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Multiple Signalling Pathways Establish Cell Fate and Cell Number in *Drosophila* Malpighian Tubules

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A unique cell, the tip mother cell, arises in the primordium of each *Drosophila* Malpighian tubule by lateral inhibition within a cluster of *achaete*-expressing cells. This cell maintains *achaete* expression and divides to produce daughters of equivalent potential, of which only one, the tip cell, adopts the primary fate and continues to express *achaete,* while in the other, the sibling cell, *achaete* expression is lost (M. Hoch *et al.,* 1994, *Development* 120, 3439–3450). In this paper we chart the mechanisms by which *achaete* expression is differentially maintained in the tip cell lineage to stabilise cell fate. First, *wingless* is required to maintain the expression of *achaete* in the tubule primordium so that *wingless* mutants lack tip cells. Conversely, increasing *wingless* expression results in the persistence of *achaete* expression in the cell cluster. Second, Notch signalling is restricted by the asymmetric segregation of Numb, as the tip mother cell divides, so that *achaete* expression is maintained only in the tip cell. In embryos mutant for *Notch* tip cells segregate at the expense of sibling cells, whereas in *numb* neither daughter cell adopts the tip cell fate resulting in tubules with two sibling cells. Conversely, when *numb* is overexpressed two tip cells segregate and tubules have no sibling cells. Analysis of cell proliferation in the developing tubules of embryos lacking Wingless after the critical period for tip cell allocation reveals an additional requirement for *wingless* for the promotion of cell division. In contrast, alteration in the expression of *numb* has no effect on the final tubule cell number. © 2000 Academic Press

Key Words: **cell fate; asymmetric division; cell proliferation; Malpighian tubule; *wingless; numb; Drosophila.***

INTRODUCTION

Cells achieve their final differentiated fate through a series of decisions, each of which may depend on multiple inputs. An example of this is the sequential activity of the proneural and neurogenic genes in *Drosophila;* a strategy that is used widely in developing tissues such as the CNS (Campos-Ortega, 1993), PNS (Campuzano and Modolell, 1992), somatic mesoderm (Corbin *et al.,* 1991; Bate *et al.,* 1993; Carmena *et al.,* 1995), and the stomatogastric nervous system (Gonzalez-Gaitan and Jäckle, 1995). Studies in the developing PNS have revealed further refinements in the mechanisms determining cell fate. Within certain proneural clusters the selection of sensory organ precursors (SOPs) is not random; patterning, involving *pannier* and genes of the *iroquois* complex, ensures the selection of a predetermined cell as the SOP (Ramain *et al.,* 1993; Gomez-Skarmata *et al.,* 1996; Simpson, 1997). Further, the precise pattern of cell fate in the progeny of the SOP requires the asymmetric localisation of a cytoplasmic determinant encoded by *numb* (*nb*), which in turn relies on the polarised distribution of the cytoskeletal-binding protein Inscuteable (Uemura *et al.,* 1989; Rhyu *et al.,* 1994; Kraut *et al.,* 1996;). Numb influences cell fate by blocking the activation of Notch, thereby biasing the outcome of signalling between potentially equivalent cells (Guo *et al.,* 1996; Spana and Doe, 1996).

The specification of cell types in the SOP lineage therefore depends on the activity of at least four groups of genes. While the molecular basis and immediate outcome of the activity of each class are increasingly well understood, how these pathways integrate, and thus ensure the reliable

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segregation of different cell types, remains a challenging question.

Normal development of the excretory system, the Malpighian tubules, of the fly depends on the segregation of a unique cell, the tip cell, in each tubule primordium. Tip cells direct and pattern cell proliferation in the developing tubules through activation of the EGF receptor (Baumann and Skaer, 1993; Kerber *et al.,* 1998), so that in the absence of tip cells (Skaer, 1989; Hoch *et al.,* 1994) or in embryos mutant for the EGF receptor or its ligand, *spitz* (Kerber *et al.,* 1998), these cell divisions fail. The allocation of tip cell fate depends on lateral inhibition within a set of cells in each primordium, which express proneural genes, such as *achaete* (*ac*), and which have the capacity to develop as tip cells. Only a single cell, the progenitor of the tip cell (the tip mother cell), continues to express proneural genes and retains tip cell potential. This restriction depends on the activity of the neurogenic genes so that multiple tip cells develop in embryos mutant for genes in this family. The tip mother cell divides once and its daughters follow separate fates. One differentiates as the tip cell and continues to express the transcription factors Achaete and Krüppel; the other as the sibling cell, which loses the expression of both markers. This second cell fate decision also depends on the activity of the neurogenic genes, *Delta* (encoding the ligand) and *Notch* (encoding the receptor) (Hoch *et al.,* 1994), but in addition on the activity of the DNA-binding protein encoded by *eyelid* (Carrera *et al.,* 1998).

In this study we explore the mechanisms underlying the specification of the tip cell further. In particular we examine the role of intercellular signalling by *wingless* (*wg*) and the asymmetric activity of *numb* (*nb*). We show that *wg* is required for the appearance of tip cells in the tubules and that *wg* expression is both necessary and, for a period, sufficient for the maintenance of *ac* expression in cells with tip cell potential. While *nb* is not required for the segregation of tip mother cells, it is necessary for the continued expression of *ac* in the tip cell and thus for the correct allocation of cell fate between the tip cell and its sibling. This activity is dependent on the expression of the cytoplasmic protein Inscuteable. Thus both mechanisms act through the maintenance of *ac* expression to confirm tip cell potential. We analyse the consequences for tubule cell proliferation of removing *wg* function and of misspecifying cell fates between the tip cells and their siblings.

MATERIALS AND METHODS

Fly Stocks

The following strains were used: Oregon R; a null allele of *wg*, wg^{CX4}; a ts allele, wg^{IL114}; two alleles of *numb*, nb^{1} (Uemura *et al.,* 1989) and nb^{796} (Buescher *et al.,* 1998), and a deficiency uncovering *nb*, Df(2L)30A-C (Lindsley and Zimm, 1992); $inscuteable^{P72}$ (Kraut *et al.,* 1996); *HS-wg*/TM3*hbLacZ* (Nordermeer *et al.,* 1992); a P-element insertion in *neuromusculin,* A37 (Kania *et al.,* 1993); *UAS-numb* (Zhong *et al.,* 1996); *HS-*N_{intra} (an activated form of *Notch,* Struhl *et al.,* 1993); and *HS-Gal4* (gift of Andrea Brand).

Manipulation of Gene Expression

Temperature-shift experiments and the ectopic expression of *wg* were carried out as described previously (Skaer and Martinez Arias, 1993). Times for temperature shifts are given as equivalent time at 25°C, so that an upshift at 5 h would be after embryos had been kept at 18°C for 10 h. Embryos carrying the ts allele or the heat shock construct were identified by the absence of β-galactosidase staining associated with the balancer chromosome. Sibling and wild-type embryos were used as controls. In addition, the efficiency of the heat shock promotor was tested both by staining embryos at the end of each heat shock regime using an antibody against Wg (*wg* was ubiquitously expressed; data not shown) and by making cuticle preparations of mature embryos (van der Meer, 1977), which showed the characteristic phenotype of naked ventral cuticle (Nordermeer *et al.,* 1992).

For the expression of N_{intra}, embryos were collected for 1 h at 25°C, aged at 18°C, dechorionated, and heat shocked for 20 min at 36°C. The heat shock was repeated after one hour at 25°C for longer periods of expression and embryos were then aged at 18 or 25°C before fixing for immunostaining.

The expression of *nb* was driven by crossing *UAS-nb* females to males carrying *HS-Gal4.* Gastrulae were selected from the resulting progeny and subjected to heat shock, as described above, so that *nb* was expressed from 3.5 to 7.5 h. Embryos were then aged at 18 or 25°C before fixing.

Malpighian tubule cells were counted in dissected stage 16 embryos (Truman and Bate, 1988), after staining immunocytochemically for Cut.

Immunocytochemistry

Embryos were stained as described previously (Hoch *et al.,* 1994) except that staining for Ac was as described in Goriely *et al.* (1991). Embryos were mounted in Araldite in capillaries to allow free rotation (Schmidt-Ott and Technau, 1992) and were photographed using a Ziess Axioplan microscope. We used the following antibodies at the dilutions shown: mAb22C10 1:200 (Zipursky *et al.,* 1984); mAbachaete 1:100 (gift of S. Carroll); anti-Krüppel 1:1000 (gift of C. Rushlow); anti-β-galactosidase 1:10,000 (Cappel); mAbcut 1:200 (gift of I. Rebay); and anti-wg 1:1000 (gift of M. van den Heuvel).

For confocal microscopy, fluorescently tagged secondary antibodies (Jackson) were used at 1:800 dilution and embryos viewed with a LeicaTCS NT laser imaging system.

The age of embryos is given either as hours after egg laying at 25°C or as stages according to Campos-Ortega and Hartenstein (1985).

RESULTS

Tip Cells Fail to Segregate in wg Mutant Embryos

In wild-type embryos, each Malpighian tubule tip cell can be recognised as a single large cell protruding from the distal end of the tubule, from stage 12 (Skaer, 1989). They express *Krüppel* and stain with the antibody 22C10 (Zipursky *et al.,* 1984; Hoch *et al.,* 1994) (Fig. 1B). Embryos carrying the wg^{cx4} mutation were stained for these markers.

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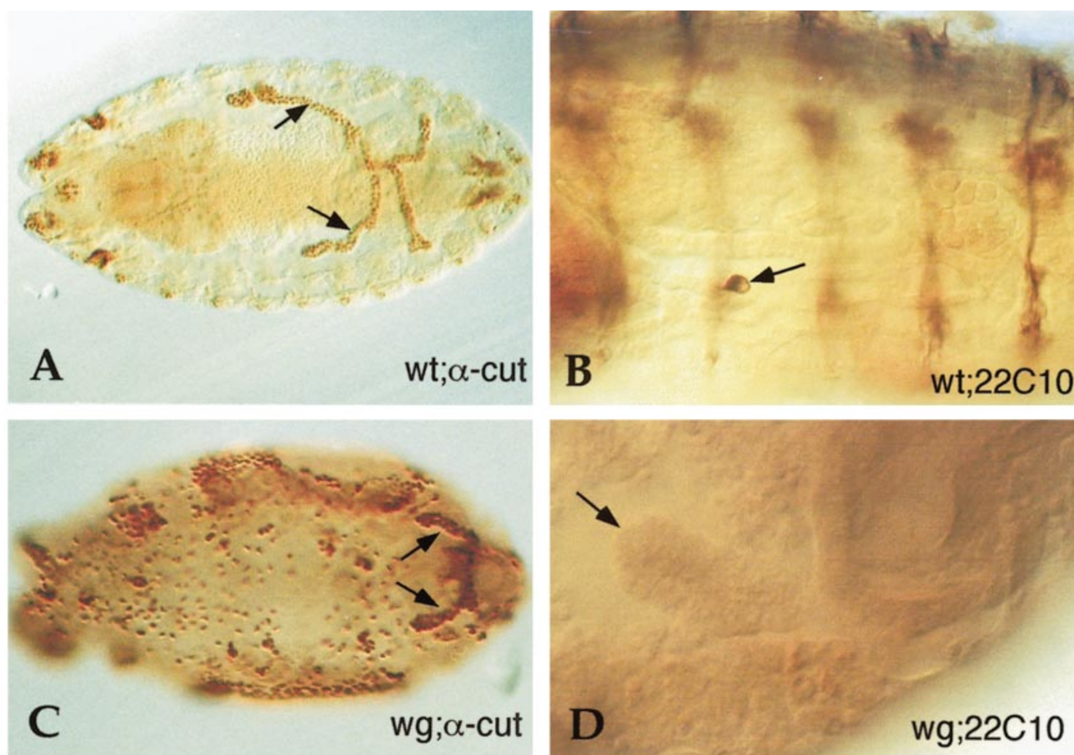

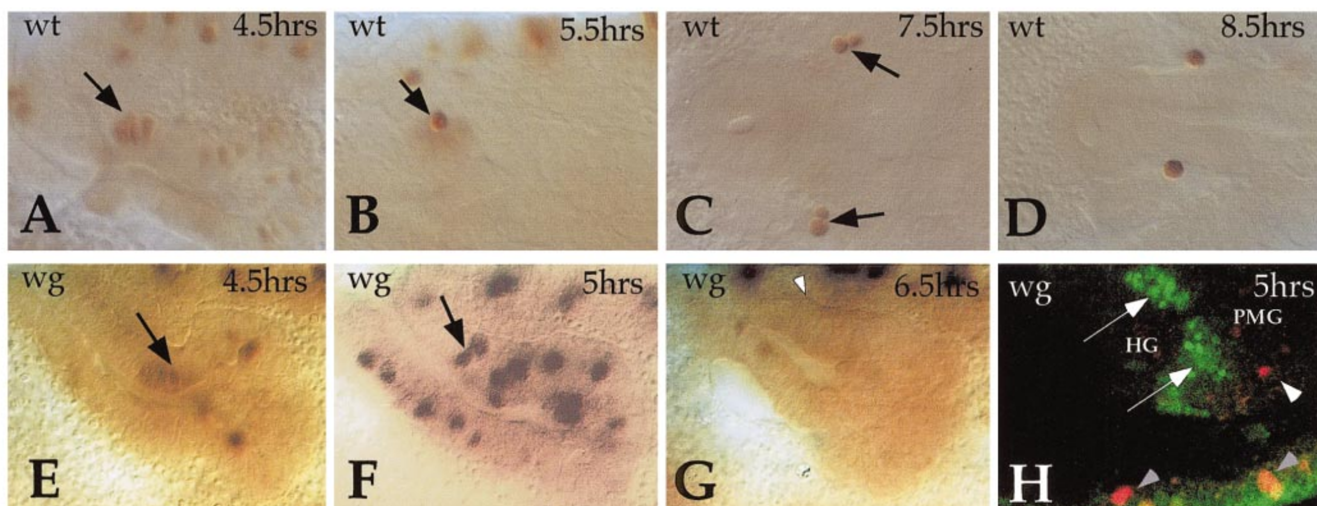

FIG. 1. (A, B). Wild-type embryos stained with anti-Cut (A) to show the Malpighian tubules (arrows, anterior tubules) and with 22C10 (B) which picks out the tip cell (arrow). (C, D) Embryos mutant for *wg*^{cx4} stained with anti-Cut (C) and 22C10 (D). The tubules (arrow in C) are much reduced in size and lack tip cells (site of tip cell arrow in D). (A, C) Dorsal views; (B, D) dorsolateral views. Anterior to the left in all cases.

FIG. 2. The expression of *ac* in wild type (A–D) and *wg*^{cx4} mutant (E–H) embryos. (A, E) Stage 10 (4.5 h); (B) stage 11 (5.5 h); (F, H) stage 10 (5 h); (G) stage 11 (6.5 h); (C) early stage 12 (7–7.5 h); (D) stage 12 (8–8.5 h). The onset of *ac* expression (arrows in A, E, F) is reduced or fails altogether in *wg* mutants compared with wild type (cf. A with E, F, H). In wild-type embryos division of the tip mother cell (arrow in B) leads to the appearance of two cells expressing *ac* (arrows in C). However, expression refines again to a single cell in each tubule (D). In mutant embryos *ac* expression fades and disappears by mid-stage 11 (white arrowheads in G). (A, B, E–G) Lateral views; (C, D) dorsal views. (H) Dorsolateral view, stained for Ac (red) and Cut (green). *ac* expression, apparent in the posterior midgut (PMG, arrowhead) and CNS (gray arrowheads), is absent in the tubule primordia (arrows). Anterior of the extended germ band embryo is to the left so that the hindgut lies to the left and the posterior midgut lies to the right.

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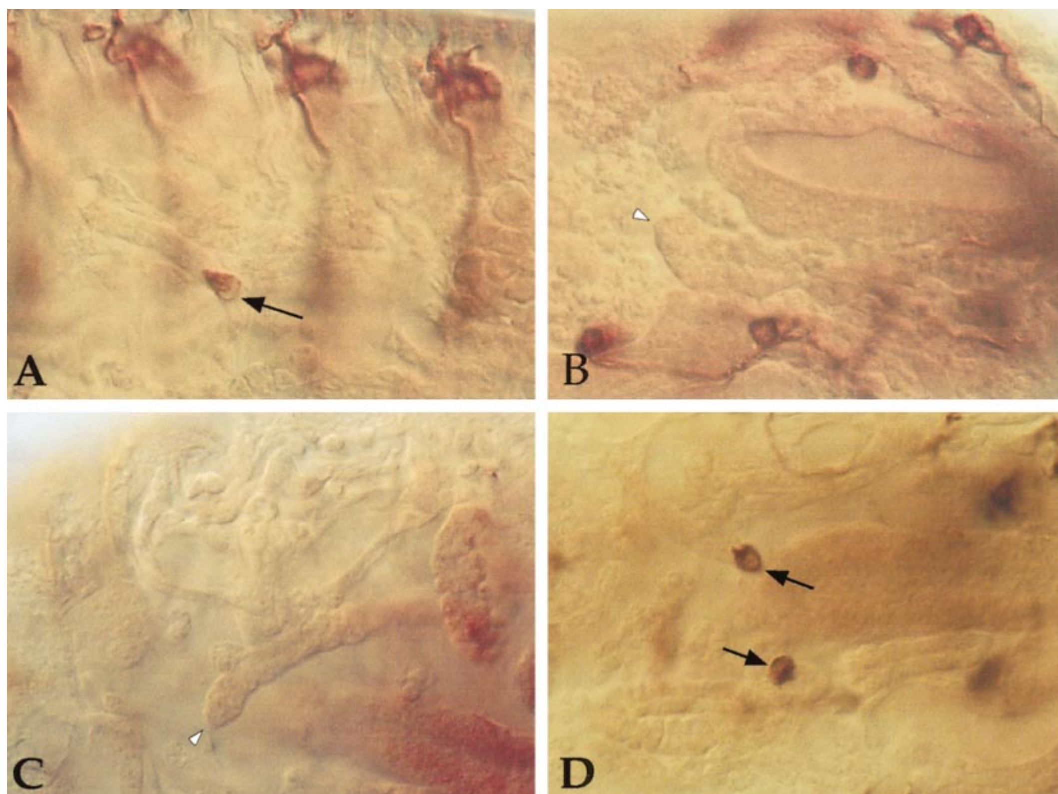

FIG. 3. In embryos homozygous for the ts allele *wg*^{IL114}, tip cells, revealed by 22C10, appear at the permissive temperature (A) but are missing in embryos raised at the restrictive temperature (B). If the expression of *wg* is removed by exposure to the restrictive temperature from 3.5 to 5.5 h, tip cells are lost (C) but restoration of *wg* expression for the same period results in their appearance (D). Arrows indicate tip cells in A and D and white arrowheads the site where they would normally appear in B and C. (A–C) Lateral views; (D) dorsal view; anterior to the left in all cases.

Cell division fails in the tubule primordia of mutant embryos, producing tubules of very reduced size (cf. Figs. 1A and 1C; Skaer and Martinez Arias, 1993). However, in these tiny tubules no tip cells develop, judged both by the absence of antibody staining and by uniform cell morphology at the tubule tip (cf. Figs. 1B and 1D).

In wild-type embryos a cluster of cells in each tubule primordium starts to express proneural genes during stage 10 at about 4.5 h (shown for Achaete (Ac) in Fig. 2A). Expression refines by 5–5.5 h to one cell, the tip mother cell, in each cluster (Fig. 2B). This cell divides between 6.5 and 7.5 h to produce two Ac-staining cells (Fig. 2C). By 8 h staining is again restricted to one cell, the tip cell, in each cluster (Fig. 2D) and *ac* continues to be expressed in this cell until stage 16 (15 h) (Hoch *et al.,* 1994). In embryos mutant for *wg, ac* expression in the tubule primordia is either absent (Fig. 2H) or weak and expressing cells are only rarely arranged in clusters (Figs. 2E and 2F). Expression weakens prematurely and disappears by 6.5 h (Figs. 2G and 2H). These results indicate that Wg is required for the normal pattern of *ac* expression in the tubule primordia, being essential for its maintenance in the tip cell lineage.

There Is a Window of Requirement for Wg between 4.5 and 5.5 h

In order to establish when the *wg* product is required for tip cell allocation, we manipulated *wg* activity using the temperature-sensitive allele, *wg*^{IL114} (Nüsslein-Volhard *et al.,* 1984). At the permissive temperature tip cells develop normally (Fig. 3A), while at the restrictive temperature Wg function is lost and tip cells fail to appear (Fig. 3B). Temperature shift experiments establish that embryos must develop at the permissive temperature between 4 and 5 h for tip cells to appear normally (Table 1). In accordance with these findings, embryos shifted to the permissive temperature for this period develop with tip cells (Fig. 3D), while a shift to the restrictive temperature results in tubules with no tip cells (Fig. 3C).

Given that the restoration of wild-type *wg* protein has been shown to take 20–30 min and that the removal of functional protein takes a similar time after shifting temperature (quoted in Skaer and Martinez Arias, 1993), these results establish the window of requirement for functional

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TABLE 1
Appearance of Tip Cells in the Malpighian Tubules of wg^{IL114} Embryos after Temperature Shifts at Different Ages

Age at temperature change, h (±30 min)	Tubules with tip cells, % of tubules analysed	Tubule cell number, average ± SEM (*n*)
Upshifts		
3	0	
4	75	
5	100	83 ± 3 (39)
6	100	107 ± 3 (24)
7	100	129 ± 4 (18)
Downshifts		
3	100	119 ± 4 (22)
4	100	68 ± 6 (12)
5	0	
6	0	
7	0	

Note. Tip cells were analysed in a minimum of 8 mutant (32 Malpighian tubules) and 8 control sibling embryos for each temperature shift. Tip cells appeared normally in control embryos for all temperature shifts and the number of cells in the tubules was not different from wild type (124 ± 3; Baumann and Skaer, 1993). Malpighian tubule cell number is given as the average for all four since the anterior and posterior pair cannot be distinguished in the strongly affected embryos.

Wg in the tubules for tip cell allocation as 4.5–5.5 h of embryogenesis.

Wg is normally expressed in the tubule primordia as they evert from the hindgut (Skaer and Martinez Arias, 1993). From 4.5 h expression is higher in the posterior region of the developing tubules than the anterior (Fig 4A). The tip mother cell segregates from this posterior region, where *wg* continues to be expressed (Figs. 4B and 4C). Wg is lost from tubules during stage 12 (Skaer and Martinez Arias, 1993).

Ectopic Expression of wg Is Sufficient to Maintain ac Expression but Not to Establish Extra Tip Cells

We used embryos carrying *wg* under the regulation of a heat shock promotor (Nordermeer *et al.,* 1992) to drive increased levels of *wg* expression throughout the tubules. We examined the expression of *ac* in the developing tubules after three different heat shock regimes, designed to drive *wg* expression for varying periods in embryos aged between 4 and 10 h (Figs. 5C–5E). In regimes which drive *wg* expression to 6–7 h, *ac* expression is maintained in the cell clusters for longer than in wild type but refines to one cell after the end of the heat shock period (Figs 5C and 5E, a–d). When *wg* expression is driven until 8.5 h the expression of *ac* refines from a cluster (Fig. 5E, e) to 2 cells (Figs. 5E, f; and 4D) but does not refine further until after the heat shock. However if *wg* expression is driven until 9–11 h, the expression of *ac* does refine to a single cell during the period of heat shock (Figs. 5E, g and h, and 5D). In summary, elevated levels of Wg in the developing tubules do not result in expansion of *ac*-expressing domains but rather in persistence of expression. There is a limit to this capacity; beyond 7 h expression refines to two cells and beyond 9 h refinement to a single cell occurs even in the presence of ectopic Wg.

After each heat shock regime examination of embryos, aged to stage 16 and stained with 22C10, revealed that a single tip cell always developed, appearing morphologically normal (data not shown). Wild-type embryos subjected to similar heat shock regimes showed the normal pattern of *ac* expression in the tubule primordia (data not shown).

There Is a Dual Requirement for wg to Maintain Cell Proliferation in the Tubules

Manipulation of *wg* expression using the ts allele, wg^{IL114}, reveals a requirement for *wg* in cell division, separate from its role in tip cell specification. If Wg is removed after 5 or 6 h AEL but before cell division ceases in the tubules, the final tubule cell number is reduced compared to wild type (Table 1). This indicates that the appearance of tip cells is insufficient, in the absence of Wg, to promote the normal pattern of division in the tubules.

Tip Cell Allocation Is Disrupted in the Absence of nb

In wild-type embryos the division of the tip mother cell is asymmetric, giving rise to the tip cell and a sibling cell (Figs. 6A and 6B; and Hoch *et al.,* 1994). Soon after this division the morphology of the tip cell alters so that it protrudes from the tubule epithelium on the basal side, though it does not delaminate from it. (Figs. 1B and 4D–4F). In contrast the sibling cell remains with(in) the tubule epithelium adjacent to the tip cell. In embryos carrying a P element in which *LacZ* is expressed in the tip mother cell (A37 in *neuromusculin;* Kania *et al.,* 1993), perdurance of β-galactosidase allows the sibling cell to be identified immunocytochemically (Fig. 6A). In embryos double stained with 22C10 and for β-galactosidase, only the tip cell stains for both antigens (Fig. 6B).

In embryos mutant for *nb* Malpighian tubules develop without tip cells (Figs. 6C–6E). The tubules of embryos carrying A37 in a *nb* mutant background contain two β-gal-positive cells that remain with(in) the tubule epithelium (Fig. 6F) and do not stain with 22C10 (Fig 6G), indicating that they both develop instead as sibling cells.

Careful examination of mutant embryos (whether nb^1, nb^{796}, or a deficiency uncovering *nb*) shows that in a proportion tip cells are allocated normally in one or two of the four tubules (approximately 8% of all progeny ($n = 115$) and Fig. 6C). This finding is confirmed by double staining A37/*nb* embryos with anti β-gal and 22C10 (Fig. 6H).

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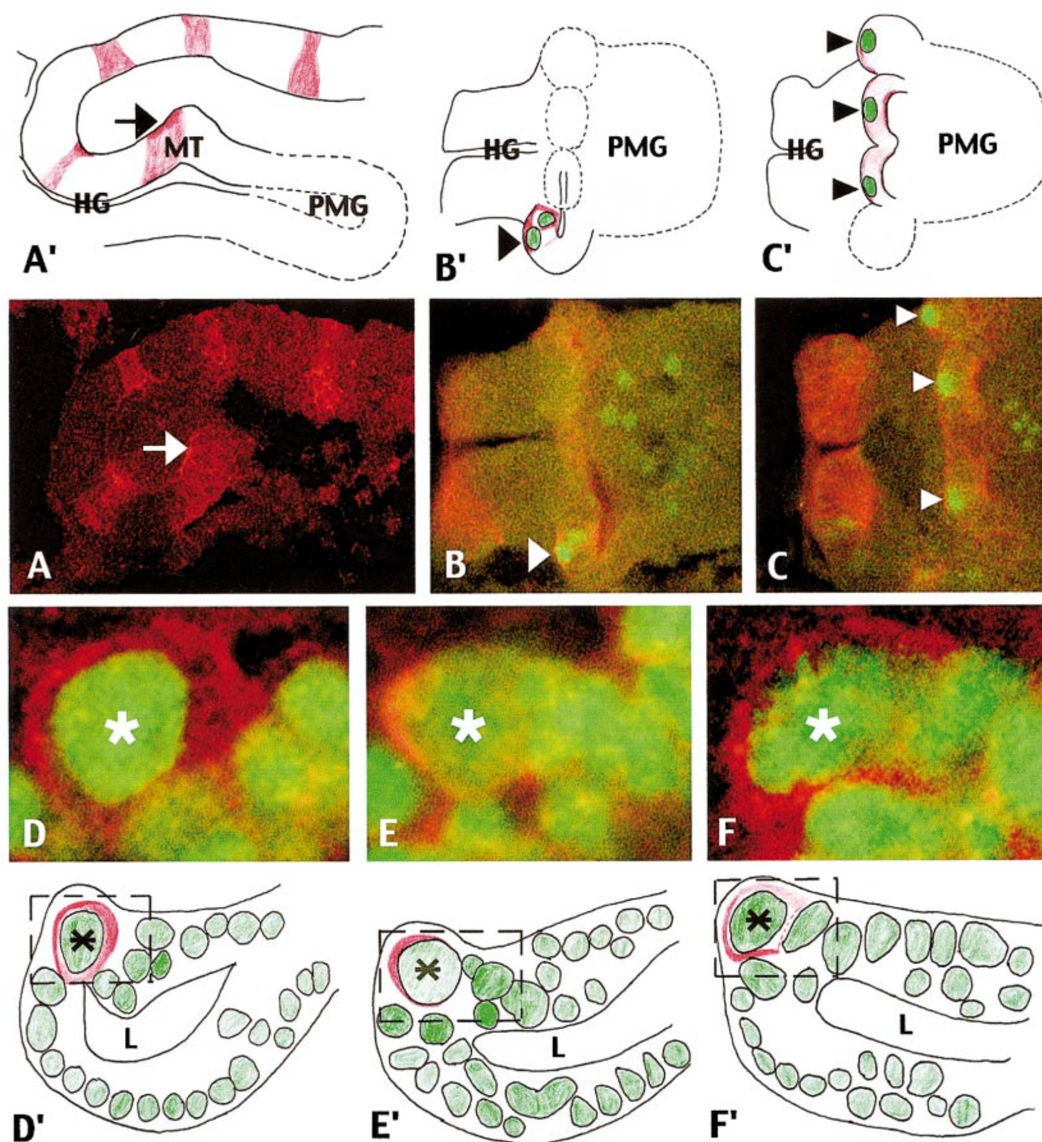

FIG. 4. (A–C) *wg* expression. (A) *wg* (red) is expressed on the posterior side (arrow) of the tubule primordia from stage 10. (B, C) Two focal planes of an early stage 11 embryo. *ac* expression (green) refines into the tip mother cells in the posterior *wg*-expressing domains, facing the hindgut, HG (arrowheads indicate tip mother cells in B, C; in the tubule shown in B refinement of *ac* expression is incomplete). (D–F) *nb* expression. (D) *nb* (red) is expressed uniformly in the tip mother cell (asterisk) at 6.5 h (mid-stage 11). (E) Staining for Cut (green) becomes diffuse in the tip mother cell (asterisk) as the nuclear envelope breaks down in early mitosis and Nb becomes asymmetrically distributed in a basal crescent. (F) Mid-stage 12. Nb is partitioned asymmetrically into the tip cell (asterisk). (A′–F′) Features illustrated in A–F (boxed areas in D′–F′). Anti-Nb, red; anti-Cut, green. Asterisks: tip mother cell (D, E); tip cell (F). HG, hindgut; MT, Malpighian tubule primordium; PMG, posterior midgut; L, tubule lumen on the apical side of the tubule cells.

Ectopic Expression of nb Directs Cells to a Tip Cell Fate

Staining with an antibody against Nb reveals that it is expressed in the tip mother cell, where initially it is distributed symmetrically (Fig. 4D). However, as the cell enters division, Nb is strikingly localised in a basal crescent (Fig. 4E), so that it segregates only to one daughter, the larger cell, which becomes the tip cell (Fig. 4F).

We investigated the effects of ectopic Nb, using a heat shock GAL4 (Brand and Perrimon, 1993) to drive its expression throughout the Malpighian tubules, from 3.5 to 7.5 h. Embryos were stained with 22C10 and those that had expressed *nb* ectopically were identified by the increased

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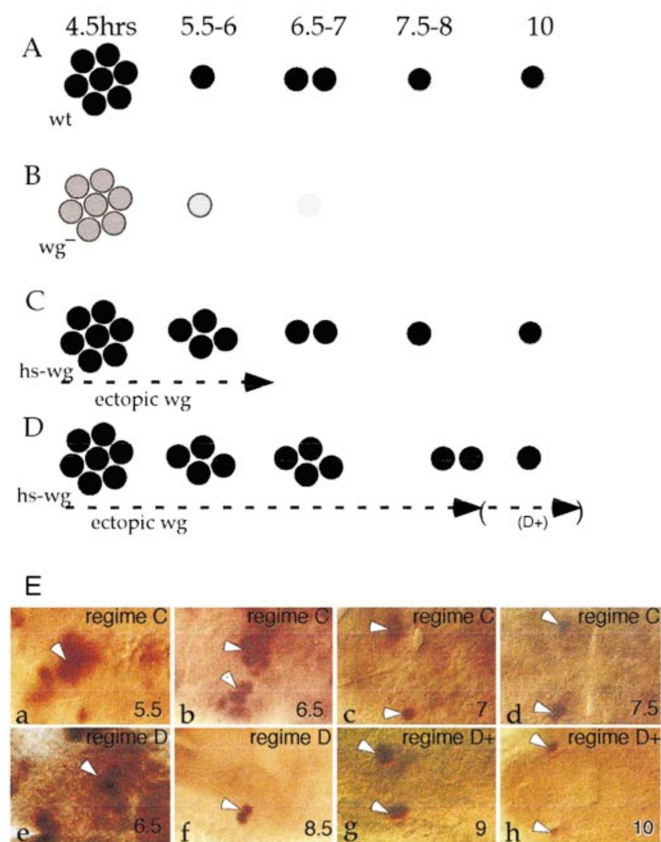

FIG. 5. The expression of *ac* in the tubules of *wg* mutant embryos and those in which *wg* is overexpressed using a heat shock construct. (A–D) Summarises expression in wild-type (A), *wg* mutant (B), and heat shocked embryos (C, D). (C and E, a–d) A regime to drive *wg* from 3.5–6.5 h. *ac* is expressed in clusters of cells at 5.5 h (a) and 6.5 h (b), but refines after the heat shock (c) to a single cell in each primordium by 7.5 h (d). (D and E, e–h) Regimes to drive *wg* expression for longer periods, until 8.5 (e, f) or up to 11 h (g, h). *ac* expression is maintained in clusters of cells at 6.5 h (e) and in two cells at 8.5 h (f). However, if *wg* is expressed up to 11 h (g, h, and dotted line in parentheses in D), *ac* expression refines into a single cell during the period of heat shock. (a–h) Figure at bottom right indicates the age of the embryo in hours.

number of neurones in the PNS (Uemura *et al.,* 1989). In these embryos the Malpighian tubules have two tip cells (Figs. 7A and 7B). Control embryos, whether siblings from the experimental cross or embryos carrying UAS-*nb* without GAL4, had only one tip cell in each tubule (Figs. 7C and 7D).

In neural and myogenic lineages, *insc* is required to direct the asymmetric segregation of Nb between daughter cells. The loss of *insc* therefore results in a phenotype resembling weak overexpression of *nb* (Kraut *et al.,* 1996; Ruiz Gomez and Bate, 1997; Buescher *et al.,* 1998). *insc* is also expressed in the Malpighian tubules (Kraut and Campos-Ortega, 1996), suggesting a role in the asymmetric distribution of Nb between the tip mother cell daughters. Analysis of 22C10 staining in embryos mutant for *insc* revealed that, although in many cases the appearance of tip cells is normal, some embryos have at least one tubule with two tip cells. Interestingly in these cases, one cell protrudes further from the distal end of the tubule than its sibling (Fig. 7E).

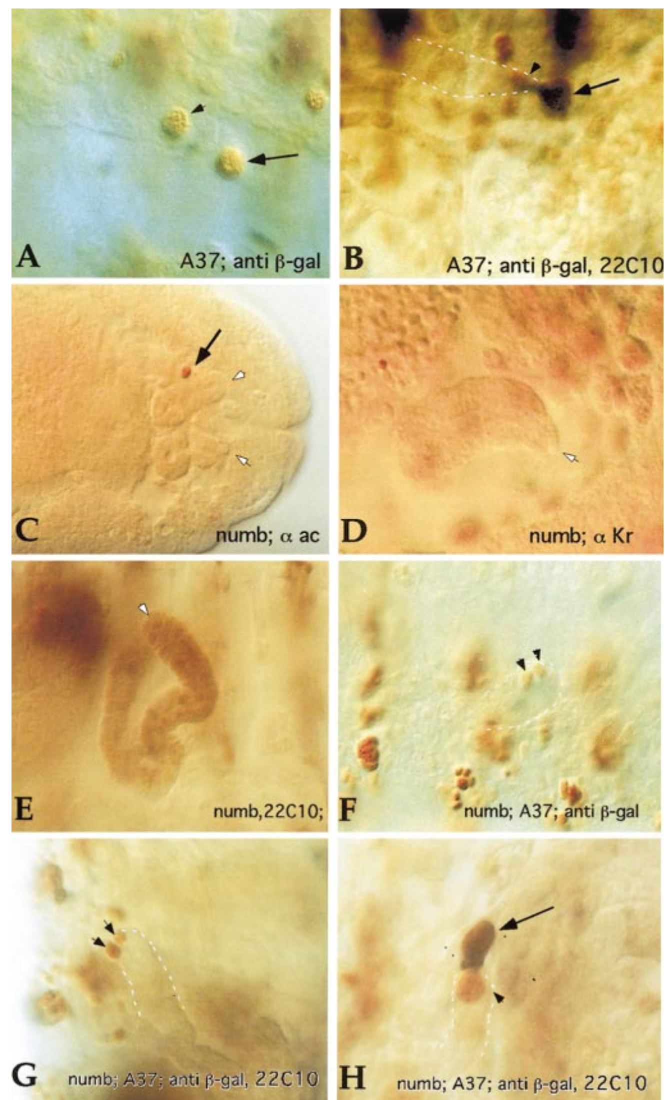

FIG. 6. Tip cell specification is perturbed in embryos mutant for *nb.* (A) Both progeny of the tip mother cell stain (brown) for β-gal in an enhancer trap line A37 (in *neuromusculin*). (B) Only one of these cells, the tip cell, stains for 22C10 (black). In embryos mutant for *nb,* staining for *ac* (C), for *Kr* (D), or with 22C10 (E) reveals the absence of tip cells, but in an A37 background, *nb* mutant tubules have two β-gal-staining cells (F), neither of which stains for 22C10 (G). In some *nb* mutant embryos the normal allocation of tip cells is found in one or two of the tubules (C stained for Ac; H, A37; *nb* embryo stained for β-gal (brown) and 22C10 (black)). Arrows indicate tip cells, arrowheads sibling cells, and white arrowheads the site where tip cells would normally segregate.

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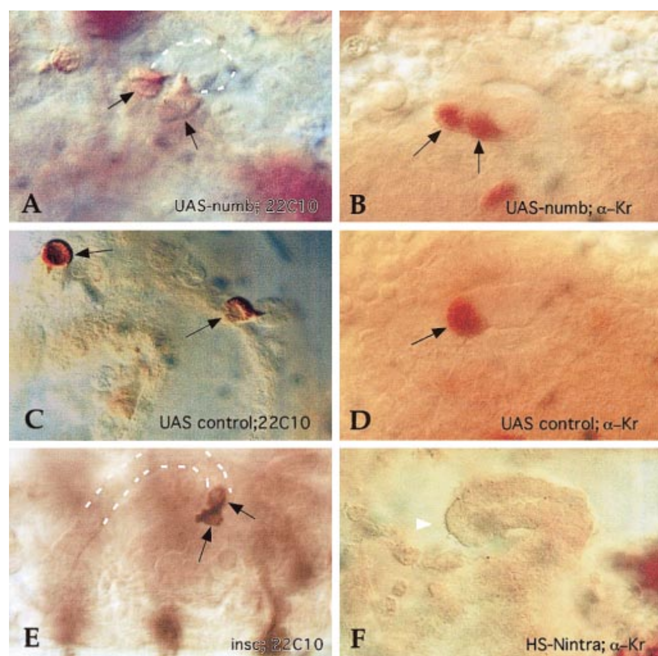

FIG. 7. In embryos expressing *nb* ubiquitously from 3.5 to 7 h and stained with 22C10 during stage 16 (A) or for *Kr* during stage 13 (B), there are two tip cells (arrows) in each Malpighian tubule. Sibling controls have only one tip cell in each tubule (arrow in C, D). Two 22C10-positive cells are occasionally found in the tubules of embryos mutant for *insc* (arrows in E). In embryos in which N_{intra} is expressed from 6 to 8 h (F), no tip cells appear (stained for *Kr* during stage 15; site of tip cell, white arrowhead).

If Nb acts by repressing signalling through Notch (Guo *et al.,* 1996; Zhong *et al.,* 1996; Spana and Doe, 1996) in the selection of tip cells, Notch loss and gain of function would be expected to show phenotypes opposite to those we have described for *nb* loss and gain of function. Both loss of *N* and the overexpression of *nb* result in the appearance of ectopic tip cells. However, in embryos lacking the zygotic function of *N* there are up to 12 tip cells (Hoch *et al.,* 1994), a more severe phenotype than the two tip cells found when the expression of *nb* is driven ectopically. This difference results from the fact that *N* is required both for the segregation of the tip mother cell from the *ac*-expressing cluster and also for the correct allocation of sibling cells (Hoch *et al.,* 1994), while *nb* is required only during the second phase of *N* signalling.

We tested the effects of *N* gain of function in the tubules by driving the expression of a constitutively active *N* construct (N_{intra}; Struhl *et al.,* 1993) under the control of a heat shock promoter. Tubules developed without tip cells (Fig. 7F) and this phenotype was found whether expression of N_{intra} was driven from 5 h, during tip mother cell specification, or from 6 or 7 h, when the allocation of the tip cell would be affected. In summary, N gain of function parallels the loss of *nb.*

Alteration in the Expression of nb Does Not Alter Tubule Cell Numbers

Interestingly, in embryos that are mutant for *nb,* and have no tip cells, there is no change in the final number of tubule cells (Table 2). This is equally true of embryos in which the overexpression of *nb* results in the specification of two tip cells. The distinction between the number of cells in the anterior and posterior tubules is also unaffected.

DISCUSSION

The segregation of tip cells depends on the coordinated activity of the proneural and neurogenic genes. In the absence of proneural genes, no tip cells develop, while in mutants lacking neurogenic gene activity all the cells in the cluster develop as tip cells; there are no sibling cells (Hoch *et al.,* 1994). Signalling through N is therefore required to establish the fate of sibling cells in the tubules; in embryos mutant for neurogenic genes supernumerary tip cells appear at the expense of siblings cells. Here we present evidence that two further processes are involved in tip cell specification, but at different stages in the series of decisions taken.

wg Is Required to Maintain ac Expression in the Tip Cell Lineage

In the absence of *wg, ac* expression is variably initiated in a few tubule primordial cells and is lost from the tip cell lineage by 6.5 h, so that tip cells fail to differentiate (Figs. 5B and 8). The temporal requirement for *wg* expression, from 4.5 to 5.5 h, and its expression in the posterior of the tubules, the region from which the tip mother cell segregates, is consistent with a role in maintaining *ac* expression in this lineage. Phillips and Whittle (1993) demonstrated a similar requirement for *wg* in the development of sensory organs in the wing.

Increasing the expression of *wg* results in the persistence but not the expansion of *ac* expression (Fig. 5C). However, the normal pattern of *ac* expression is restored later in development so that a single tip cell still segregates. These results suggest that Wg signalling is capable of maintaining *ac* expression in the pro-tip cell cluster or sibling pair but only for a limited period. Suppression of *ac* expression in all

TABLE 2
Malpighian Tubule Cell Number at Stage 15 (Average ± SEM (*n*))

	Anterior tubule	Posterior tubule
Wild type	141 ± 2 (15)	106 ± 2 (20)
nb^{796}	143 ± 2 (19)	107 ± 1 (18)
UAS-*nb*	144 ± 3 (9)	109 ± 1 (12)
Control	141 ± 2 (19)	107 ± 1 (20)

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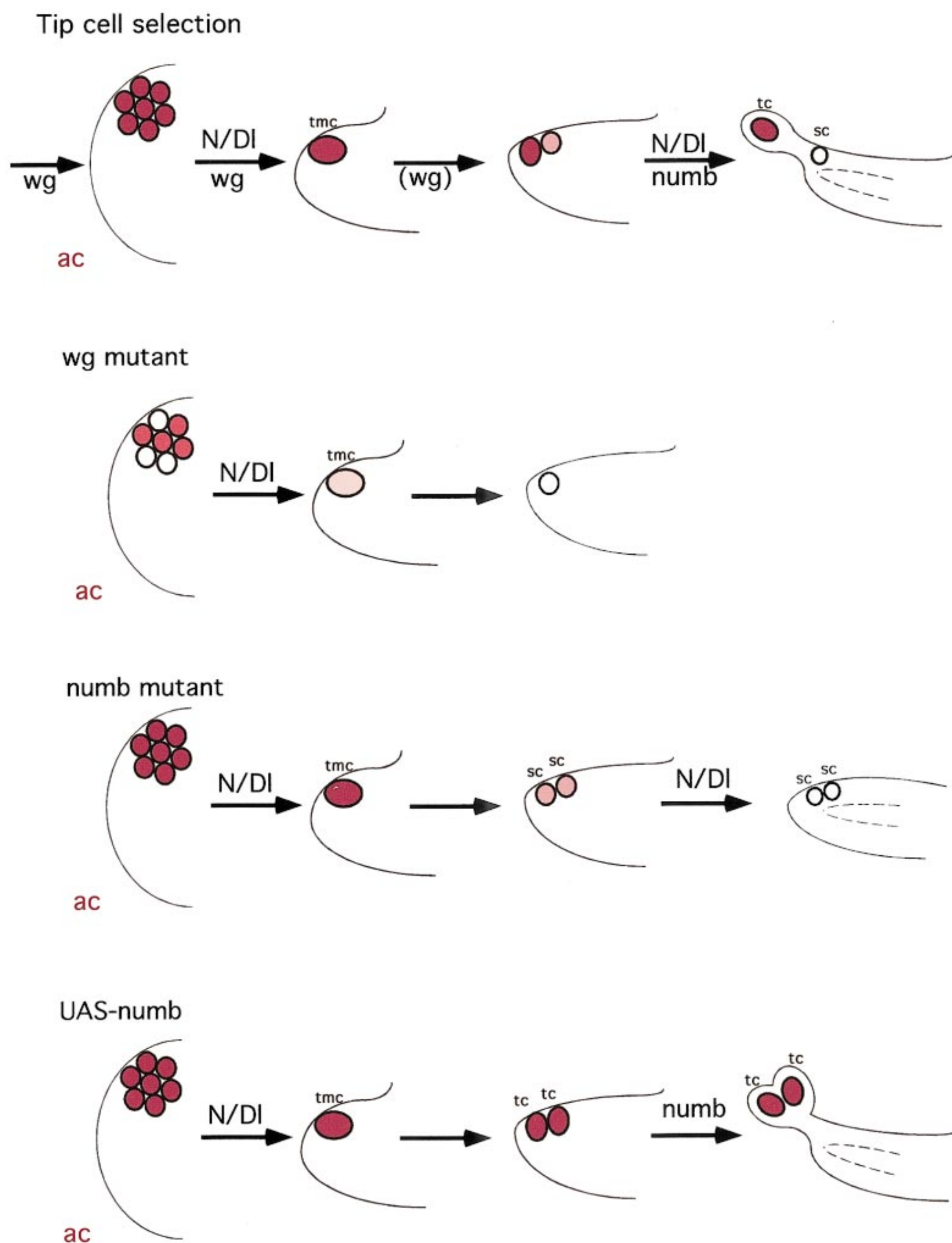

FIG. 8. Summary of tip cell allocation in wild-type, *wg,* and *nb* mutant embryos and those with ectopic *nb* expression.

but the tip cell can still occur in the presence of extra Wg, but is delayed.

The refinement of *ac* expression is a direct consequence of lateral inhibition (Brand and Campos-Ortega, 1988; Skeath and Carroll, 1992) but also requires the activity of *extra macrochaetae* (*emc*) (Cubas *et al.,* 1994; Ellis, 1994). *emc* restricts the activity of the proneural genes by forming inactive heterodimers with their products (Ellis *et al.,* 1990; Garrell and Modolell, 1990), so that in *emc* mutant tubules up to eight cells continue to express *ac* as well as the early tip cell marker, *Krüppel* (Carrera *et al.,* 1998). *emc* is expressed in the tubule primordia but is lost later from the tip cells (Cubas *et al.,* 1994). Wg could therefore act to maintain *ac* expression in the tip cell lineage either di-

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rectly, as has been suggested in the wing (Phillips and Whittle, 1993; Couso *et al.,* 1994; Neumann and Cohen, 1997; Johnston and Edgar, 1998), or indirectly through the repression of *emc.*

In summary our results show that Wg is required for the normal pattern of *ac* expression in the tubule primordia, ensuring that a single cell remains dedicated to its fate, possibly through the initiation of an *ac*-driven autoregulatory loop (Culi and Modolell, 1998).

Wg Plays a Dual Role in Regulating Cell Proliferation in the Tubules

The role of *wg* in the development of the Malpighian tubules is pleiotropic; its expression is required both for the normal eversion of tubule primordia from the embryonic hindgut and for the proliferation of these primordial cells (Skaer and Martinez Arias, 1993). The activity of tip cells also underlies normal cell proliferation in the tubules (Skaer, 1989; Hoch *et al.,* 1994). However, the loss of *wg* gives a more severe phenotype (tubules with an average of 20–25 cells) than the absence of tip cells (70–75 cells), suggesting that in addition to its role in the specification of tip cells, Wg signalling may have a direct influence on earlier cell division in the tubule primordia (Gampel, Wan, and Skaer, manuscript in preparation).

The Asymmetric Distribution of Nb Ensures Tip Cell Selection but Is Not Required for Segregation of the Tip Mother Cell

As in neural and myogenic lineages (Uemura *et al.,* 1989; Ruiz Gomez and Bate, 1997), N is involved both in the selection of precursor cells and in setting the fates of their progeny, while Nb is required only at the second stage. Thus the segregation of a single tip mother cell occurs normally in the absence of Nb but the choice of fate between the sibling cells is disrupted, so that both cells adopt the fate dictated by the activation of N. Conversely, ectopic expression of *nb* produces two tip cells, the cell fate in which N is silent (Fig. 8). These observations, and our demonstration that Nb segregates asymmetrically into the tip cell, support a role for Nb in blocking N signalling in the tip cell, possibly through a direct physical interaction with the cytoplasmic domain of N (Guo *et al.,* 1996; Zhong *et al.,* 1996). Loss of *insc* resembles a weak overexpression of *nb,* suggesting that, as in other lineages (Kraut *et al.,* 1996; Ruiz Gomez and Bate, 1997; Buescher *et al.,* 1998), Insc is an essential component of the machinery for the asymmetric distribution of Nb to the tip cell.

The silencing of N in the tip cell allows continued expression of *ac* and of its target *Kr* (Hoch *et al.,* 1994), which in turn drives the expression of tip cell markers such as 22C10 (Hoch and Jäckle, 1998). Carrera *et al.* (1998) have shown that the DNA-binding protein Eyelid acts in the tip cell lineage to repress the expression of *Kr* in the sibling cell; in the absence of *eyelid* (*eld*) two tip cells differentiate. Treisman *et al.* (1997) identified *eld* as an antagonist of Wg signalling in the eye, wing, and embryo. These observations suggest the possibility that *eld* might be a target of N activation in the sibling cell, where it acts to repress Wg-mediated activation of *ac* and therefore tip cell fate.

Sibling Cells Can Direct Tubule Cell Division

The final tubule cell number is unaltered if there are two tip cells and no siblings or vice versa. These two cell types therefore have an overlapping function in wild-type development and can substitute for each other in mutants. In the absence of proneural gene expression, the tubules contain neither a tip cell nor a sibling cell and the final tubule cell number is ca. 70, instead of ca. 120 (Hoch *et al.,* 1994). A similar result was obtained when tip cells were surgically removed (Skaer, 1989), suggesting that this procedure also damages the sibling cell.

nb Is Required to Bias Signalling between Sibling Cells

Although embryos lacking Numb always have tubules without tip cells, approximately one-third have one or two tubules in which the allocation of tip and sibling cell fate is wild type. This incomplete transformation suggests that the role of *nb* is to introduce a bias in signalling between equivalent cells. The source of the N ligand, Delta, in the tubules is the daughter cells themselves (Hoch *et al.,* 1994), so that in the absence of *nb* no outcome is guaranteed. Thus competitive signalling between the two cells could repress tip cell fate in both but, equally, a slight imbalance in equilibrium between the two cells could set up a feedback loop (Simpson *et al.,* 1993; Campos-Ortega, 1993; Heitzler *et al.,* 1996), resulting in the separation of fates as in the wild type. This contrasts with the segregation of cell types in the MP2 lineage of the CNS (Spana *et al.,* 1995) in which the Delta source is outside the lineage, so that in the absence of *nb* both daughters are always activated through N and the transformation to vMP2 is complete (Spana and Doe, 1996).

The degree of transformation of cell fate when *nb* is lost reflects its role; transformation is partial where it imposes a bias on signalling between equivalent cells, all of which initially produce Dl, but is complete where it blocks the induction of a specific cell fate through signalling from an external source. The degree of transformation in embryos lacking Nb can therefore be used as an indicator of the source of N activation in those lineages where this information is unknown.

wg and *nb* both Maintain Tip Cell Fate through *ac* Expression

The tip cell progenitor is selected from a group of competent cells by lateral inhibition and is marked out by the continued expression of *ac.* In this paper we have shown that further extrinsic and intrinsic cues, Wg signalling and

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the asymmetric distribution of Nb, operate to ensure the continued expression of *ac* and so confirm tip cell potential.

The selection of cell fate from an equivalence group by lateral inhibition alone relies on chance fluctuations in the equilibrium of signalling between cells and therefore may not be completely reliable. The activity of other genes, by biasing lateral inhibition, serve to make the selection of cells to specific fates more robust. Such mechanisms have been shown to confirm cell fate in the PNS (Cubas and Modolell, 1992; Ramain *et al.,* 1993; see Simpson, 1997) and of the anchor cell in the nematode gonad (Felix and Sternberg, 1996; Sternberg and Felix, 1997).

Our results indicate that *wg* and *nb* are required for the specification of the tip cell and sibling cell fate in the Malpighian tubules. The activity of these two genes biases the outcome of intercellular signalling at separate stages in this process, resulting in the reliable allocation of tip and sibling cell fates, suggesting that this distinction is important to the development of the tubules. However, it is clear that continued cell division in the tubules relies only on the allocation of the tip cell progenitor and not on the differentiation of fate between its daughter cells, in which *nb* plays an important role.

This result is surprising, since Nb is active where sister cells of specific lineages are allocated to separate cell fates, for example, in the PNS (Jan and Jan, 1995), in the CNS (Spana and Doe, 1996; Buescher *et al.,* 1998), and in myogenesis (Carmena *et al.,* 1995, 1998; Ruiz-Gomez and Bate, 1997; Baylies *et al.,* 1998). Separation between sister cell fates involves the maintenance of gene expression in one sibling and its repression in the other, for example, of *Kr, eve,* and *S59* in sibling muscle founder cells (Carmena *et al.,* 1995; Ruiz Gomez *et al.,* 1997; Ruiz Gomez and Bate, 1997). This pattern is also seen in the tubules; *ac, Kr,* and *Dl* continue to be expressed in the tip cell but are repressed in its sibling. In the neural and myogenic lineages the correct allocation of sibling cell fates underpins normal tissue differentiation. In the tubules, we do not yet know the separate roles of the tip cells and their siblings; they both appear to be active in regulating cell proliferation but later only the tip cell expresses genes characteristic of neuronal cells (Hoch *et al.,* 1994). The later function of both cell types has yet to be elucidated. By manipulating *nb* we can now generate tubules which lack sibling cells but have two tip cells or have two sibling cells but lack tip cells, thus providing an important tool for this analysis.

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Note added in proof. In a paper recently published, Garcia-Garcia *et al.* (1999) demonstrate a similar role for *wg* in the development of dorsocentral bristles in the adult notum of *Drosophila. Wg* is required for *ac/sc* expression in the dorsocentral proneural lineage but increasing the Wg activity does not repattern the cluster. As in the Malpighian tubules, *wg* therefore plays a permissive rather than an instructive role.

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