Development 129, 935-944 (2002)
Printed in Great Britain © The Company of Biologists Limited 2002
DEV7931

A genetic hierarchy establishes mitogenic signalling and mitotic competence in the renal tubules of *Drosophila*

Vikram Sudarsan[1,*], Sara Pasalodos-Sanchez[1,*], Susan Wan[1], Alexandra Gampel[2] and Helen Skaer[1,*,†]

[1]Centre for Developmental Genetics, BMS, University of Sheffield, UK, S10 2TN
[2]Department of Biochemistry, University of Bristol, UK, BS8 1TD
*Present address: Department of Zoology, Downing Street, Cambridge, UK, CB2 3EJ
†Author for correspondence (e-mail: hs17@cam.ac.uk)

Accepted 29 November 2001

SUMMARY

Cell proliferation in the developing renal tubules of *Drosophila* is strikingly patterned, occurring in two phases to generate a consistent number of tubule cells. The later phase of cell division is promoted by EGF receptor signalling from a specialised subset of tubule cells, the tip cells, which express the protease Rhomboid and are thus able to secrete the EGF ligand, Spitz. We show that the response to EGF signalling, and in consequence cell division, is patterned by the specification of a second cell type in the tubules. These cells are primed to respond to EGF signalling by the transcription of two pathway effectors, PointedP2, which is phosphorylated on pathway activation, and Seven up. While expression of *pointedP2* is induced by Wingless signalling, *seven up* is initiated in a subset of the PointedP2 cells through the activity of the proneural genes. We demonstrate that both signalling and responsive cells are set aside in each tubule primordium from a proneural gene-expressing cluster of cells, in a two-step process. First, a proneural cluster develops within the domain of Wingless-activated, *pointedP2*-expressing cells to initiate the co-expression of *seven up*. Second, lateral inhibition, mediated by the neurogenic genes, acts within this cluster of cells to segregate the tip cell precursor, in which proneural gene expression strengthens to initiate *rhomboid* expression. As a consequence, when the precursor cell divides, both daughters secrete Spitz and become signalling cells. Establishing domains of cells competent to transduce the EGF signal and divide ensures a rapid and reliable response to mitogenic signalling in the tubules and also imposes a limit on the extent of cell division, thus preventing tubule hyperplasia.

Key words: Cell division, Malpighian tubules, Proneural genes, EGFR signalling, *seven up*, *wingless*, *Drosophila*

INTRODUCTION

While our understanding of cell cycle regulation at the molecular level is detailed and sophisticated, our knowledge of the way in which cell division is patterned in developing tissues is relatively rudimentary. We have used a simple epithelial tissue, *Drosophila* renal tubules, to understand how cell-specific patterns of gene expression combine with intercellular signalling to regulate the spatial organisation of cell proliferation during embryogenesis.

The majority of embryonic cells in *Drosophila* go through a tightly regulated programme of cell divisions (Campos-Ortega and Hartenstein, 1997). After cellularisation of the early syncytial embryo at the end of cycle 13, most cells divide a limited number of times, in many cases no more than three. These cells then enter a variable number of endoreplicative cycles, allowing the larva to grow through increases in cell size (Foe et al., 1993). These postblastodermal divisions lack G_1 but are strikingly patterned in both space and time through the regulation of G_2 by the expression of the *Drosophila* cdc25 homologue, *string* (Edgar and O'Farrell, 1990; Edgar et al., 1994). A few tissues in the embryo undergo further divisions, with the introduction of a regulated G_1 phase (Richardson et al., 1993; Knoblich et al., 1994). These include cells set aside to construct the adult organism, the nervous system and the Malpighian tubules (Kerber et al., 1998). The four tubules differ in size, the pair that project anteriorly are longer than the posterior pair (see Fig. 3A). Clonal analysis has revealed that tubule cells undergo three to five postblastodermal divisions, which are consistently patterned and result in an average of 107 cells in each posterior and 144 cells in each anterior tubule (Janning et al., 1986; Skaer, 1989). In summary, tubule cell divisions occur in two phases; 'early' divisions, lacking G_1, that are common to all embryonic cells (known as cycles 14-16) and 'late' divisions, complete with G_1, that they share only with adult precursors and cells in the developing nervous system (cycle 17 onwards).

In this paper, we analyse how these late cell divisions in the tubules are regulated and patterned. It is already known that cell division in the tubules requires the activity of two signalling pathways, the Wingless (Wg) and the EGF receptor pathways, and is regulated by a specific cell, the tip cell (TC), which is situated at the distal tip of each growing tubule (Skaer, 1989; Skaer and Martinez-Arias, 1992; Baumann and Skaer, 1993).

Ablation of the TC, either mechanically or genetically, results in approximately half the normal number of tubule cells (Skaer, 1989; Hoch et al., 1994). Kerber et al. (Kerber et al., 1998) have shown that the TC stimulates division in its neighbours by secreting the EGF ligand, Spitz (Spi), thereby activating the transcription factors Pointed and Seven up, and leading to the expression of *string* and the G_1 regulator, *cyclin E*.

Clearly, the normal segregation of TCs, which are capable of secreting Spi, underpins this mitogenic signalling. A single TC develops in each tubule primordium from a cluster of cells that expresses proneural genes of the *achaete-scute* complex (AS-C). All the cells in this proneural cluster (PNC) have the potential to form a TC, but lateral inhibition, which is mediated by the neurogenic genes, represses expression of AS-C genes in all but a single cell in the PNC, the tip mother cell (Hoch et al., 1994). The tip mother cell divides, partitioning Numb asymmetrically between its daughters and the cell inheriting Numb becomes the TC (Wan et al., 2000). Expression of AS-C genes intensifies in the TC, it develops a striking morphology (see Fig. 2D) and later differentiates many characteristics of neuronal cells. The other daughter, the sibling cell (SC), is inhibited from taking the primary fate by further signalling through Notch (N), losing the expression of proneural genes and retaining a normal epithelial morphology (Hoch et al., 1994).

We have previously shown that the number of TCs in the Malpighian tubule can be manipulated genetically; in embryos that lack proneural gene function the tip mother cell is not specified, so tubules develop without the TC or SC. By contrast, neurogenic mutants segregate multiple TCs (Hoch et al., 1994). In the absence of Numb, tubules develop with two SCs but no TC, while the overexpression of *numb* results in the differentiation of two TCs at the expense of the SC (Wan et al., 2000). As might be predicted, the loss of TCs in embryos without the AS-C results in tubules with approximately half the wild-type number of cells. However other manipulations produce results that are not as easy to explain: alterations of Numb, whether loss or gain of function, have no effect on tubule cell number, suggesting that specification of TC versus SC fate is not important for cell division in the tubules (Wan et al., 2000). In addition we show here that, despite the presence of eight to 12 TCs in *N* mutant embryos, there is no increase in cell proliferation as would be expected from an increase in the number of signalling cells in these tubules. Instead we find a dramatic reduction in division so that *N* mutant tubules have as few cells as those that lack tip cells altogether.

We show that both SCs and TCs are sources of the ligand Spitz and that activation of the EGF pathway is required only for 'late' divisions (from cycle 17 onwards) in the tubules. We demonstrate that N acts through lateral inhibition to segregate two cell fates within the PNC; the primary TC/SC fate and a second fate in the remaining cells of the PNC; those that are competent to respond to the mitogenic signal. We show that cells are primed to respond to EGF signalling by the expression of two EGF pathway effectors, *seven up* (*svp*) and *pointedP2* (*pntP2*), even before signalling through this pathway starts in the tubules. We have analysed the factors involved in regulating the expression of *svp* and *pntP2* and show how they combine to pattern cell division in the tubules by delineating not only the source of the EGF signal but also the population of cells that is competent to respond to it.

MATERIALS AND METHODS

Drosophila genetics

Oregon R flies were used as wild type, unless otherwise specified. The following stocks were used: $numb^{796}$, N^{55e11}, *Df(1)svr* and ac^3 sc^{10-1} for loss or severe reduction in proneural gene function; top^{CO} for loss of the EGF receptor; top^{CO}*; Df(3L)H99* to protect against cell death (White et al., 1994) in an otherwise EGF receptor mutant background; *Df(1)svr;* $hs\text{-}N^{intra}$ to express activated *Notch* in a proneural mutant background; *UAS-*N^{intra} to express activated *Notch*; *hs-wg*, svp^{07842} as *svp-lacZ*; pnt^{07825} as *pntP2-lacZ*; wg^{CX4}, $argos^{I\Delta7}$, *UAS-argos*, *UAS-*λ^{top}, A37 (a p-*lacZ* insert in *neuromusculin*); and *UAS-sSpi* to overexpress the active form of Spitz. For constitutive activation of the EGF receptor (Clifford and Schüpbach, 1994) in an otherwise proneural or neurogenic mutant background, we drove *UAS-*λ^{top} in *Df(1)svr* or N^{55e11} mutants. Mutant embryos were recognised by selecting against the blue balancer. *Df(3L)H99* cell death-deficient embryos were identified by their characteristic head involution defects (White et al., 1994).

To elicit Gal4-mediated misexpression (Brand and Perrimon, 1993), the following drivers were used: *da*-Gal4 (ubiquitous); *ase*-Gal4 (TMC and its progeny, from S. Stein); fkh^{XB30}-Gal4 (Malpighian tubules, from M. Hoch); and CtB-Gal4 (Malpighian tubules (V. S. and S. P.-S., unpublished)).

Immunohistochemistry

Antibody staining of embryos was performed by following standard techniques (Hoch et al., 1994). The following antibodies were used: mouse anti-Achaete (1:10 from P. Simpson); mouse anti-BrdU (1:50, DAKO); mouse monoclonal anti-β-Gal (1:1000, Promega); rabbit polyclonal anti-β-Gal (1:10000, Sigma); mouse anti-Cut (1:100, Developmental Studies Hybridoma Bank); rabbit anti-Krüppel (1:500, from C. Rushlow); and rabbit anti-Rhomboid (1:500, from E. Bier). Primary antibody incubations were performed overnight followed by incubation with appropriate biotinylated secondaries and amplification using the Vector Elite ABC Kit (Vector Laboratories, CA). For fluorescent double labelling, we used appropriate secondary antibodies conjugated with Cy2, Cy3 or Cy5. When required, we performed an additional amplification step using streptavidin-conjugated Cy3. All fluorophores were obtained from Jackson Immunochemicals.

In situ hybridisation was performed according to Jowett (Jowett, 1997). cDNA for *svp* was obtained from M. Mlodzik and the antisense probe was made by digesting with *Kpn*I followed by synthesis with T3 RNA polymerase. A *pntP2* specific fragment was obtained from P. zur Lage. The antisense probe was made by digesting with *Bam*HI followed by synthesis with T7 RNA polymerase. Stained preparations were viewed using a Zeiss Axioplan compound microscope and images were captured using a JVC-KY55B digital camera and a Neotech Image Grabber, UK. Fluorescent preparations were viewed using Leica TCS confocal microscope, and figures were prepared using Adobe Photoshop and Canvas.

BrdU incorporation and cell counts

BrdU incorporation was performed either according to Shermoen (Shermoen, 2000) in whole-mount embryos or in dissected preparations according to Skaer (Skaer, 1989). For Malpighian tubule cell counts, embryos were either dissected as above and stained with anti-Cut antibody or tubules were dissected out of stained embryos in 70% glycerol.

RESULTS

The EGF receptor is required for the late cell divisions in the tubules

To assess when EGF pathway signalling is required for tubule cell division, we compared BrdU incorporation in wild-type

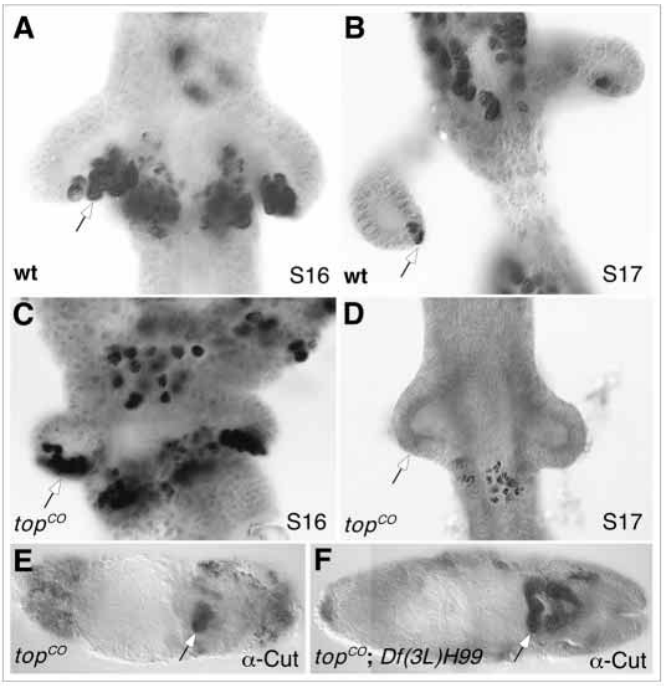

Fig. 1. Cell division in the Malpighian tubules. Tubules dissected from wild-type (A,B) and *top^{co}* mutant (C,D) embryos stained for the incorporation of BrdU to mark S phase. The third postblastodermal division (cycle 16) occurs in a domain on the posterior side of the tubules (arrow in A) and takes place normally in mutant tubules (arrow in C). Only cells in the distal part of the tubules enter cycle 17 (arrow in B). This and subsequent cycles fail in tubules from *top^{co}* mutants (arrow in D). (E,F) Mutant embryos stained for Cut. The tubules of *top^{co}* mutant embryos are very small (arrow in E) but are partially rescued in embryos that also carry a deficiency uncovering *reaper* and *hid* (tubule cells arrow in F).

and EGFR mutant embryos. In wild type, the first cycle after cellularisation, cycle 14, is common to the shared hindgut/tubule anlage. After M14, the tubule cells are specified and behave as a separate population, progressing synchronously through cycle 15 (Ainsworth et al., 2000) (A. G., unpublished). Cycle 16 is restricted to a synchronous group on the posterior side of each tubule (Fig. 1A). After M16 there are 70 (±4) cells in each tubule (Skaer, 1989). With the introduction of G_1 in cycle 17, synchrony is lost, the incorporation of BrdU is more sporadic and is limited to distal regions of the tubules (Fig. 1B). In embryos mutant for the EGF receptor (*top^{CO}*), the early divisions (cycles 14-16) occur normally (Fig. 1C) but there is no incorporation of BrdU from cycle 17 onwards (Fig. 1D). We, therefore, conclude that the EGF receptor is required for cell cycle progression from G_1 of cycle 17. Thus, it is the late cell divisions that are driven by TC release of EGF, and these divisions increase the average tubule cell number from 70 (±4) to 124 (±3) cells.

The EGF receptor is also required for tubule cell survival

As the number of tubule cells in the wild type is 70 at the beginning of cycle 17, it is surprising that the final cell number in *Egfr* or *spi* mutant embryos is much lower (23-27 cells; Table 1), despite normal progression through cycles 14-16 (Baumann and Skaer, 1993; Kerber et al., 1998) (Table 1). We were able to show that this discrepancy results from cell death: first, because the number of cells in each tubule declines after cycle 16 (from 39±2 to 26±2; Table 1) and, second, because the cell number is 74±1 in *top^{CO}* embryos that are additionally mutant at the *hid* locus, in which cell death is blocked (Fig. 1E,F; Table 1) (White et al., 1994). The difference in cell number between *top^{CO}* embryos at the end of cycle 16 (39±2)

Table 1. Number of Malpighian tubule cells

Animal	Average per tubule (all four)	Anterior tubules	Posterior tubules	Reference
Wild type	124±3 (47)	144±2 (21)	107±2 (26)	
TC ablation (early stage 12)	75±4 (12)			Skaer, 1989
AS-C$^{-/-}$ (Df(1)svr)	72±3 (25)			Hoch et al., 1994
N	89±4 (23)			
N germline clone	76±6 (15)			
AS-C$^{-/-}$/hs-*N^{intra}*	63±2 (19)			
N/*λtop*	85±1 (25)			
AS-C$^{-/-}$/*λtop*	74±1 (29)			
numb	125±1 (30)			Wan et al., 2000
UAS-*numb*	126±1 (18)			Wan et al., 2000
Control	125±1 (38)			Wan et al., 2000
Egfr (*top^{co}*) –8 hours	39±2 (19)			
Egfr (*top^{co}*) – final number	26±2 (8)			Baumann and Skaer, 1993
Df(2R)top^{18A}	27±3 (27)			Baumann and Skaer, 1993
top^{co}/*hid*	74±1 (20)			
spi^{IIT25}	23±1 (1)			
svp^{e22}	90			Derived from Kerber et al., 1998
pnt^{Δ88}	85			Derived from Kerber et al., 1998
hs-*argos*	86±5 (29)			F. Eckhardt and H. S., unpublished
UAS-*sspi*	122±3 (27)			C. Showell and H. S., unpublished
Control	124±4 (16)			C. Showell and H. S., unpublished
UAS-*svp*		158±3 (>6)		Kerber et al., 1998
Control		140±4 (>6)		Kerber et al., 1998

Figures are given ±s.e.m. with the number of tubules counted in parentheses.

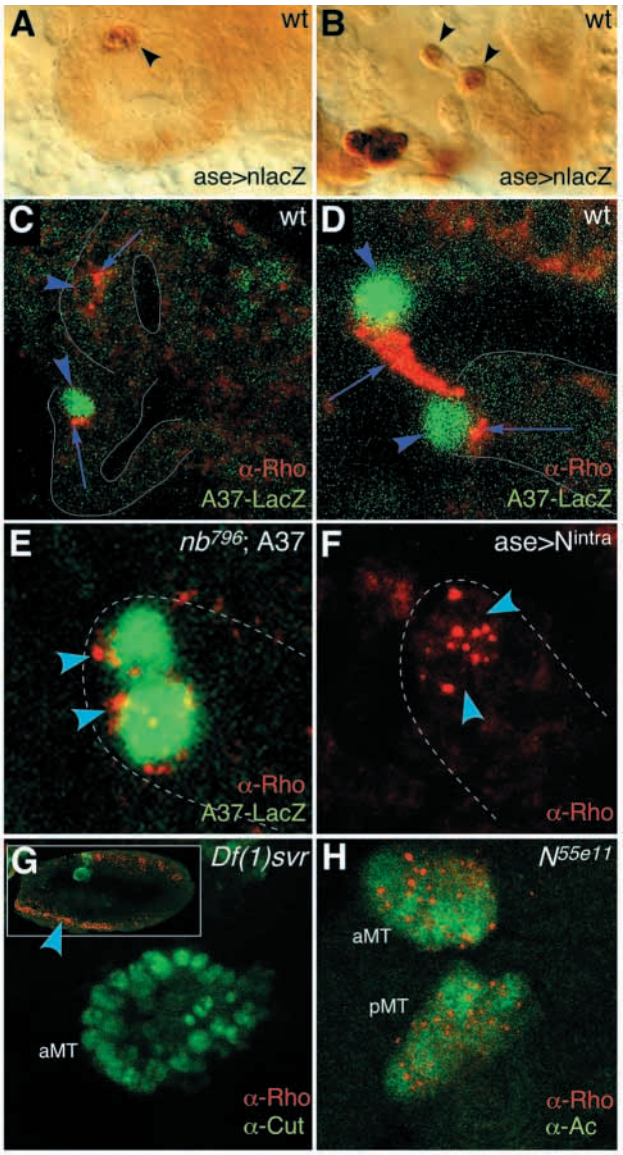

Fig. 2. Rhomboid expression in the tip cell lineage. (A,B) Embryos of the genotype *asense*-Gal4 × UAS-nlacZ and stained for β-galactosidase reveal expression in the tip mother cell at early stage 11 (arrowhead in A) and in both its daughters at stage 12 (arrowheads mark the tip and sibling cell in B). (C,D) Tubules from A37 embryos in which β-gal (green) is expressed under the regulation of the *neuromusculin* enhancer, stained with an antibody against Rhomboid (red). At stage 11, β-gal (arrowheads indicate tip mother cells) and Rho (arrows) are weakly expressed in the tip mother cells (C) and more strongly in both daughters from stage 12 onwards (D, stage 13: arrowheads, β-gal; arrows, Rho). (E,F) In *numb* mutants (E) or if an activated N construct, N^{intra} is expressed in the tip mother cell using *asense*-Gal4 (F), the TC is lost. Instead, two SCs are specified and remain fully integrated in the tubule epithelium [arrowheads and marked by β-gal (green) in E]. Rho (red) is expressed in both SCs. (G,H) In embryos mutant for the proneural (G) or neurogenic (H) genes TC specification is disrupted. (G) In embryos lacking the AS-C, the tubules (labelled for Cut in green) develop without tip and sibling cells and lack Rho, even though it is expressed in the midline (arrow in the inset). (H) In embryos mutant for *N*, the whole proneural cluster develops into TCs (eight to 12 cells in each tubule, labelled for Ac in green) and all these cells express Rho (red). aMT, anterior; pMT, posterior Malpighian tubule.

and top^{co}/*hid* double mutants (74±1) indicates, first, that cells start to die in top^{CO} embryos before the tip cell arises (Hoch et al., 1994) and, second, confirms that the EGF receptor is required for cell division only from cycle 17. Because mature top^{CO} embryos have fewer tubule cells than results simply from the loss of the TCs, and this additional loss can be rescued by blocking cell death, we deduce that EGF receptor signalling has an additional function for cell survival, during the earlier phase of cell divisions, before cycle 17.

Notch but not Numb is required for the normal tubule cell number

We have previously shown that embryos lacking Numb have tubules with no tip but two sibling cells. By contrast, the overexpression of *numb* produces two tip but no sibling cells. Strikingly, neither of these changes in cell fate alters the number of tubule cells (Wan et al., 2000) (Table 1). This suggests that SCs as well as TCs secrete the mitogenic signal Spitz. We therefore analysed the expression of Rho, as a measure of the ability of a cell to process Spi (Golembo et al., 1996; Wasserman and Freeman, 1997; Bang and Kintner, 2000; Urban, 2001). Rho is first detected in the tip mother cell (Fig. 2A,C). After the tip mother cell divides, we detect Rho in the TC and SC (Fig. 2B,D). In situations where two SCs are specified, Rho is detected in both cells (Fig. 2E,F). Thus, although the tip mother cell division is clearly asymmetric in terms of the segregation of Numb and cell fate, Rho is subsequently maintained in both daughters.

As the tip mother cell is selected by refining proneural gene expression to a single cell in the PNC (Hoch et al., 1994), we tested whether Rho expression requires proneural gene function. In embryos that lack the proneural *achaete-scute* complex (*AS-C*$^{-/-}$), Rho expression is lost in the Malpighian tubules (Fig. 2G). Conversely, in neurogenic *N* mutants, where all cells in the cluster become TCs and express *achaete* (Hoch et al., 1994), Rho is detected in all the transformed cells (Fig. 2H). We conclude that *rho* expression is initiated downstream of proneural gene function in the tip mother cell, that *rho* expression persists when the tip mother cell divides and that this is sufficient to activate secretion of the mitogen, irrespective of the daughter cell fates.

In neurogenic mutants such as *N*, where multiple Spi-secreting tip cells are specified, we expected to find a greater mitogenic response. Surprisingly, we found that in zygotic *N* mutants, the tubules fail to grow (compare Fig. 3A with 3B). In fact, there are far less than the wild-type number of cells in each tubule (89±4; Table 1). In *N* germline clone embryos, the final number of cells is the same as in the tubules of *AS-C*$^{-/-}$ embryos with no TC/SCs (76±6 compared with 72±3; Table 1). The incorporation of BrdU in *N* mutant embryos is normal until S16 but from cycle 17 is greatly reduced or entirely absent (compare Fig. 3C with 3D), indicating that the reduction in tubule cell number results from failure of the late, TC/SC-driven cell divisions.

Failure of the late cell divisions in *N* mutant embryos could be explained in several ways:

(1) The activation of N drives cell proliferation in a cell autonomous fashion.

(2) N is required for the processing of the EGFR ligand.

(3) Cells that are ‘competent’ to respond to EGF receptor activation are not specified in *N* mutant embryos.

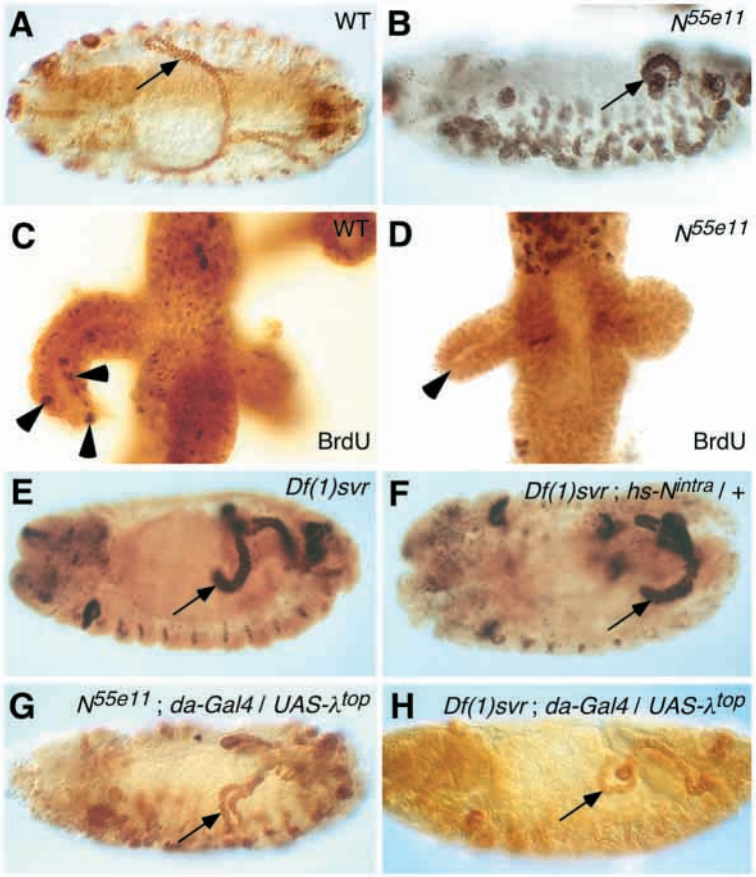

Fig. 3. Notch is necessary, but not sufficient, for tubule cell division. Embryos stained for Cut reveal that the tubules of *N* mutant embryos (B) are shorter than wild type (A) (arrows). This results from a dramatic reduction in cell division from M17, demonstrated by failure to incorporate BrdU. Compare stained cells in the distal region of dissected tubules from stage 12 wild type (arrowheads in C) with the absence of incorporation in tubules from *N* mutant embryos of the same age (arrowhead in D). Driving the expression of activated Notch (hs-N^{intra}) from 5-10.5 hours in the tubules fails to rescue cell division and tubule growth in *AS-C*$^{-/-}$ [*Df(svr)*] embryos (compare arrows in E,F). Ubiquitous expression of an activated EGF receptor (UAS-λ^{top}) also fails to rescue the tubule phenotype in either *N* (arrow in G) or AS-C$^{-/-}$ embryos (arrow in H). A,B,E-H are stained for Cut.

We tested the first possibility by overexpressing the activated form of N in the tubules of proneural gene mutants. Expression of N^{intra} in *AS-C*$^{-/-}$ embryos, throughout the period of late cell divisions (from 5 hours to 10.5 hours), does not rescue tubule growth (compare Fig. 3E with 3F) or cell division (Table 1). This result shows that N activation is not sufficient to drive proliferation in the absence of signalling cells, and suggests a more indirect role for N in wild type tubules.

We have tested the second hypothesis by driving the expression of λ^{top}, an activated form of the EGF receptor (Clifford and Schüpbach, 1994), in the tubule cells of *N* mutant embryos. Forced expression of λ^{top} fails to rescue the late (EGF receptor-driven) cell divisions (Fig. 3G, Table 1), eliminating the possibility that N exerts its function solely by affecting Spi secretion or binding. This observation also confirms that without N function the late, EGFR-driven divisions fail, suggesting that N plays a role in ensuring the response to EGF receptor activation.

In *AS-C*$^{-/-}$ embryos, expression of λ^{top} also fails to rescue the late cell divisions (Fig. 3H, Table 1). This suggests that in addition to specifying the mitogen-secreting cells, proneural gene function is required for the mitogenic response, even when the EGF receptor is activated. These data are consistent with the third hypothesis: that the proneural and neurogenic genes function not only to select the cells that will secrete Spi, but also to specify the cells that will respond to this signal.

The proneural and neurogenic genes specify a second cell fate

To understand how the proneural and neurogenic genes pattern the response to EGFR activation, we analysed the expression of genes involved in transduction of the pathway. It has previously been shown that the orphan nuclear-receptor *svp* functions downstream of the EGF receptor to promote cell divisions in the tubules (Kerber et al., 1998). In the absence of Svp function, *cycE* and *stg* transcription is abolished, with a consequent reduction in EGFR-driven cell divisions (Kerber et al., 1998). We followed these late divisions in the tubules of stage 12 wild-type embryos and found that BrdU incorporation and, hence, cell division is confined within the *svp-lacZ* domain (Fig. 4A). These results define the *svp* domain of expression as including those cells which will divide in response to EGFR activation. However, we noticed that the expression of *svp-lacZ* was initiated in a group of cells surrounding the tip mother cell, before the birth of the TC (Fig. 4B,C) (Kerber et al., 1998). This early onset of *svp* expression occurs before the late divisions start (cycle 17 onwards), when neither Svp function nor EGFR activation is required for cell proliferation. The pattern of gene expression we observe suggests that the Svp-positive cells surrounding the tip mother cell, derive from the proneural cluster.

To test this hypothesis, we observed the expression of *svp-lacZ* in embryos lacking proneural gene function. Indeed, in *AS-C*$^{-/-}$ embryos, the expression of *svp* is not initiated in the tubules (Fig. 4D). Conversely, in *N* mutants, where all cells in the cluster adopt the primary (TC) fate, the expression of *svp* is confined to the transformed cells (Fig. 4E). After the initiation of Spi signalling from the TC/SC, *svp* expression depends on EGF receptor activation (Kerber et al., 1998). However that the early expression of *svp* is not dependent on EGFR function is shown in top^{CO} mutants, where *svp-lacZ* expression is still initiated normally (Fig. 4F), but is not maintained. In *AS-C*$^{-/-}$ embryos expressing λ^{top} in the tubules, *svp* expression was not detected (data not shown). Together these data suggest that the initiation of *svp* depends on the proneural genes but is independent of EGF receptor signalling, which acts only from cycle 17 to maintain *svp* expression.

These results show that the expression of proneural genes in the tubules not only confers tip cell potential but also initiates the expression of an effector of the EGF pathway, *svp*. We suggest that this primes cells to divide in response to EGF receptor activation. Proneural genes are therefore required to

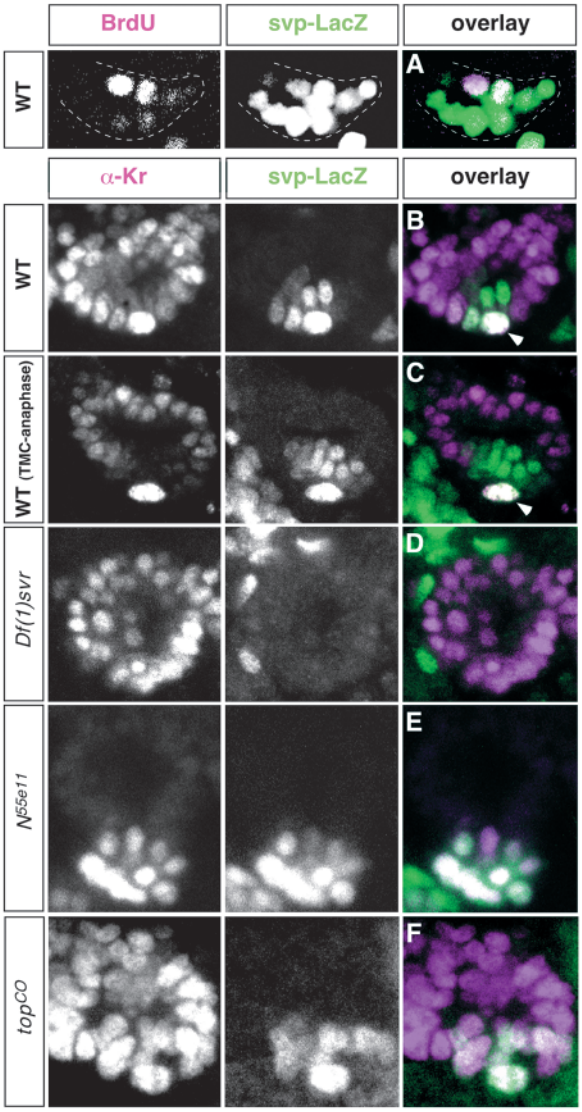

Fig. 4. *svp* expression in the proneural cluster. (A) BrdU incorporation (purple) in the tubules of stage 12 embryos expressing *svp-lacZ* (green) demonstrates that the cells in S phase also express *svp* (overlay; white). (B,C) Kr staining (purple) marks the tubule cells and is more strongly expressed in the tip mother cell at early stage 11(arrowhead, B). Slightly later, Kr expression is lost from cells surrounding the tip mother cell (C). *svp-lacZ* is expressed in wild-type embryos before the tip mother cell divides (B, in C the tip mother cell is in anaphase) and therefore before EGF receptor-driven cell divisions. (D) In $AS\text{-}C^{-/-}$ [*Df(svr)*] embryos, there is no PNC and *svp-lacZ* is not expressed during stage 11 in the tubules. (E) In N^{55e11} embryos, supernumerary TCs, which develop from the PNC, express high levels of *Kr* and *svp-lacZ*, while other tubule cells express neither. (F) In embryos mutant for EGFR (top^{CO}), *svp-lacZ* is still expressed in a cluster of tubule cells at stage 11, as in wild type (B).

specify two cell fates in the tubule PNCs; the tip mother cell and cells competent to respond to EGFR activation.

pntP2 expression is initiated in MT primordia by Wg

Svp is not the only effector of the EGF pathway. The ETS domain protein PointedP2 (PntP2) functions downstream of EGFR/Ras signalling (Brunner et al., 1994; O'Neill et al., 1994; Wassarman et al., 1995). This protein contains a single MAPK phosphorylation site (Brunner et al., 1994; O'Neill et al., 1994) and upon phosphorylation, competes with the ETS domain transcriptional repressor, Yan, to activate the expression of target genes (O'Neill et al., 1994; Rebay and Rubin, 1995). In the absence of *pnt* function, cell proliferation in the tubules is reduced in a manner similar to *svp* mutants (Table 1) (Kerber et al., 1998).

We therefore asked whether early expression of *pnt* as well as *svp* is required to prime the mitogenic response in tubule cells. *pntP2* is initiated in the posterior side of each tubule during stage 10 (Fig. 5B). This domain is characterised by high levels of *wg* expression, which are required for the normal development of *AS-C* expression in the PNC, as it develops within this domain (Wan et al., 2000). The domain of *wg* and

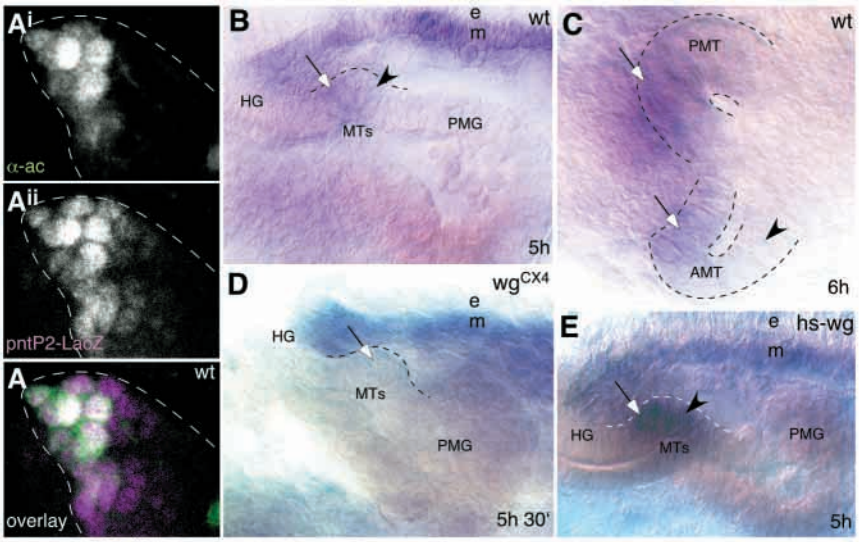

Fig. 5. *pntP2* expression in the tubules is regulated by Wg. The expression of *pnt* is driven by two promoters, P1 and P2, resulting in the expression of the isoforms PntP1 and PntP2 (Scholz et al., 1993). The isoform expressed more strongly in the tubules is PntP2. (A) A stage 10 (4.75 hours) *pntP2-lacZ* embryo stained for Ac (green) and β-gal (purple, overlay; white) shows that *pntP2* is expressed in a wider domain than the PNC. (B-E) In situ hybridisation for *pntP2*, showing expression on the posterior side of the tubules (arrow in B, late stage 10; C, stage 11; arrowheads indicate the anterior side). (D) In wg^{cx4} mutant embryos, *pntP2* expression fails in the tubules but is unaltered in the mesoderm (m; posterior side of tubule is indicated with an arrow, early stage 11). (E) Ectopic expression of *wg* by activating *hs-wg* from 3.5 to 4.5 hours results in elevated levels of *pntP2* expression throughout the tubules (arrow indicates the posterior and arrowhead the anterior side of the tubule, late stage 10). e, epidermis; m, mesoderm; AMT, anterior Malpighian tubules; HG, hind gut; MTs, Malpighian tubules; PMG, posterior midgut; PMT, posterior Malpighian tubules.

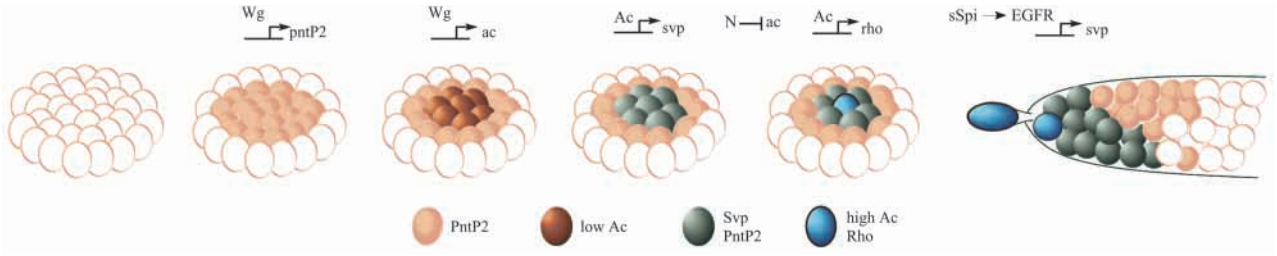

Fig. 6. Model for the establishment of cell fate in the MTs. Wg signalling activates the expression of *pntP2* in a subset of tubule cells (brown), and is required within this domain to establish proneural gene expression in the PNC (represented here by *ac*, dark brown). Moderate levels of Ac induce *svp* expression in the PNC (grey) but, as Ac strengthens in the tip mother cell, a threshold for the initiation of *rho* expression is reached (blue). The tip mother cell divides to produce the TC and SC (blue). As *ac* expression is lost from the SC by stage 12, the maintenance of *rho* expression is later independent of Ac and may depend on a positive feedback loop. The *svp*- and *pntP2*-positive cells, in grey, are descended from the PNC and are competent to respond to EGF signalling. They divide in response to Spitz secreted by the tip and sibling cells.

pntP2 expression is slightly wider than the PNC (Fig. 5A) and *pntP2* expression is initiated well before EGFR activity is required for tubule cell divisions. The expression of *pntP2* persists in this posterior domain when the tip mother cell is specified (Fig. 5C). In wg^{CX4} mutant embryos, tubule expression of *pntP2* is completely abolished (Fig. 5D), showing that Wg signalling is required to initiate its expression. Conversely, the overexpression of *wg*, using a *hs-wg* construct, results in expansion of *pntP2* expression to the anterior side of the tubule primordium and elevation of expression to high levels (Fig. 5E). Thus, Wg is necessary and sufficient to activate the expression of *pntP2* in the tubules.

DISCUSSION

EGF receptor signalling regulates tubule cell number by two routes

The importance of EGFR signalling from the TC for tubule cell division is already known (Baumann and Skaer, 1993; Kerber et al., 1998). We show that the pathway is activated to promote cell division by the production of the ligand Spi from both the tip and sibling cell, from their birth early in stage 12. EGFR activation stimulates passage through the G_1-S-G_2-M cycles in the tubules. Accordingly, EGF signalling is required for the late cell divisions in the tubules, from G_1 of cycle 17.

We show that in addition to promoting cell division, the EGF receptor is required for tubule cell survival. In *AS-C*$^{-/-}$ tubules that lack the tip and sibling cells, the number of cells never increases above 70-75 (Hoch et al., 1994). However in embryos mutant for the EGF receptor, for *spi* or doubly mutant for *rho* and *Star* (*S*) (and therefore lacking active ligand), tubules contain fewer than 70 cells [*Egfr*, 26; *spi*, 23; *rho* and *S*, 24 (Kerber et al., 1998); data for anterior tubules only]. We were able to restore EGF receptor mutant tubules to 74 cells by inactivating cell death, confirming a dual requirement for EGF signalling in both the division and survival of tubule cells. This experiment also reveals that the loss of EGFR-driven cell divisions results in the same final tubule cell number as the loss of the TC and SC in *AS-C*$^{-/-}$ embryos (72 cells), confirming that the source of the mitogenic signal is solely the TC/SCs. However, loss of active Spitz, whether by removal of the gene or failure to process inactive ligand, results in tubule cell death that is just as extensive as that caused by receptor loss. These results show that the ligand for cell survival is Spi. However the source of Spi for cell survival is not the TC/SCs, as in *AS-C*$^{-/-}$ tubules, division arrests after cycle 16 (70 cells) (Skaer, 1989) and the final cell number in the mutants is 72, indicating that tubule cells do not die in the absence of TC/SCs.

Our results correlate with the observation of Clifford and Schüpbach (Clifford and Schüpbach, 1992) that cell death plays a major role in the manifestation of the *top* (*Egfr*) final embryonic phenotype. However, Kerber et al. (Kerber et al., 1998) suggest that the EGF receptor is not required for cell survival in the MTs. Their analysis is based on TUNEL assays and Acridine Orange staining, which may not have revealed the rather gradual cell death we recorded.

Patterning cell divisions in the Malpighian tubules

Previous studies have shown that cell division in the tubules is regulated by Wg and EGF signalling, and that the segregation of tip and sibling cells by lateral inhibition within a proneural cluster of cells ensures restricted secretion of Spi (Skaer and Martinez-Arias, 1992; Hoch et al., 1994; Kerber et al., 1998; Wan et al., 2000). Deregulation of EGF signalling, whether by the overproduction of signalling cells [loss of function in neurogenic genes or *extra macrochaetae* (*emc*) (Ellis, 1994), overexpression of *AS-C* (Hoch and Jäckle, 1998)], or by hyperactivation of the pathway [removal of *argos*, overexpression of *sspi* (Kerber et al., 1998)], does not produce a great increase in tubule cell number but instead reveals remarkable stability in the regulation of cell division (Table 1). These data suggest the presence of powerful feedback restraints. The establishment of a restricted domain of cells, competent to respond to the mitogenic EGF signal and close to its source, identifies such a restraint.

In neurogenic mutants, cells in the PNC all adopt the mitogen-secreting tip cell fate at the expense of cells competent to divide. Therefore, in spite of excess signal, cell divisions fail beyond cycle 17. Strikingly, neither *AS-C*$^{-/-}$ nor neurogenic mutant tubules are rescued by the expression of an activated EGF receptor protein, confirming that the patterning of tubule cell fate within the PNC is a prerequisite for mitogenic activation in the tubules. Thus, while the primary function of the competence domain is to facilitate cell cycles in an embryo in which the majority of cells have exited the division cycle, its existence also sets a spatial limit on the ‘late’ tubule cell divisions.

These observations raise the question of whether the competence domain or a gradient of Spi activity normally

limits cell division in the tubules. Tight regulation of the EGF pathway has been demonstrated in other tissues, both through the graded distribution of ligand and the activity of negative feedback loops mediated by Argos and Sprouty (Schweitzer et al., 1995; Hacohen et al., 1998; Casci et al., 1999; Kramer et al., 1999; Reich et al., 1999). We do not know whether Sprouty acts in the Malpighian tubules, but the graded concentration of sSpi and the spatial restriction of ligand activity by Argos could act together to impose some limit on cell division in the distal region of the tubule (Kerber et al., 1998).

However, several arguments suggest that this is not the case, but that it is the size of the competence domain that limits the extent of cell division. Firstly, clonal analyis by Janning et al. (Janning et al., 1986) indicates that tubule cells divide up to five times (cycles 14-18). Division of cells in the competence domain (12-14 cells) generates 45 in the posterior tubules and 70 in the anterior. Thus, every cell in the domain would have to divide during cycles 17 and 18 in the posterior tubules and at least one further cycle, involving the division of a subset of cells, would be required in the anterior tubules (Skaer and Martinez-Arias, 1992). This argument suggests that all competent cells do divide. Second, over activation of the EGFR pathway (by expressing sSpi, λ-top, activated Ras or in *argos* mutants) produces no increase in cell proliferation in the tubules (Table 1). Again, this suggests that it is the competence domain, rather than the extent of EGF signalling, that patterns late division in the tubules. Third, the expression of *svp* throughout the tubules from stage 10 does result in an increase in tubule cell number (Kerber et al., 1998) (Table 1). Ectopic Svp at stage10/11 would result in an expansion of the number of cells expressing both *svp* and *pntP2* and therefore of the competence domain. Extra cell division would then result from cells stimulated by EGFR activation that, in a normal embryo would fail to respond.

Thus, in wild-type embryos the competence domain imposes a limit on tubule cell division and, should mitogenic signalling become deregulated, the extent of the competence domain imposes an effective brake on tubule hyperplasia.

Cell cycles in the Malpighian tubules are pre-patterned by developmental cues

We show here that several layers of pre-pattern are imposed on cell cycles in the tubules by early developmental circuitry (Fig. 6). Wg activates the expression of *pntP2* in a subset of tubule cells (encompassing the PNC), thus imposing a first restriction on cells competent to respond to the EGF pathway. Second, the expression of the proneural genes induces expression of *svp* in the PNC. Third, cells are selected from the PNC, start to express *rho* and are then able to secrete the mitogen Spi. The remaining PNC cells, a subset of *pntP2*-expressing cells that also express *svp*, are the only cells primed to respond to the mitogen.

The initiation of *svp* and *pnt2* expression is independent of EGF receptor activation; in a top^{CO} mutant, both *pntP2* and *svp* are expressed in a subset of tubule cells from stage 11 (V. S., unpublished and Fig. 4F, respectively). *pntP2* expression is dependent on Wg. *svp* expression is promoted by moderate levels of proneural gene expression, while *rho* expression depends on high levels of proneural gene activity. We propose that lateral inhibition represses the expression of proneural genes, thus ensuring that *svp* but not *rho* is activated in the competence domain. We also show that Wg, by activating *pntP2* expression, acts together with N and the proneural genes to prime cells close to the source of secreted Spi to be responsive to it.

While the initiation of *rho* expression in the TC and SC depends on high levels of proneural gene expression, its maintenance must be independent of the activity of these genes, at least in the SC, which loses proneural gene expression soon after it is born (Hoch et al., 1994). In other systems, activation of the EGF receptor from a primary source has been shown to stimulate the secretion of the EGF ligand from neighbouring cells (Golembo et al., 1996; Golembo et al., 1999; Wasserman and Freeman, 1998). A similar positive-feedback loop could maintain signal amplification in the tubules with Spi from the TC activating *rho* expression in the neighbouring SC. However, as both the TC and SC are born expressing *rho* and tubule cell division is unaffected by the specification of two TCs (UAS-*numb*) or SCs (*numb* mutants), it seems more likely that signalling between the TC and SC is reciprocal and acts to maintain *rho* expression in both cells, thereby ensuring continued high levels of secreted signal.. Indeed, results obtained from TC ablation (Skaer, 1989) support this model. The TC was removed mechanically and resulted in tubules with only 75 cells (Table 1), suggesting complete loss of secreted Spi so that all the late cell divisions failed.

We do not suggest that the expression of *ac* and the establishment of the PNC are alone sufficient for cell division. Indeed in other situations both the SOP and PNC are mitotically quiescent while they express *ac* (Usui and Kimura, 1992; Cubas et al., 1991), and in the developing wing, Ac acts to repress the transcription of *string*, thus ensuring that cells do not divide (Johnston and Edgar, 1998). Rather, we suggest that initial, moderate expression and subsequent repression of proneural gene expression is a precondition for cell division in the tubules, but only in the presence of mitogenic signal.

A common strategy for patterning cell fate?

The selection of founder cells, which organise tissue development by signalling to recruit their neighbours has been demonstrated in *Drosophila* by mosaic analysis of developing leg bristles (Tobler, 1966) and has since been shown to occur in the developing eye (Cagan and Ready, 1989; White and Jarman, 2000), PNS (Hartenstein and Posakony, 1989; Reddy et al., 1997; zur Lage et al., 1997) and muscles (Bour et al., 2000; Ruiz-Gomez et al., 2002). In each case, the signalling properties are established by the expression of specific proneural genes. In the eye (Tio et al., 1994; Freeman, 1996) and chordotonal organs (zur Lage and Jarman, 1999), though not necessarily in other systems, signalling is through the EGFR pathway, resulting from the initiation of *rho* expression in the founder cells. We have shown, in the Malpighian tubules, that responsive cells, as well as the progenitors of the signalling cells, segregate from the PNC. This pattern has also been demonstrated in adult chordotonal organs (zur Lage and Jarman, 1999) and in the recruitment of multiple cell fates in the developing eye (Cagan and Ready, 1989; Baker and Zitron, 1995; Flores et al., 2000; White and Jarman, 2000; Xu et al., 2000). Evidence from the pattern of recruitment, as well as from changes in cell fate in mutants, indicates the existence of a similar mechanism in the development of the embryonic

chordotonal organs (zur Lage et al., 1997), somatic muscles (Bour et al., 2000; Ruiz-Gomez et al., 2002) and adult olfactory sensilla (Reddy et al., 1997).

These results suggest that, while the segregation of single cells from an equivalence domain is a unifying theme in the generation of tissues from a wide range of organisms (Sternberg, 1993; Simpson, 1997), PNCs in specific tissues have developed an additional function: to establish a second cell fate that cooperates with the first to implement the subsequent programme of tissue differentiation.

We thank the following for kindly providing us with flies and reagents: Alfonso Martinez-Arias, Ethan Bier, Michael Hoch, Andrew Jarman, Petra zur Lage, Chris Rushlow, Trudi Schüpbach, Pat Simpson, Stefan Stein, Ursula Weber and the Developmental Studies Hybridoma Bank. We are grateful to Michael Bate, Barry Denholm, Peter Lawrence and Pat Simpson for helpful discussions and comments on the manuscript, and to Phil Ingham for advice. V. S. thanks Sasikanth Acharya for initiating an interest in *Drosophila* development. This work was funded by The Wellcome Trust (S. P.-S., A. G. and H. S.), the MRC (S. W.) and an INLAKS Foundation scholarship to V. S.

REFERENCES

Ainsworth, C., Wan, S. and Skaer, H. (2000). Coordinating cell fate and morphogenesis in *Drosophila* renal tubules. *Philos. Trans. R. Soc. London B Biol. Sci.* **355**, 931-937.

Baker, N. E. and Zitron, A. E. (1995). *Drosophila* eye development: Notch and Delta amplify a neurogenic pattern conferred on the morphogenetic furrow by *scabrous*. *Mech. Dev.* **49**, 173-189.

Bang, A. G. and Kintner, C. (2000). Rhomboid and Star facilitate presentation and processing of the Drosophila TGF-alpha homolog Spitz. *Genes Dev.* **14**, 177-186.

Baumann, P. and Skaer, H. (1993). The *Drosophila* EGF receptor homologue (DER) is required for Malpighian tubule development. *Development Supplement*, 65-75.

Bour, B. A., Chakravarti, M., West, J. M. and Abmayr, S. M. (2000). *Drosophila* SNS, a member of the immunoglobulin superfamily that is essential for myoblast fusion. *Genes Dev.* **14**, 1498-1511.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brunner, D., Ducker, K., Oellers, N., Hafen, E., Scholz, H. and Klambt, C. (1994). The ETS domain protein pointed-P2 is a target of MAP kinase in the *sevenless* signal transduction pathway. *Nature* **370**, 386-389.

Cagan, R. L. and Ready, D. F. (1989). Notch is required for successive cell decisions in the developing *Drosophila* retina. *Genes Dev.* **3**, 1099-1112.

Campos-Ortega, J. A. and Hartenstein, V. (1997). *The Embryonic Development of* Drosophila melanogaster. Berlin: Springer-Verlag.

Casci, T., Vinos, J. and Freeman, M. (1999). Sprouty, an intracellular inhibitor of Ras signaling. *Cell* **96**, 655-665.

Clifford, R. and Schüpbach, T. (1992). The torpedo (DER) receptor tyrosine kinase is required at multiple times during *Drosophila* embryogenesis. *Development* **115**, 853-872.

Clifford, R. and Schüpbach, T. (1994). Molecular analysis of the *Drosophila* EGF receptor homolog reveals that several genetically defined classes of alleles cluster in subdomains of the receptor protein. *Genetics* **137**, 531-550.

Cubas, P. de Celis, J. F., Campuzano, S. and Modolell, J. (1991) Proneural clusters of achaete-scute expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Dev.* **5**, 996-1008.

Edgar, B. A. and O'Farrell, P. H. (1990). The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G2 by *string*. *Cell* **62**, 469-480.

Edgar, B. A., Sprenger, F., Duronio, R. J., Leopold, P. and O'Farrell, P. H. (1994). Distinct molecular mechanism regulate cell cycle timing at successive stages of *Drosophila* embryogenesis. *Genes Dev.* **8**, 440-452.

Ellis, H. M. (1994). Embryonic expression and function of the *Drosophila* helix-loop-helix gene, *extramacrochaetae*. *Mech. Dev.* **47**, 65-72.

Flores, G. V., Duan, H., Yan, H., Nagaraj, R., Fu, W., Zou, Y., Noll, M. and Banerjee, U. (2000). Combinatorial signaling in the specification of unique cell fates. *Cell* **103**, 75-85.

Foe, V. E., O'Dell, G. M. and Edgar, B. A. (1993). Mitosis and morphogenesis in the *Drosophila* embryo: point and counterpoint. In *The Development of* Drosophila melanogaster. Vol. 1 (ed. M. Bate and A. Martinez-Arias), pp. 149-300. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Freeman, M. (1996). Reiterative use of the EGF receptor triggers differentiation of all cell types in the *Drosophila* eye. *Cell* **87**, 651-660.

Golembo, M., Raz, E. and Shilo, B. Z. (1996). The *Drosophila* embryonic midline is the site of Spitz processing, and induces activation of the EGF receptor in the ventral ectoderm. *Development* **122**, 3363-3370.

Golembo, M., Yarnitzky, T., Volk, T. and Shilo, B. Z. (1999). Vein expression is induced by the EGF receptor pathway to provide a positive feedback loop in patterning the Drosophila embryonic ventral ectoderm. *Genes Dev.* **13**, 158-162.

Hacohen, N., Kramer, S., Sutherland, D., Hiromi, Y. and Krasnow, M. A. (1998). *sprouty* encodes a novel antagonist of FGF signaling that patterns apical branching of the *Drosophila* airways. *Cell* **92,** 253-263.

Hartenstein, V. and Posakony, J. W. (1989). Development of adult sensilla on the wing and notum of *Drosophila melanogaster*. *Development* **107**, 389-405.

Hoch, M. and Jäckle, H. (1998). *Krüppel* acts as a developmental switch gene that mediates Notch signalling-dependent tip cell differentiation in the excretory organs of *Drosophila*. *EMBO J.* **17**, 5766-5775.

Hoch, M., Broadie, K., Jäckle, H. and Skaer, H. (1994). Sequential fates in a single cell are established by the neurogenic cascade in the Malpighian tubules of *Drosophila*. *Development* **120**, 3439-3450.

Johnston, L. A. and Edgar, B. A. (1998). Wingless and Notch regulate cell-cycle arrest in the developing *Drosophila* wing. *Naure.* **394**, 82-84.

Janning, W., Lutz, A. and Wissen, D. (1986). Clonal analysis of the blastoderm anlage of the Malpighian tubules in *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **195**, 22-32.

Jowett, T. (1997). *Tissue In Situ Hybridization*. New York: John Wiley & Sons.

Kerber, B., Fellert, S. and Hoch, M. (1998). *seven-up*, the *Drosophila* homolog of the COUP-TF orphan receptors, controls cell proliferation in the insect kidney. *Genes Dev.* **12**, 1781-1786.

Knoblich, J. A., Sauer, K., Jones, L., Richardson, H., Saint, R. and Lehner, C. F. (1994). Cyclin E controls S phase progression and its down-regulation during *Drosophila* embryogenesis is required for the arrest of cell proliferation. *Cell* **77**, 107-120.

Kramer, S., Okabe, M., Hacohen, N., Krasnow, M. A. and Hiromi, Y. (1999). Sprouty: a common antagonist of FGF and EGF signaling pathways in *Drosophila*. *Development* **126**, 2515-2525.

O'Neill, E. M., Rebay, I., Tjian, R. and Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. *Cell* **78**, 137-147.

Rebay, I. and Rubin, G. M. (1995). *Yan* functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. *Cell* **81**, 857-866.

Reddy, G. V., Gupta, B., Ray, K. and Rodrigues, V. (1997). Development of the *Drosophila* olfactory sense organs utilizes cell-cell interactions as well as lineage. *Development* **124**, 703-712.

Reich, A., Sapir, A. and Shilo, B. (1999). *Sprouty* is a general inhibitor of receptor tyrosine kinase signaling. *Development* **126**, 4139-4147.

Richardson, H. E., O'Keefe, L. V., Reed, S. I. and Saint, R. (1993). A *Drosophila* G1-specific cyclin E homolog exhibits different modes of expression during embryogenesis. *Development* **119**, 673-690.

Ruiz-Gomez, M., Coutts, N., Suster, M. L., Landgraf, M. and Bate, M. (2002) *myoblasts incompetent* encodes a zinc finger transcription factor required to specify fusion competent myoblasts in *Drosophila*. *Development* **129**, 133-141.

Scholz, H., Deatrick, J., Klaes, A. and Klambt, C. (1993). Genetic dissection of *pointed*, a *Drosophila* gene encoding two ETS-related proteins. *Genetics* **135**, 455-468.

Schweitzer, R., Howes, R., Smith, R., Shilo, B. Z. and Freeman, M. (1995). Inhibition of *Drosophila* EGF receptor activation by the secreted protein Argos. *Nature* **376**, 699-702.

Shermoen, A. W. (2000). BrdU labeling of chromosomes. In Drosophila *Protocols* (ed. W. Sullivan, M. Ashburner and R. S. Hawley), pp. 57-65. New York: Cold Spring Harbor Laboratory Press.

Simpson, P. (1997). Notch signalling in development: on equivalence groups and asymmetric developmental potential. *Curr. Opin. Genet. Dev.* **7**, 537-542.

Skaer, H. (1989). Cell division in Malpighian tubule development in *Drosophila melanogaster* is regulated by a single tip cell. *Nature* **342**, 566-569.

Skaer, H. and Martinez-Arias, A. (1992). The wingless product is required for cell proliferation in the Malpighian tubule anlage of Drosophila melanogaster. *Development* **116**, 745-754.

Sternberg, P. (1993). Falling off the knife edge. *Curr. Biol.* **3**, 763-765.

Tio, M., Ma, C. and Moses, K. (1994). *spitz*, a *Drosophila* homolog of transforming growth factor-alpha, is required in the founding photoreceptor cells of the compound eye facets. *Mech. Dev.* **48**, 13-23.

Tobler, H. (1966). Zellspezifische Determination und Beziehung zwischen Proliferation und Transdetermination in Bein- und Flügelprimordien von *Drosophila melanogaster. J. Embryol. Exp. Morphol.* **16**, 609-633.

Urban, S., Lee, J. R. and Freeman, M. (2001). *Drosophila* Rhomboid-1 defines a family of putative intramembrane serine proteases. *Cell* **107**, 173-182.

Usui, K. and Kimura, K. I. (1992). Sensory mother cells are selected from among mitotically quiescent cluster of cells in the wing disc of *Drosophila*. *Development* **116**, 601-610.

Wan, S., Cato, A. M. and Skaer, H. (2000). Multiple signalling pathways establish cell fate and cell number in *Drosophila* Malpighian tubules. *Dev. Biol.* **217**, 153-165.

Wassarman, D. A., Therrien, M. and Rubin, G. M. (1995). The Ras signaling pathway in Drosophila. *Curr. Opin. Genet. Dev.* **5**, 44-50.

Wasserman, J. D. and Freeman, M. (1997). Control of EGF receptor activation in *Drosophila*. *Trends Cell Biol.* **7**, 431-436.

Wasserman, J. D. and Freeman, M. (1998). An autoregulatory cascade of EGF receptor signaling patterns the *Drosophila* egg. *Cell* **95**, 355-364.

White, K., Grether, M. E., Abrams, J. M., Young, L., Farrell, K. and Steller, H. (1994). Genetic control of programmed cell death in *Drosophila*. *Science* **264**, 677-683.

White, N. M. and Jarman, A. P. (2000). *Drosophila atonal* controls photoreceptor R8-specific properties and modulates both receptor tyrosine kinase and *hedgehog* signalling. *Development* **127**, 1681-1689.

Xu, C., Kauffmann, R. C., Zhang, J., Kladny, S. and Carthew, R. W. (2000). Overlapping activators and repressors delimit transcriptional response to receptor tyrosine kinase signals in the *Drosophila* eye. *Cell* **103**, 87-97.

zur Lage, P., Jan, Y. N. and Jarman, A. P. (1997). Requirement for EGF receptor signalling in neural recruitment during formation of *Drosophila* chordotonal sense organ clusters. *Curr. Biol.* **7**, 166-175.

zur Lage, P. and Jarman, A. P. (1999). Antagonism of EGFR and Notch signalling in the reiterative recruitment of *Drosophila* adult chordotonal sense organ precursors. *Development* **126**, 3149-3157.