes to turning off ISC identity in the differentiating cell (Fig. 7). The specificity of ISC-specific E-box expression might be due to the ISC-specific expression of a bHLH family member. Although our array analysis raised the possibility that Scute may be specifically expressed in ISCs, our genetic analysis indicates that *scute* function is not essential for ISC maintenance. Alternatively, specificity of gene expression might result from inhibition of bHLH activity in the EB and differentiating daughters, possibly by E(spl)-bHLH factors, rather than by the ISC-specific expression of a Da partner. It is also possible that a non-bHLH, ISC-specific factor restricts the Da-dependent bHLH activity to ISCs in a manner similar to the synergism observed in wing margin sensory organ precursors (SOPs) between the Zn-finger transcription factor Senseless and Da (Acar et al., 2006; Jafar-Nejad et al., 2006).

Recently, a role for the Da homologs E2A (Tcf3) and HEB (Tcf12) has been found in mammalian ISCs marked by the expression of Lgr5 and, in this context, E2A and HEB are thought to heterodimerize with achaete-scute like 2 (Ascl2), which is essential for the maintenance and/or identity of Lgr5$^+$ ISCs (van der Flier et al., 2009). In *Drosophila*, however, *AS-C* genes are not essential for ISC maintenance, but appear to play a role in enteroendocrine fate specification. The observation that Da bHLH activity is required for the identity of both *Drosophila* ISCs and mammalian Lgr5$^+$ ISCs suggests that there might be conservation at the level of the gene expression program. Additionally, the bHLH genes *Atoh1* (*Math1*) and *Neurog3* are both important for differentiation of secretory cells in the mammalian intestine (Lee et al., 2002; Yang et al., 2001). Clearly, further analysis of the control of Da/E2A bHLH activity, as well as of the gene networks downstream of Da/E2A, will be of great interest.

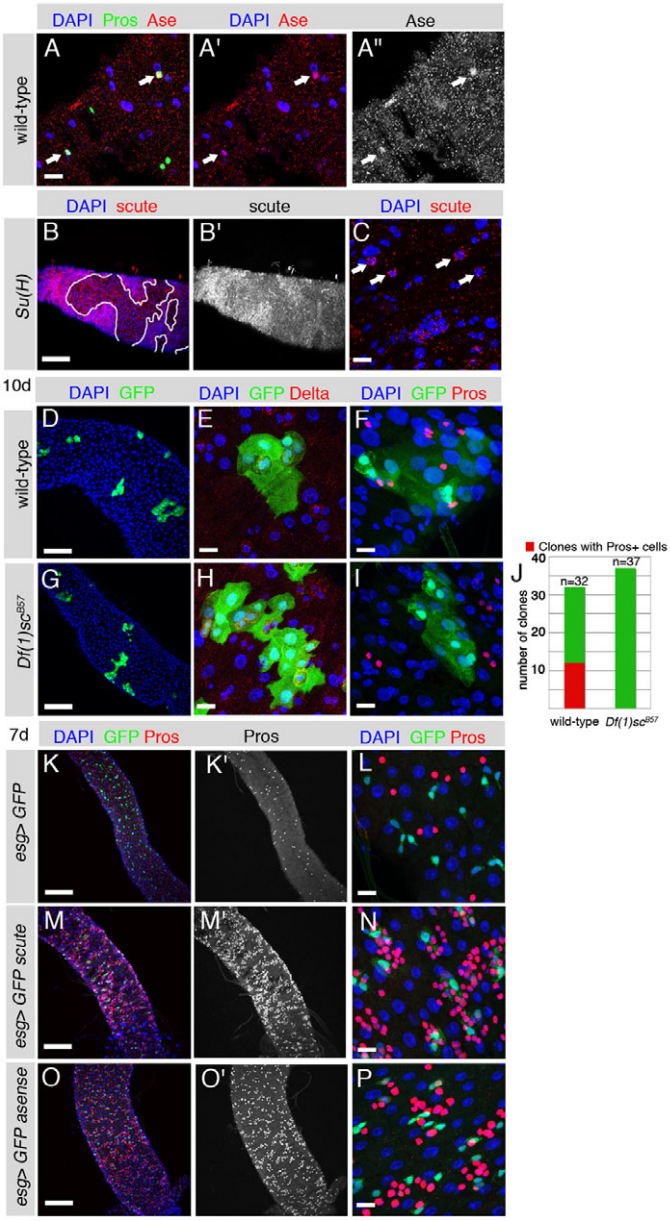

Fig. 6. *achaete-scute* complex genes are dispensable for ISC fate, but act in enteroendocrine fate. (**A-A″**) Expression of the bHLH protein Asense (Ase, red in A,A′, white in A″) was specifically detected in a subset of Pros$^+$ cells (arrows; DAPI, blue; Pros, green). (**B-C**) *scute* RNA (red) was detected by fluorescent in situ hybridization in small nuclei cells both within and outside (arrows in C) of the *Su(H)* mutant clone area (identified by DAPI staining and outlined in B). (**D-J**) *Df(1)sc^{B57}* mutant clones (G-I) grew similarly to wild-type control clones (D-F) and contained Delta$^+$ ISCs as well as polyploid ECs (Delta, red in E,H; DAPI, blue; GFP, green). However, *Df(1)sc^{B57}* mutant clones did not contain Pros$^+$ cells (red). (J) Quantification revealed that one-third of wild-type clones at 10 days AHS contained at least one Pros$^+$ cell (red bar, 12/32 clones), whereas *Df(1)sc^{B57}* (0/37) did not contain Pros$^+$ cells (P=0.00003, Fisher's exact test; see Materials and methods for statistics). (**K-P**) Expression of *scute* (M-N) and *asense* (O-P) in ISCs and EBs of adult flies using *esgGAL4Gal80ts* produced ectopic Pros$^+$ ee cells, as compared with control flies (K-L; GFP, green, Pros, white or red; DAPI, blue). Scale bars: 100 μm in B,B′,D,G,K,K′,M,M′,O,O′; 10 μm in A-A″,C,E,F,H,I,L,N,P.

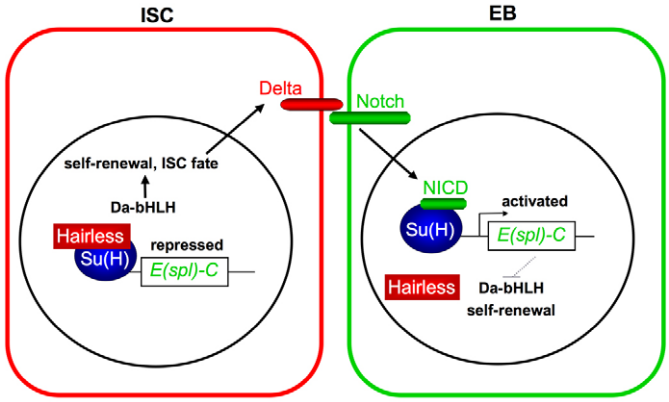

Fig. 7. Model for ISC maintenance. We propose that Hairless prevents ISC loss by repressing expression of Notch target genes, including the *E(spl)-C* genes. We further propose that Da-dependent bHLH activity promotes ISC identity, including the ability to self-renew and to express Delta. Delta, in turn, activates Notch in the adjacent EB (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Ohlstein and Spradling, 2007), releasing the intracellular domain of Notch (NICD). We speculate that, in response to Notch activation, the E(spl)-bHLH repressors downregulate Da-dependent bHLH activity in EBs as described in other systems (reviewed by Kageyama et al., 2007; Alifragis et al., 1997; Gigliani et al., 1996; Heitzler et al., 1996; Oellers et al., 1994), thereby shutting off ISC identity and promoting differentiation. The solid lines represent interactions for which we provide evidence, whereas the dashed line represents a proposed mechanism based on interaction data from other systems.

Our data suggest that ISC fate is promoted both by inhibition of Notch target genes through Hairless/Su(H) repression and by activation of ISC-specific genes through bHLH activity. How then is asymmetry in Notch activity eventually established between the two ISC daughters to allow one cell to remain an ISC and one cell to differentiate? We can envisage three types of mechanism that would allow for asymmetry of Notch signaling.

First, the binary decision between the ISC and EB fates might result from a competition process akin to lateral inhibition for the selection of SOPs (Heitzler and Simpson, 1991). In this process, feedback loops establish directionality by amplifying stochastic fluctuations in signaling between equivalent cells into a robust unidirectional signal. Our finding that the Da activator and E(spl)-bHLH repressors are important to properly resolve ISC/EB fate is consistent with this type of model. Activation of the Notch pathway in one of the daughter cells could then lead to the changes in nuclear position previously noted (Ohlstein and Spradling, 2007).

Second, the asymmetric segregation of determinants could bias Notch-mediated cell fate decisions. The cell fate determinants Numb and Neur are asymmetrically segregated in neural progenitor cells to control Notch signaling (Bardin et al., 2004; Knoblich, 2008). However, we find no evidence for the asymmetric segregation of these proteins in dividing ISCs (A.J.B., unpublished). Additionally, our data indicate that Numb is not important to maintain ISC fate. We cannot exclude, however, the possibility that another, unknown Notch regulator is asymmetrically segregated to regulate the fate of the two ISC daughters.

A third possibility is that after ISC division, one of the two daughter cells receives a signal that promotes differential regulation of Notch. Indeed, it has been noted that the axis of ISC division is tilted relative to the basement membrane, resulting in one of the

progeny maintaining greater basal contact than the other (Ohlstein and Spradling, 2007). An extracellular signal coming either basally or apically could bias the Notch-mediated ISC versus EB fate decision. For instance, Wg secreted by muscle cells could act as a basal signal to counteract Notch receptor signaling activity in presumptive ISCs (Lin et al., 2008). This could be accomplished by Wg promoting bHLH activity or gene expression. Indeed, Wg has been demonstrated to promote proneural bHLH activity in *Drosophila* (Couso et al., 1994; Phillips and Whittle, 1993; Tomoyasu et al., 1998).

These models are not mutually exclusive, however, and proper control of ISC and differentiated cell fates during tissue homeostasis might involve multiple mechanisms.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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