© 2016. Published by The Company of Biologists Ltd | Development (2016) 143, 3024-3034 doi:10.1242/dev.134387

RESEARCH ARTICLE

Escargot and Scratch regulate neural commitment by antagonizing Notch activity in *Drosophila* sensory organs

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ABSTRACT

During Notch (N)-mediated binary cell fate decisions, cells adopt two different fates according to the levels of N pathway activation: an N_{off}-dependent or an N_{on}-dependent fate. How cells maintain these N activity levels over time remains largely unknown. We address this question in the cell lineage that gives rise to the *Drosophila* mechanosensory organs. In this lineage a primary precursor cell undergoes a stereotyped sequence of oriented asymmetric cell divisions and transits through two neural precursor states before acquiring a neuron identity. Using a combination of genetic and cell biology strategies, we show that Escargot and Scratch, two transcription factors belonging to the Snail superfamily, maintain N_{off} neural commitment by directly blocking the transcription of N target genes. We propose that Snail factors act by displacing proneural transcription activators from DNA binding sites. As such, Snail factors maintain the N_{off} state in neural precursor cells by buffering any ectopic variation in the level of N activity. Since Escargot and Scratch orthologs are present in other precursor cells, our findings are fundamental for understanding precursor cell fate acquisition in other systems.

KEY WORDS: Bristle lineage, E(spl) genes, Neural cell commitment, Notch pathway, Snail factors, Transcription repression

INTRODUCTION

During nervous system formation, the Notch (N) signaling pathway regulates neuronal specification at two levels: first, by regulating the number of neural precursor cells; and second, by conditioning sibling cell fates in neural cell lineages (Guruharsha et al., 2012; Jukam and Desplan, 2010; Simpson, 1997). In the latter case, N signaling is differentially activated in pairs of newly divided sibling cells and, as such, restricts the acquisition of a given cell fate to only one daughter cell. The molecules and regulatory interactions involved in triggering such N-dependent asymmetric processes have been investigated in depth, but the factors involved in maintaining N-related cell fate commitment are unknown. Here, we shed light on one mechanism that maintains the N_{off} state in neural precursor cells in the *Drosophila* mechanosensory organ (mSO).

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Received 18 December 2015; Accepted 12 July 2016

Drosophila mSOs are formed by a neuron and three accessory cells. All these cells arise from a primary precursor cell, called pI, which undergoes a stereotyped sequence of oriented asymmetric cell divisions. The pI cell division produces the secondary precursor cells pIIb and pIIa. The pIIa cell gives rise to the outer terminal cells, the socket and the shaft cells. The pIIb cell produces the neural type cells, the glial cell (which disappears as a result of apoptosis) and the tertiary precursor cell pIIIb, which then generates the sheath cell and the neuron (Fichelson and Gho, 2003; Gho et al., 1999; Lai and Orgogozo, 2004). At each of these divisions, each daughter cell acquires a different identity due to the differential activation of the N pathway. This differential activation of the N pathway between daughter cells has been well documented during pI division: one daughter cell, pIIb (the N_{off} cell), acts as an N signal sender and its sibling, pIIa (the N_{on} cell), as an N signal receiver (Guo et al., 1996). The bias in the activation of the N pathway is mainly assured by the stereotyped segregation of determinant factors such as Numb and Neuralized (Neur) in one daughter cell (the future pIIb cell) (Guo et al., 1996; Le Borgne and Schweisguth, 2003; Morin and Bellaiche, 2011).

Snail transcription factors are involved in a wide range of cellular functions and are essential in metazoan development (Barrallo-Gimeno and Nieto, 2005; Nieto, 2002; Thiery et al., 2009). Two gene families compose the Snail superfamily: Snail and Scratch. These two families encode transcription factors that contain a divergent N-terminal region and a highly conserved C-terminal region containing four to six zinc fingers that recognize sequence-specific DNA binding motifs similar to the E-box, the core binding site of basic helix-loop-helix (bHLH) transcription factors (Nieto, 2002). In *Drosophila*, *snail* (*sna*), *escargot* (*esg*) and *worniu* (*wor*) compose the Snail gene family, and *scratch* (*scrt*) and two putative genes (*scratch-like 1* and *scratch-like 2*) compose the Scratch family (see Nieto, 2002). *Snail, esg, wor* and *scrt* encode factors involved in neurogenesis. For instance, the ventral nerve cord is severely underdeveloped in *Drosophila* embryos mutant for Snail-related factors (Ashraf and Ip, 2001; Ashraf et al., 1999; Cai et al., 2001). Moreover, *sna*, *esg* and *wor* redundantly control the segregation of cell fate determinants during asymmetric neuroblast divisions (Ashraf and Ip, 2001; Cai et al., 2001). Although these data indicate that Snail/Scratch factors play central roles in neurogenesis, little is known about the molecular basis of their involvement.

A growing body of evidence supports the involvement of transcription factors from the Snail superfamily in synergy with the N pathway in cell identity specification (Cowden and Levine, 2002; Morel et al., 2003). In vertebrates, during development as well as in pathological situations, N signaling and several Snail factors interact in a complex crosstalk during the epithelial-mesenchymal transition (Wang et al., 2010). In addition, direct interaction between the intracellular domain of the N receptor and Snail has been reported in hepatocellular carcinoma cells (Kim et al., 2013; Lim et al., 2011).

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The present study shows that Esg and Scrt are specifically expressed in the neural/pIIb sublineage and act redundantly to maintain the binary neural/non-neural fate decision by downregulating N pathway activity. Our results indicate that Esg and Scrt, acting redundantly, are necessary and sufficient for the acquisition of the neural precursor pIIb cell fate. Moreover, these factors repress N pathway activation; they probably bind to the promoter of N target genes and downregulate their transcription. We present evidence suggesting that Snail factors downregulate N target gene transcription by competing with activators as proneural factors. Since Esg and Scrt orthologs are present in other precursor cells, our findings on the interaction between Snail factors and the N pathway are fundamental to understanding precursor cell fate acquisition in other systems.

RESULTS

Esg and Scrt, acting redundantly, are necessary and sufficient to maintain neural type fate decisions

We studied members of the *Drosophila* Snail gene superfamily in a screen designed to identify factors involved in neural commitment in the mSO. After individual *esg* or *scrt* downregulation, external sensory structures were unaffected (Fig. 1A-C). By contrast, when both *esg* and *scrt* were downregulated, mSOs with two bristles were observed in 48% of the flies (n=83, Fig. 1D). Similar double-shaft structures were also observed in *esg* null clones induced in the heteroallelic $scrt^{jo11/jo16}$ background (Fig. 1G). The double-shaft phenotype could result from either a cell transformation or from extra divisions in the outer cell sublineage. In pupae at 24 h after puparium formation (APF), mSOs composed of no more than four cells were observed after *esg* and *scrt* downregulation (Fig. 1F,F′,H-H″), as in the control (Fig. 1E). Moreover, except for the apoptosis of glial cells, we never observed other cells in the process of fragmentation (not shown). These observations rule out the possibility that double-shaft mSOs arose from extra divisions of outer cells, and suggest instead that they resulted from a cell fate transformation. Indeed, after *esg* and *scrt* downregulation, mSOs were formed exclusively of four outer cells [Pdm1 (Nubbin) positive, Fig. 1F′], two of which were Su(H)-positive socket cells (Fig. 1H′), whereas no inner cells marked by Elav (neurons) or Prospero (Pros) (sheath cells) were observed (Fig. 1F″). Taken together, these observations show that the extra set of external structures observed after downregulation of both *esg* and *scrt* results from a cell transformation. This shows that *esg* and *scrt* are involved in neural cell fate decisions in the mSO cell lineage.

We next studied whether Esg and Scrt are sufficient to induce neural type cell fates. Overexpression of *scrt* (*HS-scrt*) at 16 h APF produced flies with severe bristle loss (Fig. 1I). As shown in Fig. 1J, this phenotype was associated with mSOs formed exclusively of neural type cells (identified by the lack of Pdm1 staining, Fig. 1J′), two of which were neurons (Elav-positive cells, Fig. 1J″). Similar bristle loss phenotypes (Fig. 1K) and mSOs composed exclusively of neural type cells (two Elav-positive cells or one Elav and one Pros cell, Fig. 1L-L″) were also observed when *esg* was overexpressed (using an *HS-Gal4/UAS-esg* fly line heat shocked at 16 h APF). These observations show that overexpression of *esg* or *scrt* is sufficient to induce a cell transformation whereby all sensory cells adopt a neural identity.

Taken together, our data indicate that *esg* and *scrt*, acting redundantly, are necessary and sufficient to maintain neural type fate decisions.

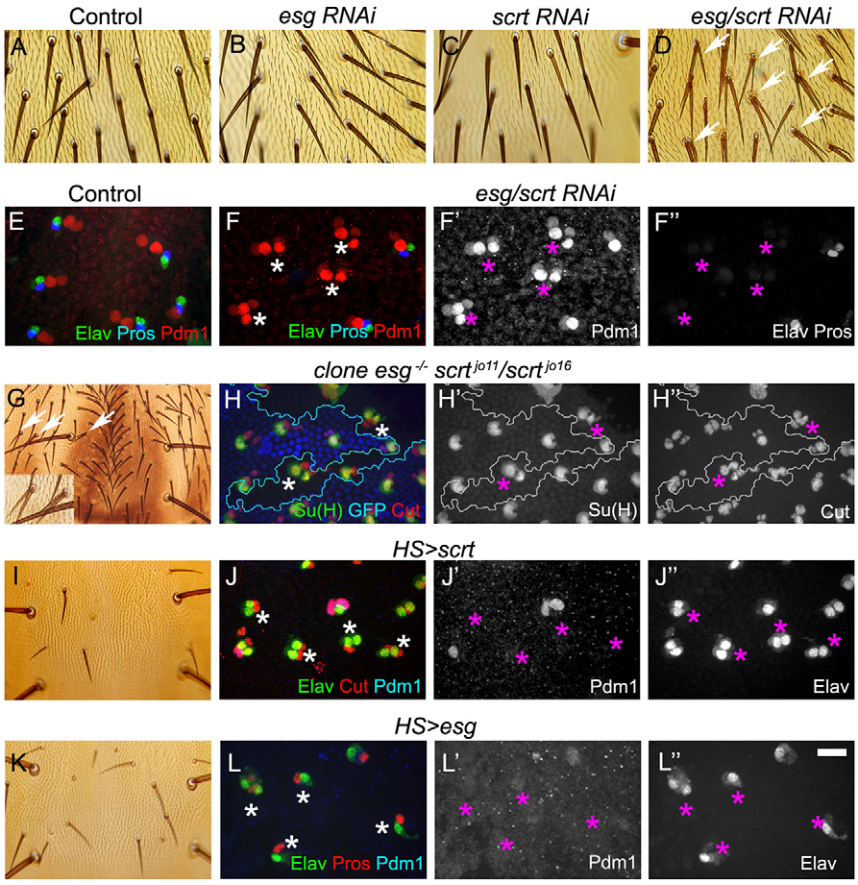

Fig. 1. Esg and Scrt redundantly control neural cell fate commitment. (A-D) Esg and/or Scrt were depleted in the medio-dorsal thorax by expressing RNAi constructs using a *pnr-Gal4* driver at 25°C. Details are shown of adult thorax from flies of the indicated genotypes. Arrows indicate double-shaft mSOs. (E,F,H,J,L) Immunofluorescence analysis in which sensory cells were identified by Cut, sheath cells by Pros, neurons by Elav, outer cells by Pdm1 and socket cells by Su(H). mSO (*pnr-Gal4* alone) is shown in control (E) and after downregulation of both *esg* and *scrt* by RNAi (F-F″). Asterisks (F-F″) indicate mSOs composed of outer cells only. (G-H″) mSOs in *esg* mutant clones in an *scrt* heteroallelic background. (G) Details of an adult thorax showing mSO with a double shaft (arrows and inset). (H-H″) *esg* clones were detected by the absence of GFP (blue; line indicates clonal border). Asterisks indicate mSOs harboring double socket cells. (I) Detail of a thorax after ectopic expression of Scrt (*HS-scrt* pupae, heat shocked at 37°C for 60 min at 16 h 30 min APF). (J-J″) mSO cells after overexpression of *scrt*. Asterisks show mSOs devoid of Pdm1-positive outer cells. (K) Details of a thorax after ectopic expression of Esg (*HS-Gal4/UAS-esg* pupae, heat shocked at 37°C for 60 min at 16 h 30 min APF). (L-L″) mSO cells after overexpression of *esg*. Asterisks show mSOs devoid of Pdm1-positive outer cells. Pupae in E,F,H and J are at 30 h APF; pupa in L at 28 h APF. Scale bar: 10 µm for E, F,H,J,L.

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Esg and Scrt are necessary and sufficient for neural precursor/pIIb cell fate acquisition

The most likely explanation for the phenotype observed after downregulation of *esg* and *scrt* is that pIIb neural precursor cells are transformed into pIIa non-neural precursor cells. To address this possibility, we monitored the expression of *tramtrack* (*ttk*), a non-neuronal fate determinant expressed in pIIa cells (Audibert et al., 2005), following *esg* and *scrt* downregulation. In non-clonal heteroallelic $scrt^{jo11/jo16}$ tissue at 19 h APF, two-cell sensory clusters were composed of pIIb and the Ttk-positive pIIa cell (Fig. 2A,A′, arrowheads). By contrast, two-cell mSOs inside esg^{G66B} null clones induced in the heteroallelic $scrt^{jo11/jo16}$ background were composed of two Ttk-positive pIIa cells (Fig. 2A,A′, asterisks; 14 out of 84 mSOs analyzed). As such, downregulation of *esg* and *scrt* leads to a cell transformation whereby the neural precursor cell pIIb acquires a non-neural Ttk-positive pIIa cell fate.

Next, we studied whether this cell transformation was associated with an ectopic activation of the N pathway in the presumptive pIIb cell. To this end, in two-cell sensory clusters, we monitored N receptor activation by detecting nuclear translocation of the N intracellular domain (NICD) (Couturier et al., 2012). In control conditions, NICD was detected in the nucleus of the posterior pIIa cells (Fig. 2B,B′,D), whereas after *esg* and *scrt* downregulation NICD was also detected in the anterior cell (Fig. 2C,C′) and at a level comparable with that found in full N_{on} Ttk-positive pIIa cells (Fig. 2D). This level of NICD in the anterior cell was associated with Ttk staining (not shown). Altogether, these observations show that, after downregulation of *esg* and *scrt*, the N pathway is ectopically activated in the anterior presumptive pIIb cells. This led to the expression of non-neuronal fate determinants such as *ttk*, and the presumptive pIIb cell consequently adopted a pIIa cell fate.

Similarly, the phenotype observed after overexpression of *esg* or *scrt* might be explained by a cell transformation of pIIa non-neural precursor cells into pIIb neural precursor cells. We analyzed the expression of *pros* to identify pIIb cells following *esg* or *scrt* overexpression. As shown in Fig. 2E,F, we observed two-cell mSOs where both cells expressed cortical Pros, whereas in the control mSO only one precursor cell, i.e. the neural precursor pIIb cell, expressed Pros. These observations show that the presumptive non-neural precursor pIIa cell acquired a neural precursor pIIb cell identity after overexpression of *esg*.

We show here that the secondary precursor cell fate decision is sensitive to the level of activity of both Esg and Scrt. Moreover, Esg and Scrt are necessary and sufficient for the acquisition of the neural precursor pIIb cell fate.

Esg and Scrt are expressed in the neural branch of the bristle lineage

We next explored the expression of *esg* and *scrt* during the completion of the bristle lineage (from 16 h to 24 h APF). As revealed by specific Esg polyclonal antibodies, a nuclear-located

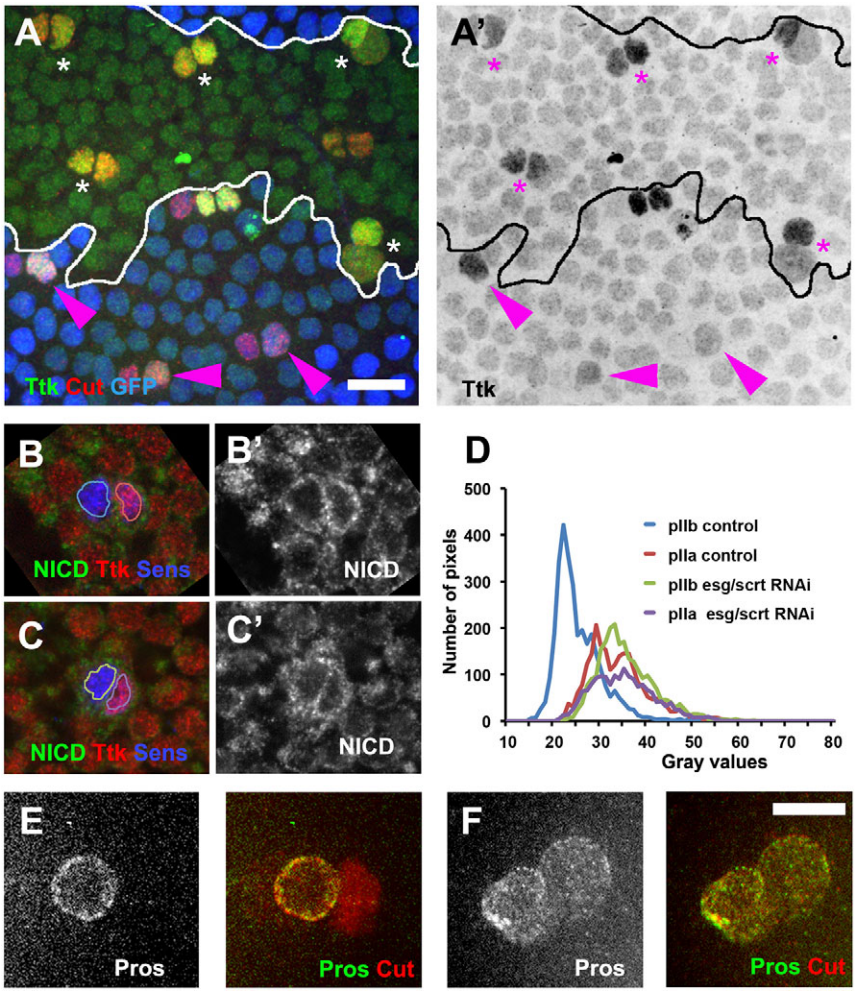

Fig. 2. Esg and Scrt are necessary and sufficient for neural precursor pIIb cell fate acquisition. (A) *esg* clone (detected by the absence of GFP, blue; white line indicates clonal border) in an *scrt* heteroallelic background at 18 h APF. Sensory cells were identified by Cut immunoreactivity (red). Ttk immunoreactivity is in green, with inverse fluorescence in A′. Arrowheads indicate wild-type two-stage mSOs, located outside the *esg* clone, with only one Ttk-positive cell. Asterisks indicate two-cell stage mutant mSOs, located inside the *esg* clone, where both cells are Ttk positive. (B-D) Endogenous NICD (green; single channel in B′ and C′) in two-cell mSO in control (B) and after *esg* and *scrt* downregulation (C). Ttk immunoreactivity is in red; mSOs are identified by Sens immunoreactivity (blue). (D) The distribution of gray values for the NICD channel in areas selected in B and C. (E,F) Pros expression in mSO at the two-cell stage in control (E) and following overexpression of *esg* using a *neur-Gal4* promoter expressing Gal4 in all mSO cells (F). Scale bars: 10 µm in A; 5 µm in E,F.

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signal was clearly observed in some pIIb cells (3 out of 9 two-cell organs), and this staining vanished at later stages (Fig. 3A,A′). *In vivo* recordings of the Esg protein-trap fly line (*esg*^{p1986}*::eGFP*) revealed that Esg::GFP was absent in pI cells and was turned on progressively in pIIb cells, 30 min prior to their division (Fig. 3C, Movie 1). This was consistent with the low proportion of Esg-positive pIIb cells observed by immunofluorescence. Thereafter, Esg::GFP was detected in all pIIb descendants: the glial, pIIIb and sheath cells as well as in the neuron (Movie 1). According to the expression pattern obtained with antibodies, we believe that the GFP signal in pIIb descendants is due to the persistence of GFP molecules.

Using anti-Scrt polyclonal antibodies, a signal was clearly observed in the nucleus of pIIb as well as in pIIIb but was undetectable later on (Fig. 3B,B′). Scrt expression was turned on progressively in pIIb, since we observed many two-cell mSOs in which Scrt was absent or barely detectable (4 out of 15, second frame in Fig. 3B). In addition, Scrt was only expressed in neural progenitor cells and was not detected in pIIa. Using a protein-trap *scrt::GFP* line and an enhancer-trap *scrt*^{439}*-lacZ* reporter line we confirmed the expression of Scrt in pIIb (Fig. 3D, Fig. S1). Furthermore, the low proportion of pIIb staining for Scrt suggests that the onset of Scrt expression occurred late in these cells.

We also analyzed the expression of *sna* and *wor*, the other two members of the Snail family. Using specific antibodies, we failed to observe *wor* expression in bristle cells at any period in the lineage (not shown). By contrast, *sna* was strongly expressed in terminal glial cells prior to entering into apoptosis (Fig. S2).

Taken together, the results show that, in the bristle lineage, Esg and Scrt are specifically co-expressed in late pIIb cells and in early pIIIb (Fig. 3E). This specific expression in both neural precursor cells is compatible with the possibility that *esg* and *scrt* control neural precursor cell fate in this lineage.

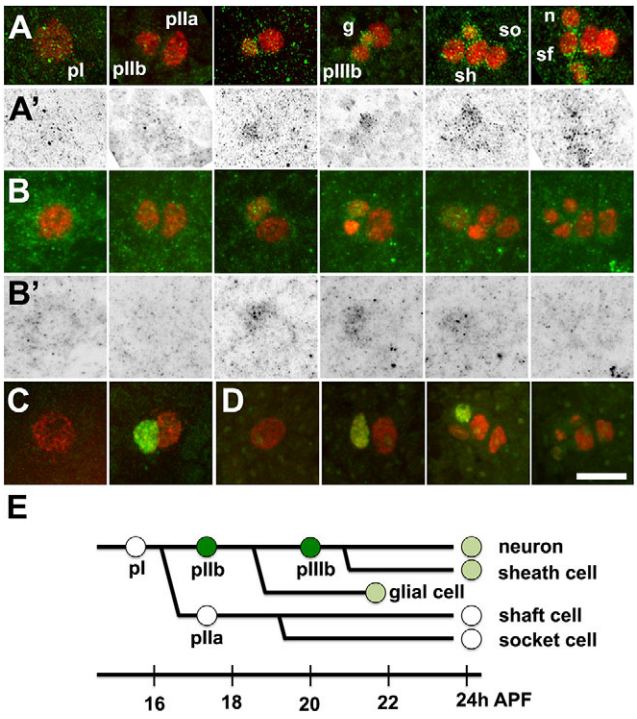

Fig. 3. Esg and Scrt are expressed in the neural sublineage of bristle cells. Expression pattern of Esg (A) and Scrt (B) revealed by immunodetection (green in A,B, inverse fluorescence in A′,B′) at consecutive stages of development. so, socket cell; sf, shaft cell; n, neuron; sh, sheath cell; g, glial cell. (C) Expression pattern of Esg using the protein-trap *esg::GFP* line (green) at two consecutive stages of development. (D) Expression pattern of Scrt using the protein-trap *scrt::GFP* line (green) at consecutive stages of development. In all panels, sensory cells were identified by Cut immunoreactivity (red). (E) Schematic tree of the bristle lineage showing the expression pattern of Esg and Scrt (green). Light green denotes the maintenance of signal in the reporter lines. Scale bar: 10 µm.

Esg or Scrt counteracts N pathway activation

The neural to non-neural cell transformation induced after *esg* and *scrt* downregulation is reminiscent of that observed following overactivation of the N pathway. Conversely, overexpression of *esg* or *scrt* mimics N pathway downregulation. This prompted us to test whether *esg* and *scrt* function in the same genetic pathway as N in the control of fate determination in bristle cells. We analyzed the penetrance of the double-shaft phenotype observed after depletion of both *esg* and *scrt* in conditions where N activity was slightly dysregulated (Fig. 4A). Using the *pannier* (*pnr*)*-Gal4* driver to express *esg* RNAi and *scrt* RNAi in the median region of the notum, we observed around four duplicated organs per notum when pupae were maintained at 30°C (n=77). This value was significantly reduced to two double-double bristles per notum when *esg* and *scrt* were downregulated in an *N* heterozygous background ($N^{ts\text{-}1}/N^{+}$ pupae maintained at 30°C; n=76, P<0.001). Conversely, a significant increase in the number of duplicated organs (around six double-double bristles per notum) was observed when *esg* and *scrt* were downregulated in a background in which N activity was slightly increased (*HS-N*^{intra} pupae maintained at 30°C; n=63, P<0.001). Since we never observed double bristles under these two mild N dysregulation conditions alone, this indicates that Esg and Scrt are functionally related to N in the control of neural cell fate decisions in mSO cells. Furthermore, and more importantly, our data indicate that Esg and Scrt act on neural cell fate determination by counteracting N activity.

Where in the N pathway do Esg and Scrt act? To analyze whether they act on the reception of the N signal, we overexpressed *esg* or *scrt* in the context of ligand-independent N pathway activation by overexpressing the activated form of the N receptor N^{intra}. Overexpression of *esg* alone induced mSOs formed almost exclusively of Elav-positive neurons (Fig. 4B-B″), whereas overexpression of N^{intra} alone induced the formation of mSOs formed exclusively of Pdm1-positive outer cells (Fig. 4C-C″). Strikingly, co-expression of N^{intra} with Esg induced the formation of mSOs with a low level of Pdm1 (Fig. 4D,D′). Moreover, some of these cell clusters were formed exclusively of small strongly Elav-positive cells (Fig. 4D″). Similar results were obtained using Scrt instead of Esg to counteract N pathway activation (Fig. S3). Altogether, these data show that Esg or Scrt overrides the differentiation towards outer fate that results from enforced N^{intra} expression. Thus, Esg or Scrt prevents direct activation of the N pathway by N^{intra} overexpression, suggesting that they counteract N pathway activation by acting downstream of N signal reception.

Overexpression of N^{intra} and Esg from the beginning of puparation (0 h APF) resulted in similar clusters composed of Elav-positive cells (not shown). This rules out the alternative possibility that the preponderance of *esg* or *scrt* over *N*^{intra} could be due to a differential delay in the expression of *esg*, *scrt* and *N*^{intra}. It is relevant to note that mSO cells were not totally transformed to an external or internal fate. We believe that this was due to the mild overexpression procedure rather than to differences in cell sensitivity. Indeed, to avoid any interference of *esg* and *scrt* overexpression with pI specification, pupae were shifted after

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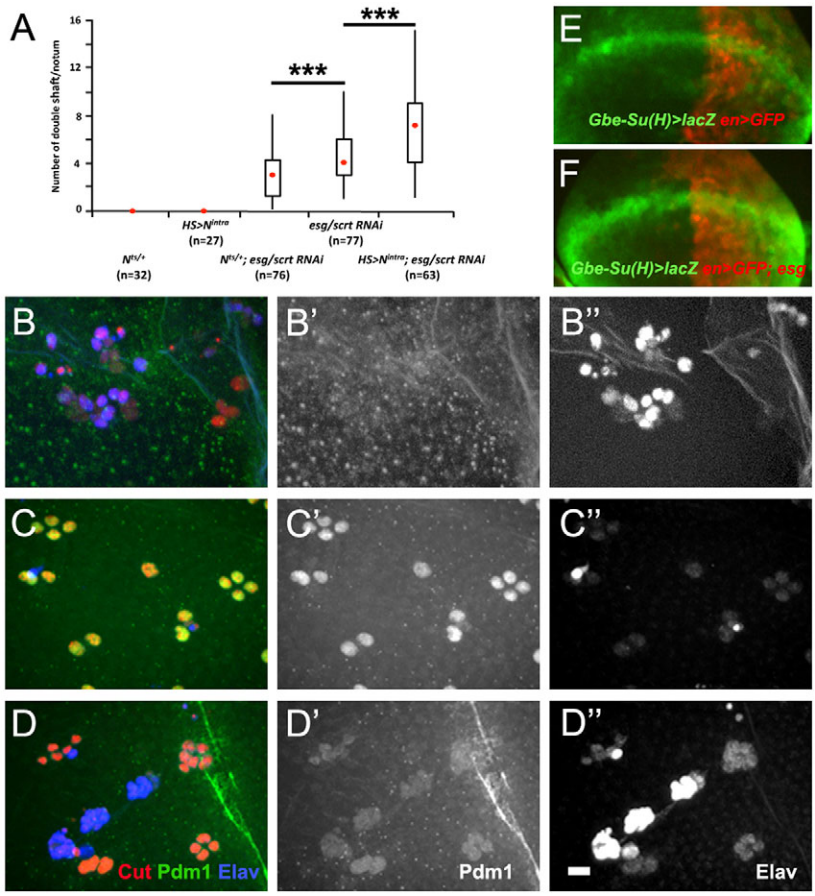

Fig. 4. Esg and Scrt counteract N pathway activation. (A) Genetic interaction between *esg*, *scrt* and *N*. Box plot of the number of duplicated external cells per notum in females. Esg and Scrt were depleted in the dorsal thorax by expressing RNAi constructs using the *pnr-Gal4* driver at 30°C alone, in a mild N loss-of-function background ($N^{ts\text{-}1}$/+ at 30°C) and in a mild N gain-of-function background (*HS-*N^{intra} at 30°C). Note that under these conditions no double bristles were observed in $N^{ts\text{-}1}$ and *HS-*N^{intra} pupae alone. ***P<0.001, Mann–Whitney test. Red dots indicate median values. (B-D″) Overexpression of *esg* (B-B″), N^{intra} (C-C″) and *esg* and N^{intra} (D-D″) 15 h APF onwards using the conditional driver *neur>Gal4 Tub-Gal80*^{ts}. Immunodetection of Elav (blue), Pdm1 (green) and Cut (red) at 22 h APF. Note that mSOs were composed mainly of Elav-positive inner cells (B″,D″) and Pdm1-positive external cells (C′). (E,F) β-Gal expression of a synthetic N reporter construct (*Gb-Su(H)-lacZ*, green) and GFP expression under the control of an *engrailed* driver (red) in a control (E) and after *esg* overexpression (*en-Gal4*/*UAS-esg, UAS-GFP*, red, F). Late third instar larvae. Scale bar: 10 µm in B-D.

specification and maintained for a short period of time at the restrictive temperature.

To investigate the possibility that Esg binds directly to N^{intra} and thereby neutralizes its transcriptional action (Kim et al., 2013; Lim et al., 2011), we analyzed the expression of the N reporter construct *Gbe-Su(H)-lacZ* (Furriols and Bray, 2001). This synthetic promoter is composed exclusively of Su(H) and Grainyhead binding sites. In wing discs, this reporter was strongly expressed in the wing margin (Fig. 4E) and was unaffected after expression of Esg driven by *engrailed* or *patched* (Fig. 4F; data not shown). These data indicate that Esg does not interact directly with N^{intra} to antagonize N signaling. Our results support the idea that the N pathway inhibition induced by Esg impairs N-mediated transcriptional activation and requires native N target promoters.

Esg and Scrt downregulate the transcription of an N target gene reporter in a sequence-specific manner

To test whether Esg and Scrt negatively control N activity by downregulating the transcription of N target genes, we analyzed the effects of these factors on transcription of *E(spl)m4*, a well-described N target gene, (Bailey and Posakony, 1995; Nellesen et al., 1999).

First, using a DNA-mediated Esg pull-down assay, we studied whether Esg binds to the proximal 305 bp of the *E(spl)m4* promoter as bait. This fragment harbors two E-boxes: E1, a canonical binding site for the proneural factors Achaete (Ac) and Scute (Sc), and E2, a canonical Esg binding site (Bailey and Posakony, 1995; Maeder et al., 2007). The data presented in Fig. 5A reveal the efficient retention of Esg by the *E(spl)m4* promoter fragment (lane 2), whereas almost no retention was observed when beads were coated with E-box-free promoter fragments (lane 1, *ftz* promoter). Interestingly, very little binding was detectable when beads were coated with modified *E(spl)m4* promoter fragments in which the canonical E-box E2 was replaced by an unrelated sequence (lane 3). These data indicate that Esg binds to the *E(spl)m4* promoter through a sequence-specific mechanism.

Second, to test whether Esg can repress N target gene transcription *in vivo*, we used a construct in which the expression of β-Gal is under the control of the 510 bp proximal region of the *E(spl)m4* promoter (Bailey and Posakony, 1995). In the wing pouch of the *E(spl)m4::lacZ* wing imaginal disc, β-Gal was detected in sensory cells at each side of the anterior wing margin, as well as in groups of chordotonal mSOs located in the hinge (arrowhead and asterisks, respectively, in Fig. 5B) (see also Bailey and Posakony, 1995). As shown in Fig. 5C,C′, this β-Gal staining was absent in sensory cells in the margin (arrow) and in chordotonal organs (bracket) located inside Esg-expressing patches (identified by the expression of GFP). This β-Gal suppression was not associated with an anti-neurogenic phenotype since Senseless-positive precursor cells were still detected in the region of Esg overexpression (not shown). These data show that overexpression of Esg blocks the expression of the *E(spl)m4* reporter without affecting the selection of mSO precursor cells. A similar blockage of *E(spl)m4-lacZ* expression was observed when Scrt was overexpressed, suggesting that Esg and Scrt have similar repressive properties on N target gene expression (Fig. S4).

In an *E(spl)m4* mutant reporter line in which both E1 and E2 were mutated [*E(spl)-2m::lacZ* (Bailey and Posakony, 1995)], β-Gal expression was only maintained in chordotonal sensory cells (Fig. 5D). Interestingly, this expression was not affected by

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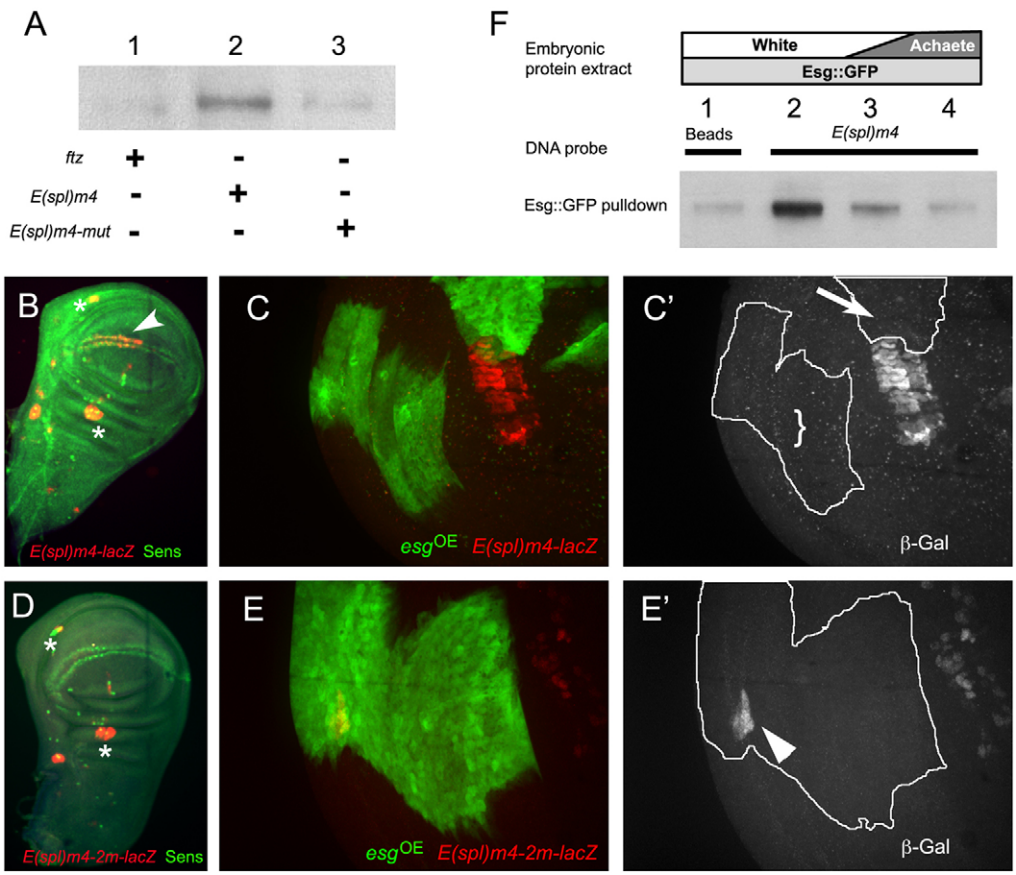

Fig. 5. Esg specifically binds to the *E(spl)m4* promoter and downregulates its transcriptional activity *in vivo*. (A) DNA-mediated Esg pull-down assay of a protein extract from embryo expressing Esg::GFP. Magnetic beads were coated with: lane 1, *ftz* promoter as a control E-box-free promoter fragment; lane 2, *E(spl)m4* promoter bearing the canonical E-box sequence ACAGGTG; lane 3, mutant *E(spl)m4* promoter fragments in which this canonical E-box was replaced by unrelated sequence. (B) β-Gal expression in wing imaginal discs driven by a native *E(spl)m4* promoter (*E(spl)m4-lacZ*) in wing margin (arrowhead) and chordotonal organs (asterisks). (C,C′) Flip-out Esg overexpression clone covering the region of the ventral chordotonal organ in control conditions. Note that in the Esg overexpression regions, β-Gal expression disappears either in the margin (arrow) or in the chordotonal organ (bracket). (D) β-Gal expression in wing discs driven by a mutant form of the *E(spl)m4* promoter (*E(spl)m4-2m-lacZ*). Note the remaining two β-Gal-positive chordotonal organs (asterisks). (E,E′) Flip-out Esg overexpression clone covering the region of the ventral chordotonal organ in mutant conditions (*E(spl)m4-2m-lacZ*). Note that Esg overexpression does not affect β-Gal expression in the ventral chordotonal organ (arrowhead). (F) Competition between Esg and Ac to bind to the *E(spl)m4* promoter. Lane 1: non-coated beads. Lanes 2-4: beads coated with *E(spl)m4* promoter. Note that Esg::GFP pull down was reduced by increasing the amount of Ac-expressing embryo extract. Western blots were revealed with anti-GFP antibodies.

overexpression of Esg (Fig. 5E,E′). Together, these results indicate that Esg prevents transcription of *E(spl)m4* by binding to the promoter in a sequence-dependent manner. As such, these results suggest that Esg and Scrt downregulate N pathway activity by acting as transcriptional repressors of N target genes.

Esg may outcompete proneural activator factors on N target enhancers

Since Esg and Scrt and proneural factors Ac and Sc bind E-boxes in a restricted domain of the *E(spl)m4* promoter, we asked whether Esg or Scrt and Ac or Sc compete with each other for the control of *E(spl)m4* transcription. To address this question, we studied Esg binding to the proximal 305 bp of the *E(spl)m4* promoter in the presence of increasing amounts of Ac. We assayed extracts that consisted of a fixed amount of *esg::GFP*-expressing embryo extract complemented with a mixture of *white* embryo and *ac*-expressing embryo extracts at variable proportions such that the total amount of protein remained constant. We observed that the binding of Esg to beads coated with *E(spl)m4* was displaced by increasing the amount of Ac extract (Fig. 5F, compare lanes 2-4). This displacement was almost complete since the level of Esg binding was comparable to the non-specific binding observed (Fig. 5F, compare lanes 4 and 1). These results suggest that Esg and Ac mutually interfere in binding to the *E(spl)m4* promoter. As such, the downregulation of *E(spl)m4* transcription by Esg is probably due, first, to the displacement of an activator complex brought by proneural factors, such as Ac, and second, to a repressor complex conveyed by Esg as a DNA-binding factor.

Esg and Scrt are not neural precursor cell determinants

We have shown that *esg* and *scrt* are involved in the control of neural precursor cell identity by preventing N-mediated activation of target gene expression. To test whether Esg and Scrt also act as cell determinants in the control of neural precursor cell identity, we studied the identity of mSO cells when *esg* and *scrt* were downregulated in a context in which N pathway activity was also impaired. Transient inhibition of the N pathway (using $N^{ts\text{-}1}$) during the period of pI cell division induced mSO composed exclusively of Pros- and Elav-positive internal cells due to transformation of pIIa into pIIb cells (Fig. 6B). However, mSOs were composed exclusively of external cells (no Elav and Pros staining) when *esg* and *scrt* were downregulated (Fig. 6C). Interestingly, in pupae in

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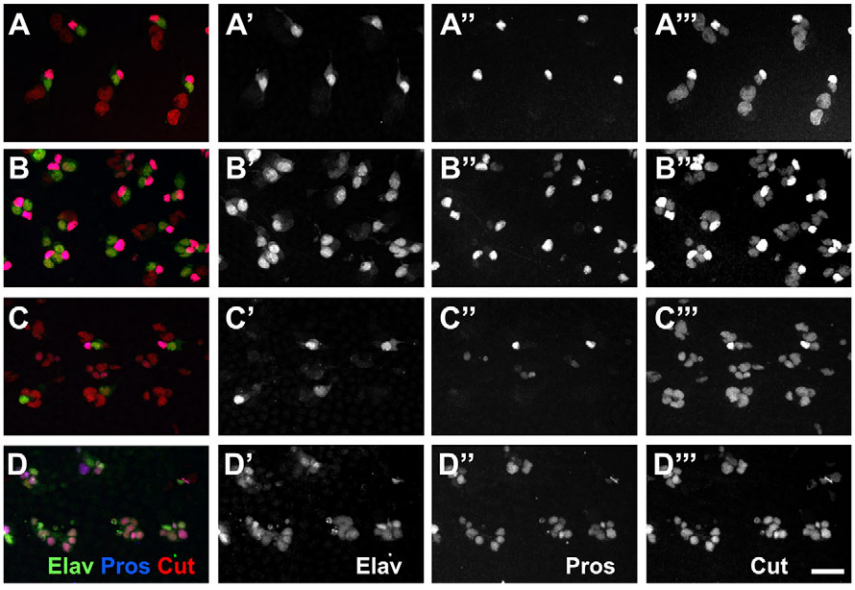

Fig. 6. Epistatic relationships between Esg, Scrt and the N pathway. Esg and Scrt functions were downregulated using an RNAi construct expressed via the *pnr>Gal4* driver. The N pathway was impaired using the $N^{ts\text{-}1}$ thermosensitive allele and shifting pupae to restrictive temperature (30°C) during the period of pI division. mSO cells were identified by Cut (A‴-D‴), neurons by Elav (A′-D′) and sheath cells by Pros (A″-D″) immunoreactivity. (A) mSOs from control $N^{ts\text{-}1}$/+; +/*UAS-RNAi scrt; UAS-RNAi esg*/*TM6Tb* pupae showing a normal set of cells. (B) Transformed mSO from $N^{ts\text{-}1}$/Y; +/*UAS-RNAi scrt; UAS-RNAi esg*/*TM6Tb* pupa in which pIIa cells acquired a pIIb cell identity due to the transient impairment of the N pathway during pI cell division. (C) Transformed mSO from $N^{ts\text{-}1}$/+; +/*UAS-RNAi scrt; pnr>Gal4*/*UAS-RNAi esg* pupae in which pIIb cells acquired a pIIa cell identity due to *esg* and *scrt* RNAi-mediated downregulation. (D) mSO from $N^{ts\text{-}1}$/Y; +/*UAS-RNAi scrt; pnr>Gal4*/*UAS-RNAi esg* pupae after *esg* and *scrt* RNAi-mediated downregulation and transient impairment of the N pathway during pI cell division. Note mSO cells co-expressed Elav and Pros, although at a lower level than observed in fully differentiated neuron and sheath cells (as observed in A-C). Scale bar: 10 µm.

which the N pathway and *esg* and *scrt* were simultaneously downregulated, mSO cells acquired an internal precursor cell identity (Fig. 6D). These cells co-expressed Pros and Elav at a lower level than those observed in terminal neurons or sheath cells (compare Fig. 6A-D), a distinctive molecular profile of neural precursor cells in this system. Strikingly, although cells adopted a neural-like fate, no clear distinction between neuron and sheath cells was observed under these conditions. This suggests that Esg and Scrt were also required in pIIIb progeny to implement the neuron sheath identities. Nonetheless, the acquisition of neural precursor cell fate was not impaired when *esg*, *scrt* and the N pathway were downregulated. Moreover, these data show that the external cell fate adopted by sensory cells after *esg* and *scrt* downregulation requires N signaling. Altogether, our results show that *esg* and *scrt* do not determine neural precursor cell fate. Rather, they are required to maintain an N_{off} state in these cells, an essential condition to implement the neural precursor cell identity.

DISCUSSION

Using a combination of genetic and cell biology analyses, we show that Esg and Scrt, which belong to the Snail gene superfamily, control neural cell fate commitment in the *Drosophila* mSO. Both transcription factors are expressed at early stages in the bristle lineage, particularly in the sublineage that gives rise to neural type cells. We show that these factors redundantly control the secondary precursor fate decision and, in particular, they maintain the N_{off} cell fate, i.e. the neural precursor pIIb fate. In addition, our results indicate that Esg and Scrt counteract the activity of the N pathway. Using the *E(spl)m4* promoter as a paradigm of an N target gene, we present data suggesting that Esg and Scrt downregulate the transcription of N target genes by binding to the promoter. In addition, Esg and Scrt seem to act as proneural antagonists by outcompeting proneural transcription activators for binding to N target gene promoters. As such, we propose that Esg and Scrt, by repressing N target gene transcription, lock the neural precursor pIIb identity by absorbing any potential variation in N activity.

Esg and Scrt act redundantly to control cell identity

Cumulative evidence obtained in both vertebrate and invertebrate models indicates that Snail factors can function redundantly and compensate for each other's loss during essential developmental processes such as neurogenesis, hematopoiesis or chondrogenesis (Ashraf and Ip, 2001; Chen and Gridley, 2013; Pioli and Weis, 2014). We show that when both *esg* and *scrt* were downregulated, 48% of flies harbored mSOs composed only of outer cells (the double-shaft phenotype). This phenotype was not observed in flies in which *esg* or *scrt* was downregulated individually. To our knowledge, this is the first report showing that Esg and Scrt act redundantly and compensate for one another in the control of cell fate. Since Esg and Scrt are two distantly related members of the Snail superfamily, this redundancy suggests that ancestors of Snail factors were already involved in the control of cell fate identity.

Interestingly, *esg* is expressed in several stem cell types in *Drosophila*, including histoblasts, intestinal stem cells and male germ stem cells (Fuse et al., 1994; Micchelli and Perrimon, 2006; Streit et al., 2002). Several studies performed on these cell types have shown that Esg is required to maintain stem cell fate. Indeed, loss of *esg* drives stem cells to differentiate (Hayashi, 1996; Korzelius et al., 2014). An interesting question is whether *scrt* is expressed in these stem cells. If so, the phenotype observed in the *esg* mutant could be partially penetrant due to an *scrt*-mediated redundancy similar to that described here in neural progenitor cells. Since members of the Snail and Scrt families are also expressed in neural progenitors in vertebrates (Itoh et al., 2013; Vieceli et al., 2013; Zander et al., 2014), our results are likely to be of general relevance and might help improve our understanding of the mechanisms underlying vertebrate neurogenesis.

Interaction between N and Snail factors

Downregulation of *esg* and *scrt* induced a transformation from N_{off} pIIb to N_{on} pIIa cells resulting in double-shaft mSOs. Interestingly, the number of transformations induced under *esg* and *scrt* downregulation conditions changed significantly with mild variations in the activity of the N pathway – variations that do not induce changes in cell fate by themselves. As such, we favor the idea that Esg and Scrt act redundantly to control neural precursor pIIb cell identity by downregulating N pathway activity. In agreement with this idea, we observed that Esg or Scrt overrides sustained activation of the N pathway. Furthermore, we show that Esg or Scrt antagonizes transcriptional activity of the *E(spl)m4*

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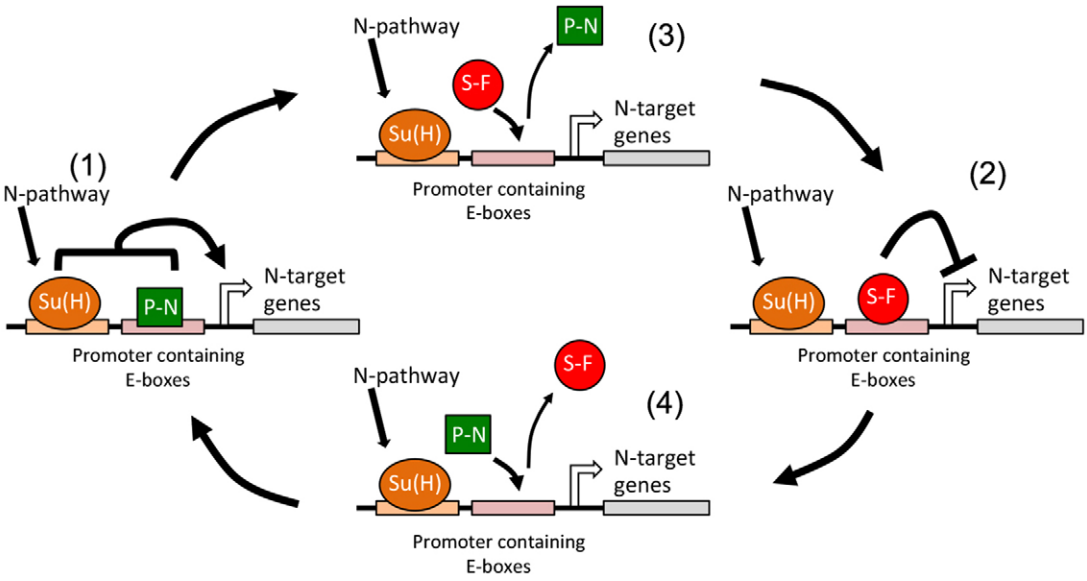

Fig. 7. Working model of the repression exerted by Snail factors on N target gene transcription. (1) Activation of N target gene transcription mediated by both proneural factors (P-N) and the N pathway through Su(H). (2) Repression of N target gene transcription mediated by Snail factors (S-F), such as Esg. (3,4) Since Snail transcriptional repressors and proneural factors bind to the same region of the promoter containing E-boxes, these factors compete and displace each other. The occupancy of E-box DNA binding sites by proneural factors will activate transcription, whereas occupancy by Snail factors will repress it.

promoter. *E(spl)m4* is part of the *E(spl)* complex that contains direct N target genes encoding transcription factors that inhibit neural development (Delidakis and Artavanis-Tsakonas, 1992; Lai et al., 2000). This promoter is subject to two modes of transcriptional activation: one mediated by Ac and Sc proneural factors that operate by binding to E-box binding sites and the other mediated by the N pathway through Su(H) binding sites (Bailey and Posakony, 1995). We have shown here that Esg binds specifically to the *E(spl)m4* promoter and negatively controls the transcriptional activity of this promoter *in vivo*. Our data also show that Esg and Ac/Sc compete for binding to the *E(spl)m4* promoter. We do not know whether this reflects direct competition for similar E-box binding sites or is an allosteric interference between the factors binding to two different E-boxes. In any case, the result seems to be that Ac/Sc binding to E-box binding sites activates transcription, whereas Esg occupancy represses it. As such, we propose a mechanism for the transcriptional repression exerted by Esg on N target genes: first, it displaces the transcriptional activator brought by proneural factors, such as Ac, and second it brings a transcriptional repressor complex to the promoter (Fig. 7). Since E-box and Su(H) binding sites are frequently associated in promoters of *E(spl)* genes and other N target genes (Bernard et al., 2010; Cave et al., 2005, 2011; Maeder et al., 2007; Nellesen et al., 1999; Singson et al., 1994), it is expected that the Esg-mediated negative control of *E(spl)m4* expression shown in the present work will reveal a more general control of the N signaling response exerted by Snail factors.

It is relevant to note that *in vivo* mapping of Esg binding sites on DNA using the DamID technique in *Drosophila* midgut cells has identified a number of N target genes. These include several *E(spl)* genes, such as *E(spl)mα* and *E(spl)mβ*. As such, these results show that Esg, and probably Scrt, binds the endogenous promoters of N target genes (Loza-Coll et al., 2014). Accordingly, transcriptomic studies have shown that dysregulation of Esg expression in *Drosophila* intestinal stem cells is associated with the upregulation or downregulation of N target genes. In agreement with our data, *E(spl)m4* expression was upregulated when *esg* expression was downregulated (Korzelius et al., 2014). These results support our proposition that Esg or Scrt binds to the promoter of some N target genes and represses their transcription. The fact that a similar mechanism seems to be employed in other systems suggests that this function of Snail family transcription factors is widespread in diverse precursor cell types.

That Snail factors and proneural transcription activators may compete for similar DNA binding sites has already been suggested in a pioneer study on the Esg binding site (Fuse et al., 1994). A comparable mechanism in which Snail factors antagonize proneural activity has been proposed for the loss of bristles phenotype observed in *Scutoid* mutants, in which Snail and Esg are ectopically expressed in mSO cells. Moreover, consistent with our observations, overexpression of *esg* suppressed the excess of bristles observed after expression of the proneural factor *asense* alone (Fuse et al., 1999). Interestingly, it has been suggested that proneural factors and bHLH proteins encoded by *E(spl)-HLH* genes can compete for binding to E-box sequences resulting in transcriptional repression rather than activation (Jennings et al., 1999). We have shown that Esg and Scrt regulate the transcription of *E(spl)m4*, which is a Bearded factor rather than a bHLH factor. However, the fact that the promoter of several *E(spl)* genes harbors E-boxes raises the exciting possibility that Esg and Scrt downregulate N signaling at two different levels: first by downregulating transcription of N target genes, such as *E(spl)* genes (this work), and second by outcompeting E(spl) factors for common DNA sites. The existence of these potential interactions would depend on the heterogeneity of E-box sequences, on the sequence flanking the consensus core, and on the spatial configuration of E-boxes present in particular promoters (Chang et al., 2015).

N activation in secondary precursor cell determination is a rapid process, occurring during the first 30 minutes after birth (Couturier et al., 2012; Remaud et al., 2008). In addition, the onset of *esg* and *scrt* expression occurs in late pIIb cells. Furthermore, when *esg*, *scrt* and N signaling were downregulated, sensory cells acquired a neural cell fate. As such, these data indicate that Esg and Scrt are not

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determining the neural pIIb precursor cell identity per se. Additionally, these results show that cell transformation from N_{off} pIIb to N_{on} pIIa cells observed after downregulation of *esg* and *scrt* requires functional N signaling. Therefore, our results indicate that Esg and Scrt are involved in the maintenance of the N_{off} state. We propose that Esg and Scrt fix pIIb identity by absorbing any variation in N activity in the normally N_{off} pIIb precursor cell. An increasing number of reports indicate that stochastic fluctuations in biochemical reactions are the cause of variations in cellular behavior (Sanders et al., 2009; Süel et al., 2006). Our results suggest that Esg and Scrt, by downregulating N target gene transcription, may act as a buffer against these potential fluctuations in N signaling activity.

The data shown in Fig. 6 raise the possibility that Esg and Scrt might be needed to assure the identity of pIIIb progeny. However, because of their expression pattern, we favor the idea that the role of Esg and Scrt in maintaining cell fate is specific to pIIb. Other cell determinants also appear to act cell specifically in the bristle lineage. In particular, the N_{off} state of neural progenitor cells seems to be assured by diverse mechanisms. Hamlet, a transcriptional regulator that is involved in the fate decision of the two pIIIb daughter cells, also acts by reducing N signaling. Hamlet appears to modify the chromatin landscape and controls the accessibility of transcriptional activator complexes at the promoter of N target genes (Endo et al., 2011; Moore et al., 2004). Moreover, several reports suggest that N transduction involves different sets of mediators, Su(H) being essential to determine pIIa but not pIIIb cell fate decisions (Le Gall et al., 2008; Nagel et al., 2000; Wang et al., 1997). Although N signaling is involved in each binary decision in the bristle lineage, this pathway does not specify each cell identity. Thus, other factors must come into play. In the case of Esg and Scrt, these factors will act in secondary precursor cell decisions to maintain the N_{off} state of the pIIb neural precursor cells.

In conclusion, Esg and Scrt act redundantly to block N activity in secondary neural precursor cells. This effect seems to be mediated by downregulation of N target gene transcription. We propose that Esg and Scrt are involved in a cell-specific mechanism that maintains an N_{off} state in neural precursor cells. Since Esg and Scrt binding sites are known to be present in the promoters of several N target genes, we believe that this mechanism assures N activity damping in a wide variety of cell types.

MATERIALS AND METHODS

Fly stocks

Fly crosses were carried out at 25°C except where otherwise stated. Using the Gal4/UAS expression system (Brand and Perrimon, 1993) the following constructions were expressed: UAS-histone H2B::RFP (H2B::RFP) (Bellaïche et al., 2001), UAS-*esg* (gift from Shigeo Hayashi, Riken Center for Developmental Biology, Kobe, Japan), UAS-*scrt* (F0001921 FlyORF), UAS-$scrt^{RNAi}$ [No 105201, Vienna Drosophila RNAi Center (VDRC)], and UAS-esg^{RNAi} (No 9794 and 9793, VDRC). As drivers, we used *neuralized* ^{p72}*-Gal4* (*neur-Gal4*), *pannier-Gal4* (*pnr-Gal4*), *engrailed-Gal4* (*en-Gal4*) and *HS-Gal4*. The protein-trap line esg^{P01986}*::GFP* (FlyTrap), *scrt::GFP* (#318073, VDRC; Sarov et al., 2015) and the enhancer-trap *scrt::lacZ* (Bloomington Drosophila Stock Center) were used to monitor *esg* and *scrt* expression. *HS-scrt* transgenic flies were generously provided by E. Bier (University of California San Diego, La Jolla, USA).

Somatic clones were obtained by means of the FLP/FRT recombination system (Xu and Rubin, 1993) using *Ubx-FLP* or *HS-FLP* lines. The *w; FRT40A* esg^{G66B}*/CyO* line was used to generate *esg* null clones. Escapers of the strong allelic combination $scrt^{jo11}/scrt^{jo16}$ (gift from E. Bier) were used to analyze the *scrt* mutant phenotype. Flip-out overexpression clones were obtained using the *HS-FLP; tubulin FRT-stop-FRT Gal4; UAS::GFP* line. The *E(spl)m4::lacZ* and mutant form *E(spl)-2m::lacZ* lines were obtained from J. Posakony (University of California San Diego, La Jolla, USA). For wing disc clones, larvae maintained at 25°C were heat shocked at 24 h and 48 h after egg laying (37°C, 1 h) and dissected at late third instar. For intralineage clones in the wing margin, pupae at 11 h APF were heat shocked twice (37°C at 11 h and 12 h APF) and dissected at 24 h APF.

Transformation of pIIb into pIIa was realized by ectopic expression of the active form of N (N^{intra}) via an HS promoter. *White* pupae were collected, kept for 17 h at 25°C and then heat shocked for 1 h at 37°C. We dissected them 1 h 30 min after heat shock. Transformation of pIIa into pIIb was realized by downregulation of N using a thermosensitive allele ($N^{ts\text{-}1}$). In this case, fly crosses and embryonic and larval development were carried out at 18°C. *White* male pupae were then collected, kept for 17 h at 25°C and then at 30°C for 1 h before dissection.

The conditional *neur-Gal4, tub-Gal80*^{ts} and *pnr-Gal4, tub-Gal80*^{ts} were used to overexpress both N^{intra} and Esg or Scrt. To avoid the lethality induced by overexpression of these factors at earlier stages of pupal development, pupae were maintained at 18°C and shifted to 30°C at 15 h APF.

To test epistasis between *esg*, *scrt* and the N pathway, pupae from the cross of females ($N^{ts\text{-}1}/N^{ts\text{-}1}$*;;pnr>Gal4/TM6Tb*)× males (*+/Y; UAS-RNAi scrt/ UAS-RNAi scrt; UAS-RNAi esg/UAS-RNAi esg*) were maintained at 18°C from 0 h to 28 h APF (equivalent to 14 h APF at 25°C), shifted to 30°C for 4 h and returned to 18°C for 12 h until dissection (equivalent at 24 h APF at 25°C).

Immunohistology

Pupal nota were dissected between 17 h and 35 h APF and processed as previously described (Gho et al., 1996). Antibodies are described in the supplementary Materials and Methods.

Polyclonal anti-Scrt antibodies were immune purified from rabbit serum immunized with a mix of two Scrt peptides (IYRPYSLDDKPAHGYR and YTYEAFFVSDGRSKRK) mixed with Freund's complete adjuvant and boosted three times by injection with Freund's incomplete adjuvant (Covalab).

Images were obtained on an Olympus BX41 fluorescence microscope (40× or 60× oil immersion objective) equipped with a Yokogawa spinning disc and a CoolSnapHQ2 camera driven by Metaview software (Universal Imaging). Images were processed with ImageJ (NIH).

Esg::GFP pull-down

All experiments were performed with the same batch of *Drosophila* protein extract obtained from *daughterless-Gal4/UAS-esg::GFP* (*da>esg::GFP*) embryos taken 20 h after egg laying, aliquoted and stored at −80°C, 1 mg of devitellinized embryos in 5 µl de lysis buffer. DNA templates were generated by PCR using 5′ biotinylated primers. An *ftz* template was obtained using 5′-GGGAGTTGCGCACTTGCT-TG-3′ and 5′-GTGCACGCAACGCTGGTGAG-3′ primers. An *E(spl)m4* template was obtained using 5′-AAGGATCCTTTCGA-ACCGAAACTGTG-3′ and 5′-AGAA-CCCGAAGCCGAGCAG-G-3′, which correspond to the proximal 305 bp of the *E(spl)m4* promoter bearing the canonical (E2) E-box ACAGGTG. In the *E (spl)m4* mutated promoter the more proximal canonical E-box sequence was replaced by a TAGAATT sequence. Biotinylated DNA was coupled to streptavidin-coated magnetic beads (M280, Dynal Biotechnology), with 0.1 mg beads per 200 ng DNA, overnight at 4°C. The beads were washed three times in B&W buffer (as recommended by Dynal Biotechnology) and streptavidin-immobilized DNA was saturated for 1 h in PBS containing 20% horse serum before incubation for 1 h with the *da>esg::GFP* embryo protein extract in PBS containing 0.15% Triton X-100. Protein extract and beads were separated according to the manufacturer's instructions and washed four times with 100 mM NaCl, 25 mM NaH_2PO_4. Each fraction was then processed for SDS-PAGE. GFP was revealed using rabbit anti-GFP (1/800; Roche) and horseradish peroxidase coupled to an anti-rabbit antibody (1/1000; Promega).

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Competition experiments were performed by incubating beads coated with *E(spl)m4* templates with a fixed amount of protein extract from *da>esg::GFP* embryos complemented with the same volume of a protein extract from *da-Gal4/UAS-achaete* and *white* embryos at a ratio of 0:2, 1:1 and 2:0. As such, the total amount of protein in the final protein extract mixture was relatively constant.

Acknowledgements

We specially thank James W. Posakony for providing *E(spl)* fly stocks; Yohanns Bellaiche for generous aid; the fly community for fly strains and antibodies; Heather McLean, Fred Bernard and Yohanns Bellaiche for critical reading of the manuscript; and Carine Torset for technical help.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Designed the experiments: A.R., A.A., M.G.; performed the experiments and data analysis: A.R., A.A., S.L.-V., P.F., M.G.; technical support: F.S.; prepared and edited the manuscript: A.A., S.L.-V., M.G.

Funding

This work was supported by the Centre National de la Recherche Scientifique; the Université Pierre et Marie Curie; and by a specific grant from GEFLUC Paris-Ile de France [1 h/260]. A.R. was financed by grants from the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche and the Fondation pour la Recherche Médicale for her last year of graduate training.

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.134387.supplemental

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Supplementary Materials and Methods

Primary Antibodies:

Mouse anti-Cut (DSHB, #2B10, 1:500); rat anti-ELAV (1:10, DSHB, #7E8A10); rat anti-Esg (1:500, gift from S. Hayashi, Riken Center for Developmental Biology, Kobe, Japan); rabbit anti-β-Galactosidase (1:500, Cappel, #55976); rabbit anti-GFP (1:1000, Santa Cruz Biotechnology, #sc-8334); rat-anti-Sens (1:1000, gift of Y. Bellaiche, Institute Curie, Paris, France); mouse anti-Pros (1:5, gift from C. Doe, Institute of Neuroscience, Eugene, USA); rabbit anti-Ttk (1:300, gift from F. Schweisguth, Institut Pasteur, Paris, France); rat anti-Su(H) (1:500, gift from F. Schweisguth, Institut Pasteur, Paris, France); rabbit anti-Pdm1 (1:2000, gift from T. Préat, École supérieure de physique et de chimie industrielles, Paris, France); mouse anti-Snail (1:2500, gift from A. Alberga, Laboratoire de Génétique Moléculaire des Eucaryotes, Strasbourg, France); mouse anti-Worniu (1:1000, gift from Y. Cai, Temasek Lifesciences Laboratory, Singapore, Singapore); mouse anti-NICD (1:100, DSHB, #C17 9C6).

Secondary Antibodies:

Alexa 488-conjugated secondary anti-mouse (#A11029), anti-rat (#A11006), anti-rabbit (#A11034), anti-guinea pig (#A11073), Alexa 568-conjugated secondary anti-mouse (#A11031), anti-rat (#A11077), anti-rabbit (#A11011), anti-guinea pig (#A11075) were purchased from Molecular Probe and used at 1:1000. Cy5 conjugated antibodies anti-mouse (#715-175-151), -rat (#712-175-153) or -rabbit (#711-175-152) were purchased from Jackson Immunoresearch and were used at 1:2000.

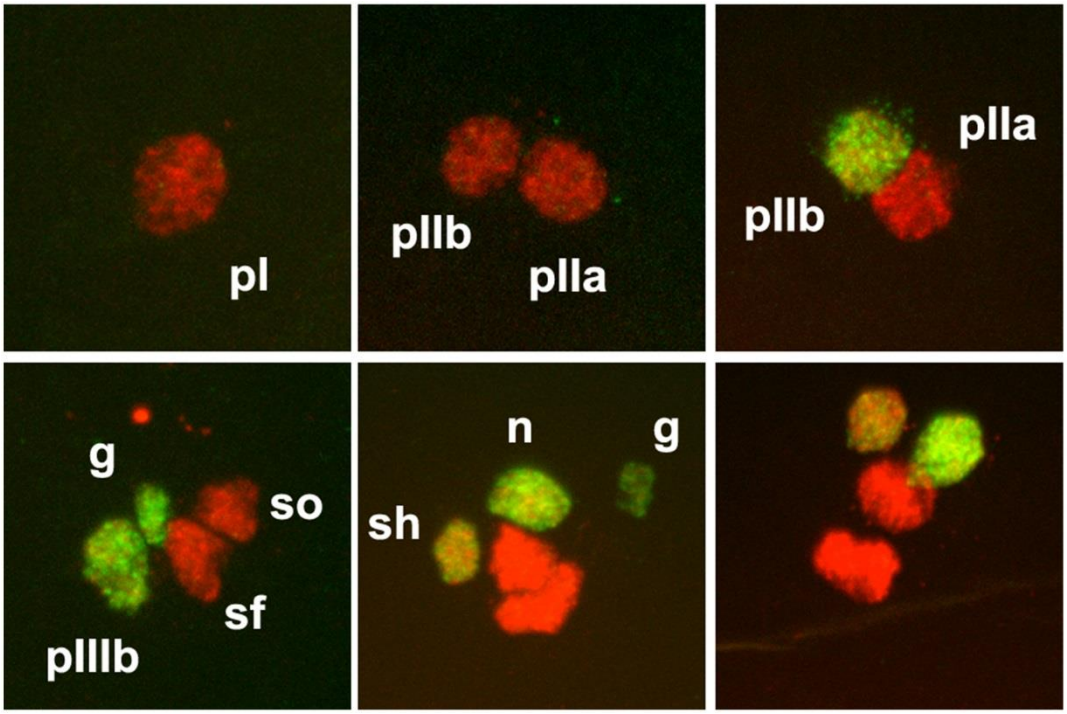


Figure S1. Scrt is expressed in the neural sublineage of the bristle cells.

Expression pattern of Scrt inferred from an enhancer trap *scrt-lacZ* fly line (green) at consecutive stages of development. Sensory cells were identified by the expression of Cut-immunoreactivity (red). Probably due to a persistence effect, the β-Gal signal was observed in all pIIb descendants, with a particularly high level in neurons.

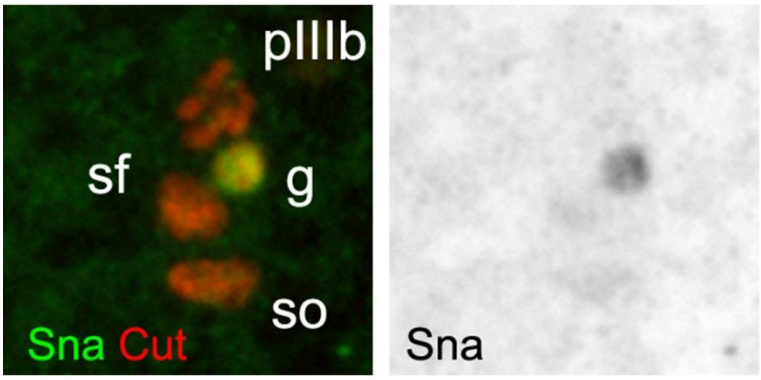


Figure S2. Snail was expressed in the glial cell.

mSO cells identified by Cut immunoreactivity (red) stained against Snail. Note that only the glial cell expressed Snail. pIIIb cell was identified since is in division. sf: shaft cell, so: socket cell, g: glial cell.

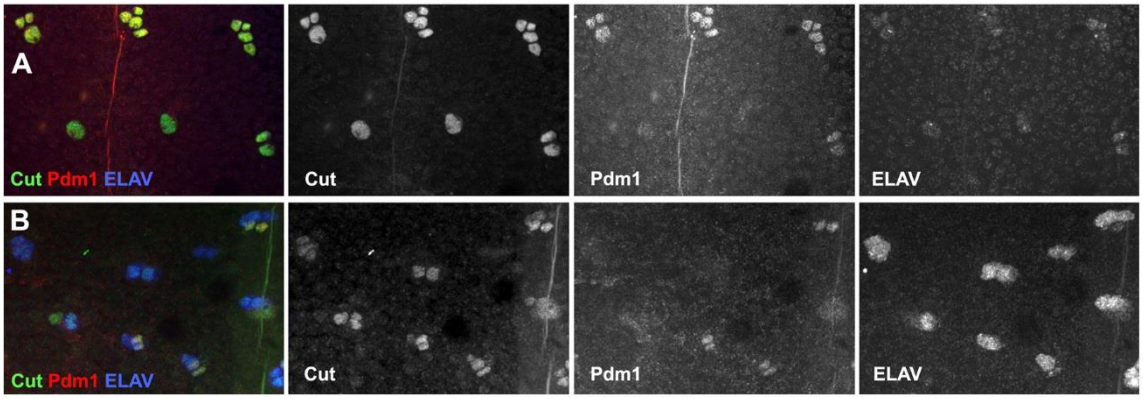


Figure S3. Scrt counteracts ligand-independent activation of the N-pathway by overexpression of N^{intra}.

(A) N^{intra} overexpression induced mSO formed exclusively by Pdm1 positive external cells (C'). (B) mSO composed exclusively of *ELAV*-positive inner cells were observed when N^{intra} together with Scrt were overexpressed (B''). Pupae at 22h APF. N^{intra} and *esg* overexpression were induced at 15h APF by shifting to 30°C using the conditional driver *pnr>Gal4 Tub-Gal80^{ts}*. mSO cells identified by cut immunoreactivity (red).

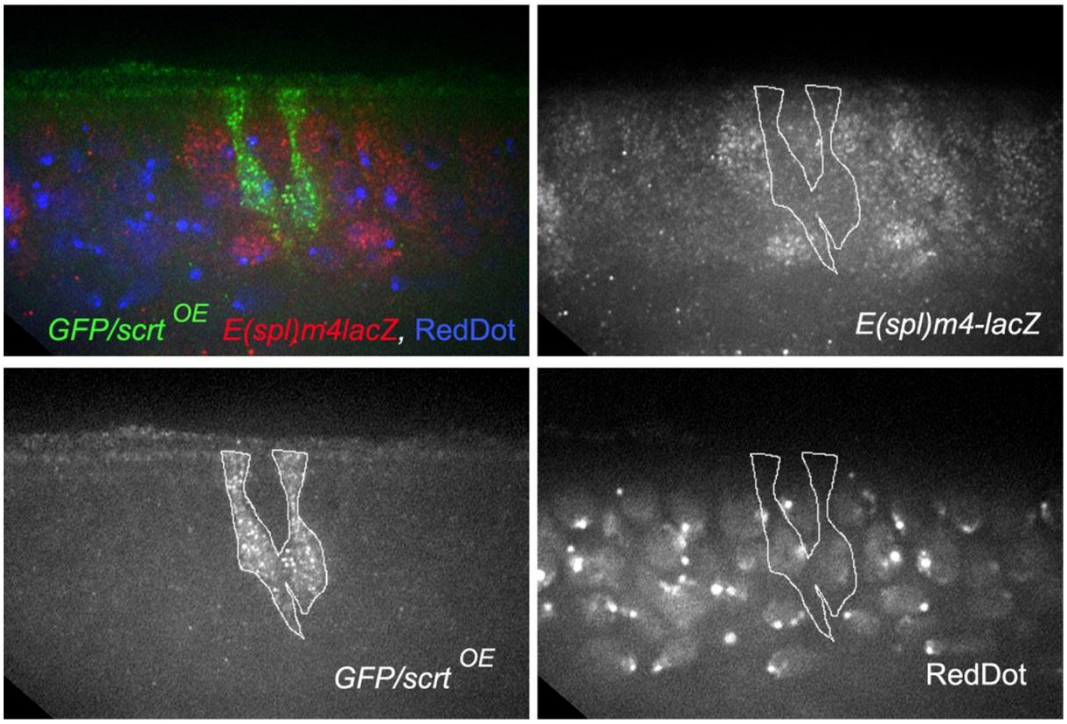
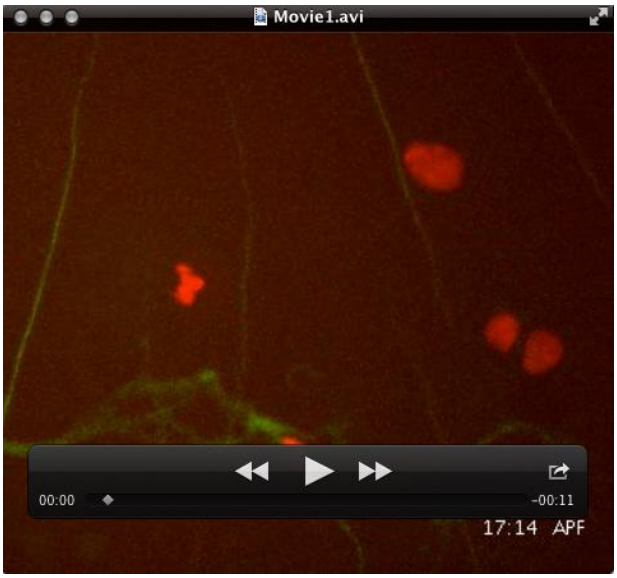


Figure S4. Scrt downregulates transcription activity of *E(spl)m4* promoter *in vivo*.

Flip-out Scrt overexpression in an intralineage clone in the wing margin. *HS-FLP; tubuline::FRT-STOP-FRT GAL4, UAS-GFP/UAS-scrt; E(spl)m4-lacZ* fly line heat-shocked at 11h and 12h APF and fixed at 24h APF. mSO expressing *E(spl)m4-lacZ* (red) in which an intralineage *scrt*-overexpression clone (identified by GFP, green) has been induced. Note that β-Gal expression disappears in clonal cells. All nuclei were marked by RedDot™. The image corresponds to one confocal plane.



Movie 1.

In vivo observation of mSO of a protein-trap *esg::GFP; neur-Gal4/UAS-RFP* pupae. The posterior is towards the right and the view is dorsal.