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Krüppel acts as a developmental switch gene that mediates Notch signalling-dependent tip cell differentiation in the excretory organs of *Drosophila*

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Cell proliferation in the excretory organs of *Drosophila*, the Malpighian tubules (MT), is under the control of a neural tip cell. This unique cell is singled out from equivalent MT primordial cells in response to Notch signalling. We show that the gene *Krüppel* (*Kr*), best known for its segmentation function in the early embryo, is under the control of the Notch-dependent signalling process. Lack-of-function and gain-of-function experiments demonstrate that *Kr* activity determines the neural fate of tip cells by acting as a direct downstream target of proneural basic helix–loop–helix (bHLH) proteins that are restricted in response to Notch signalling. We have identified a unique *cis*-acting element that mediates all spatial and temporal aspects of *Kr* gene expression during MT development. This element contains functional binding sites for the restricted proneural bHLH factors and Fork head protein which is expressed in all MT cells. Our results suggest a mechanism in which these transcription factors cooperate to set up a unique cell fate within an equivalence group of cells by restricting the activity of the developmental switch gene *Kr* in response to Notch signalling.

Keywords: Fork head/*Krüppel*/Malpighian tubules/Notch signalling/proneural bHLH factors

Introduction

The generation of cell diversity in multicellular organisms involves numerous local signalling events that are required for the determination of cell fates during development. One type of signalling that plays a pivotal role for cell fate determination in a wide variety of different tissues and organisms involves the evolutionarily conserved Notch signalling pathway. Notch signalling is required for the selection of a single cell or a group of cells from the surrounding, equivalent cells in a process which has been termed lateral inhibition (for review see Artavanis-Tsakonas *et al*., 1995). Once selected, a specific differentiation programme is triggered in the singled-out cells, and distinct cell types arise.

Well-studied examples of lateral inhibition in *Drosophila* are the segregation of neural and epidermal precursor cells in the neuroectodermal region of the *Drosophila* embryo and the patterning of the fly's sensory organs (for review see Campos-Ortega, 1993). Both developmental processes require the four genes of the *Achaete–Scute-Complex* (AS-C), *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*), which confer to cells the ability to become neural precursor cells or sensory organ precursor cells (SOPs). The proneural AS-C genes are first expressed in so-called proneural cell clusters. Local cell interactions mediated by a Notch-dependent signalling cascade which involves the transmembrane receptor Notch, its activating ligand Delta and several intracellular components that lead to the induction of basic helix–loop–helix (bHLH) transcription factors of the Enhancer of split [*E*(*spl*)] gene complex repress the transcription of proneural genes. This process causes a gradual restriction of proneural gene activity to a single cell within the equivalence group, the neuroblast and the SOP, respectively. The surrounding epidermoblasts cease to express the AS-C genes and subsequently take an epidermal fate (for reviews see Campos-Ortega, 1993; Artavanis-Tsakonas *et al*., 1995).

Whereas the genetic components underlying Notch signalling are known in some detail, only fragmentary information exists on target genes which are regulated in response to Notch signalling and bHLH activity in the singled-out cells. The few candidate target genes include *scabrous*, *neuralized*, *cut* and *hunchback* (reviewed in Campuzano and Modolell, 1992). For *hunchback*, which is expressed in all neuroblasts, it could be shown that its promoter contains binding sites for proneural proteins that mediate gene expression in response to proneural bHLH proteins in yeast cells (Cabrera and Alonso, 1991). Furthermore, experiments with imaginal discs identified *Bearded* and *E*(*spl*) genes as targets of the proneural bHLH factors (Singson *et al.*, 1994). However, most of the embryonic target genes that cause neural fate in response to singled-out bHLH activities as well as the molecular mechanisms underlying the selection process are still unknown.

The segregation of the neural tip cell during *Drosophila* Malpighian tubule (MT) development offers a simple model system to study the mechanistics of neural cell fate specification in response to Notch signalling and proneural bHLH activity. The MTs consist of two pairs of blind-ending tubes, representing the kidney-like excretory organ of the fly (Wessing and Eichelberg, 1978). The precursor cells of the MT are specified at the posterior pole of the blastoderm embryo in response to the local activity of the gene *fork head* (*fkh*) which encodes a key transcription factor for gut and MT development (Weigel *et al.*, 1989a,b; Gaul and Weigel, 1991; Pankratz and Hoch, 1995; Hoch and Pankratz, 1996). Genetic evidence suggests that *fkh*, in turn, is required for the activation of *Kr* at the posterior pole of the blastoderm embryo and later in a ring of MT primordial cells at the hindgut/midgut boundary of the developing alimentary canal. In the absence of *Kr* activity, the MT precursor cells become integrated into the hindgut epithelium and no MTs arise (Gloor, 1950; Gaul *et al*., 1989; Harbecke and Janning, 1989; Gaul and Weigel,

1991). During tubule eversion, *Kr* expression becomes gradually restricted to a single cell, the 'tip mother cell', in each of the four tubule precursors (Hoch *et al.*, 1994).

Genetic studies indicate that the tip mother cell is singled out via *Notch*-dependent lateral inhibition (Campos-Ortega, 1993; Artavanis-Tsakonas *et al.*, 1995; Young and Wesley, 1997) from a group of equivalent cells which express proneural genes (Hoch *et al.*, 1994). It subsequently divides asymmetrically and gives rise to two daughter cells which express proneural proteins and *Kr*. The smaller cell—termed 'satellite cell'—discontinues the expression of proneural genes and *Kr*, and it becomes part of the tubule epithelium. In contrast, the larger daughter cell continues to express proneural genes and *Kr* and becomes located at the ending of each tubule (Hoch *et al.*, 1994). This cell has been called the 'tip cell' (Skaer, 1989) and has a dual function during tubule development in differentiating as a neural cell and controlling the proliferation of neighbouring cells by sending out a mitogenic signal (Skaer, 1989; Hoch *et al.*, 1994). In mutants for the AS-C genes or *daughterless* (*da*), no *Kr*-expressing tip cell is formed, whereas in mutants affecting components of the Notch signalling cascade, multiple *Kr*-expressing cells are generated (Hoch *et al.*, 1994).

Here we show that the *Drosophila* gene *Kr* is a direct target of the proneural bHLH factors and that its activity specifies a single cell with neural fate within an equivalence group of cells during MT development. The proneural bHLH proteins are capable of direct interaction with a singular *Kr cis*-acting element (*Kr*MT element) which mediates gene expression throughout the MT primordium and *Notch*-dependent restriction of gene expression to the tip cell. The results suggest that the proneural bHLH proteins function in combination with the transcription factor Fork head (Fkh), present in all MT cells, first to restrict *Kr* expression to the tip mother cell and subsequently to maintain *Kr* expression in the tip cell only. Furthermore, the data indicate that *Kr* functions as a developmental switch gene that causes neural tip cell differentiation in one of the two otherwise equivalent daughters of the tip mother cell.

Results

Control of Kr expression during MT development

Kr expression initially defines the MT anlage at the posterior pole of the blastoderm embryo (Figure 1A) in response to *fkh* activity (Gaul *et al.*, 1987; Gaul and Weigel, 1991). During gastrulation and germ band extension, *Kr* expression becomes confined to a ring of MT precursor cells at the midgut/hindgut junction (Figure 1B). During the eversion of the four MT protuberances, *Kr* expression becomes first restricted to the tip mother cell and subsequently to the tip cell (Figure 1C–F). The tip cell maintains *Kr* expression until late stages of embryogenesis (Hoch *et al.*, 1994).

The selection of the *Kr*-expressing tip mother cell is a *Notch*-dependent process that involves the restriction of proneural gene activity within an equivalence group of cells (Hoch *et al.*, 1994). These cells express bHLH transcription factors encoded by the *AS-C* genes and by the ubiquitously expressed gene *da* (Figure 1G and H). In the absence of the zygotic *Notch* activity (or of any other component of the *Notch* signalling pathway such as the Notch ligand Delta or downstream effectors encoded by the *E*(*spl*)-Complex; for reviews, see Campos-Ortega, 1993; Artavanis-Tsakonas *et al.*, 1995; Young and Wesley, 1997), multiple *Kr*-expressing tip cells arise (Figure 1I). Conversely, in mutants lacking the *AS-C* genes, *Kr* expression is not maintained and no tip cell is formed (Figure 1K). A further regulator of *Kr* expression in the tubules is the proneural bHLH antagonist Extramacrochaete (Emc). During SOP allocation, Emc was found to suppress the function of proneural bHLH proteins by sequestering them as heterodimers that are incapable of binding to DNA (Van Doren *et al*., 1991). Thereby, Emc interferes with a positive regulatory feedback loop which is part of the control of the *AS-C* genes (Cabrera *et al*., 1994). As a result, AS-C activity is maintained above threshold levels only within one cell of the equivalence group that initially express the *AS-C* genes (reviewed in Campuzano and Modolell, 1992). In the MTs, EMC is expressed in all tubule cells except in the tip cell (Cubas *et al.*, 1994; Ellis, 1994). In *emc* mutant tubules, multiple *Kr*-expressing tip cells arise (Carrera *et al.*, 1998; M.Hoch, unpublished results) which co-express proneural bHLH factors (Cubas *et al.*, 1994). This observation provided additional evidence for the proposal that the presence of functional proneural bHLH factors is the cause of restricted *Kr* expression, and of the capability of forming a tip cell within a cluster of equivalent MT precursor cells.

Cis-acting requirement for Kr expression during MT development

In order to elucidate the mechanisms underlying *Kr* expression in the MT primordium and its *Notch*-dependent final restriction to the tip cell, we have isolated a *cis*-acting element which mediates functional MT expression of *Kr in vivo* (Figure 2A). Previous studies of the regulatory region of the *Kr* gene had localized the MT enhancer element to a 9 kb fragment in the *Kr* upstream region (Figure 2A; Hoch *et al.*, 1990). No other element had been found within the *cis*-acting region of the *Kr* gene that conducts gene expression during MT development. We used the 9 kb DNA fragment to identify a minimal element that mediates all aspects of *Kr* gene expression during MT development. For this, we generated reporter gene fusion constructs containing overlapping subfragments of the 9 kb fragment (see Materials and methods). These were integrated into the germline by P-element-mediated transformation (Rubin and Spradling, 1982) and their expression patterns examined during MT development. Only one fragment, a unique 650 bp element ~14 kb upstream of the *Kr* transcription start site (Figure 2A), conducted reporter gene expression identical to the endogenous *Kr* pattern during MT development (see below). This indicates that the *cis*-acting region of *Kr* contains a single and separate enhancer element that is responsible for the spatiotemporal aspects of *Kr* expression during MT development.

We next asked whether the 650 bp element of *Kr* contains the regulatory sequences necessary to mediate functional *Kr* expression *in vivo.* For this, we constructed a mini-gene containing the *Kr cis*-acting element in front of a *Kr* coding region (see Materials and methods) and generated transgenic flies bearing this construct in their

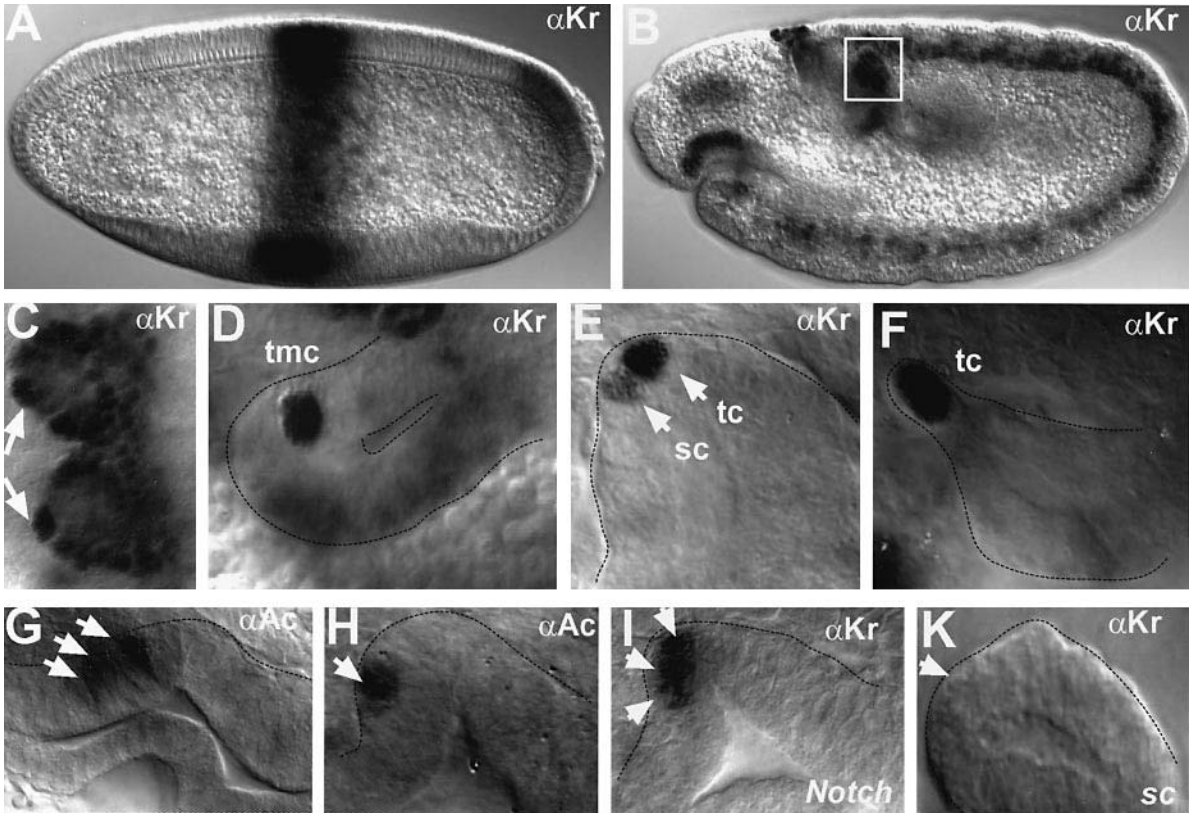

Fig. 1. Singling out of *Kr*-expressing tip cells in the MT primordium. Top, dorsal; left, anterior. (A–F) *Kr* expression in wild-type and (I, K) in mutant embryos monitored by anti-Kr antibody stainings. (**A**) During cellular blastoderm stage, *Kr* is expressed in the middle region of the embryo and in the combined anlagen of the MT and the posterior midgut at the posterior pole. (**B**) During germ-band extension stage, *Kr* is expressed in a variety of tissues including the MT primordium [the white box marks an outgrowing tubule which is magnified in (C–F)**]**. Restriction of *Kr* expression to the precursor cell of the tip cell, the tip mother cell, has been initiated in (**C**) (arrows) and is completed in (**D**). The tip mother cell then divides asymmetrically (E, arrows) to give rise to the satellite cell (**E**) which loses *Kr* expression and the *Kr*-expressing tip cell (E, **F**) at the distal end of each tubule. (**G**, **H**) Expression of the proneural bHLH protein Ac in the tip mother cell (arrow in G) and the tip cell (arrow in H), visualized by anti-Ac antibody stainings. (**I**) In zygotic *Notch*^{e55} mutants, multiple tip cells develop (arrows; three of six tip cells in this focal plane) which all express high levels of *Kr*, as in wild-type. (**K**) In proneural *sc*^{B57} mutants, *Kr* is not expressed in the tubules and the tip cells are absent (arrow). tc, tip cell; sc, satellite cell; tmc, tip mother cell.

germline. This transgene restores partial MT development in otherwise *Kr*-deficient embryos. A full rescue (Figure 2B–D) was consistently obtained with four copies of the transgene. We therefore suspect that although the 650 bp element contains the sequences that are sufficient to mediate the temporal and spatial aspects of *Kr* gene expression, it may lack elements that are necessary to conduct sufficiently high levels of *Kr* expression required for normal MT development.

A 420 bp subelement, termed *Kr*MT (Figure 2A), maintains the ability to mediate reporter gene expression covering all aspects of *Kr* expression during MT development, including its restriction to the tip cell (Figure 3A–E). We used the *Kr*MT element to determine the *trans*-acting factor requirement needed for the activation of the gene and for its final restriction to the tip cell. In accordance with genetic analysis, mutant embryos which lack the transcription factor Fkh (Weigel *et al.*, 1989a; Weigel and Jäckle, 1990) fail to express the reporter gene (Figure 3F). Furthermore, the restriction of reporter gene expression to the tip cell is not achieved in embryos lacking proneural gene activities (Figure 3G), meaning that *Kr*MT-mediated reporter gene expression is initiated normally but discontinues in the everting tubules. Conversely, ectopic *Kr*MT-mediated reporter gene expression was observed in MT protuberances of *Notch* mutants. In such mutant tubules, bHLH expression is also maintained in all the cells of the equivalence group (Figure 3H). These results indicate that the *Kr*MT element is capable of decoding the information for the initial activation in the MT anlage and of singling out of the tip cell, a process which involves Fkh and the *Notch*-dependent restriction of proneural bHLH proteins. Autoregulation of *Kr* can be excluded, as the *Kr*MT element does not respond to ectopic *Kr* activity provided in all the MT cells (data not shown).

Functional Fkh and bHLH protein binding sites on the KrMT element

In order to examine whether Fkh and proneural bHLH proteins are able to interact with the *Kr*MT element, we performed *in vitro* DNaseI footprinting analysis using bacterially expressed Fkh, proneural bHLH proteins and *Kr*MT DNA (see Materials and methods). The results (summarized in Figure 4A) showed that Fkh binds to one strong site (Kaufmann *et al.*, 1994). Homodimers of the bHLH proteins Ac (or Sc), L'sc and Ase as well as heterodimer combinations between Da and these factors are able to bind to one strong and a weaker E-box binding site within the *Kr*MT element (Figure 4A).

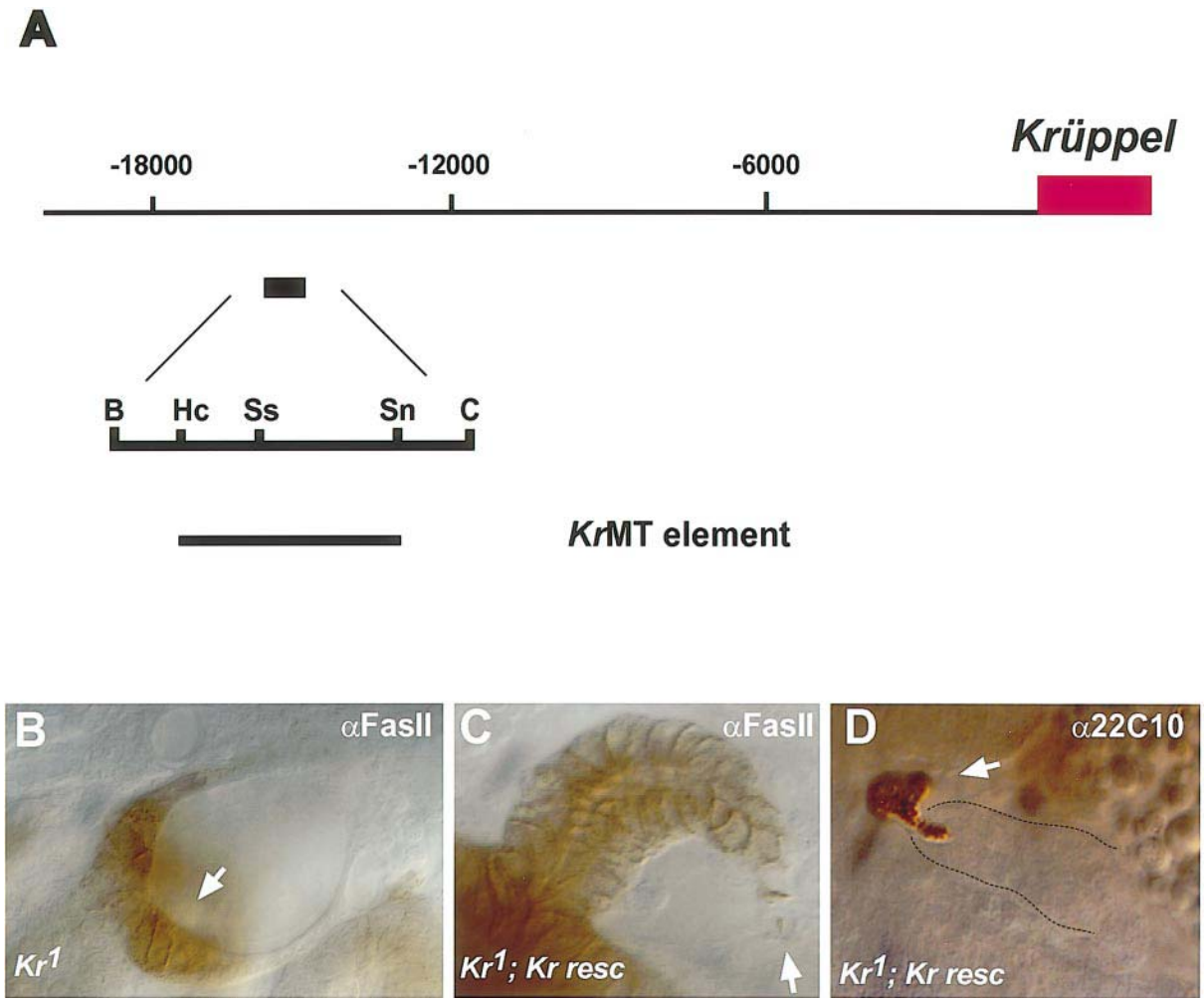

Fig. 2. Identification of the *Kr*MT *cis*-acting element. (**A**) Physical map of the *Kr* upstream region on top. The 650 bp *cis*-acting element is localized ~14 kb upstream of the start site of the *Kr* gene (black box and magnified bar; B, *Bam*HI; Hc, *Hin*cII; Ss, *Ssp*I; Sn, *Sna*BI; C, *Cla*I). The *Kr*MT element (sequence in Figure 4A) is a 420 bp subelement of the 650 bp rescue fragment which mediates reporter gene expression that covers all spatial and temporal aspects of *Kr* expression during MT development, including its restriction to the tip cell (see Figure 3A–E). (**B**) A minirescue construct containing the 650 bp element [see (A)] in front of the *Kr* coding region is capable of completely rescuing the tubule phenotype of Kr^{1} amorphic mutants, as can be visualized by anti-FasII (B, **C**) and anti-22C10 antibody stainings (**D**; arrow marks neural tip cell).

To establish the functional significance of the Fkh and bHLH binding sites *in vivo*, we generated reporter gene constructs under the control of mutant *Kr*MT elements. In these mutant elements single sites and/or combinations of Fkh and bHLH bindings sites were altered in a site-specific manner such that the corresponding proteins were unable to bind *in vitro* (Figure 4B). Mutated *Kr*MT-driven fusion genes were inserted into the germline of flies by P-element-mediated transformation and their expression patterns were analysed in transgenic embryos.

Altering the Fkh-binding site resulted in a strong reduction of reporter gene activation (Figure 4D, compare with C). In contrast, when both E-box binding sites for the proneural factors were eliminated, the initial reporter gene activation in the MT anlage at the posterior pole of the embryo was normal (Figure 4E). However, reporter gene expression was already decreased in the primordium at the eversion stage (Figure 4F) and faded away when the singling out of the tip cell occurred (Figure 4G). These results show that the *Kr*MT element contains functional Fkh and proneural bHLH proteins binding sites which regulate gene expression *in vivo*, implying that *Kr* is a direct target gene of Fkh and of proneural bHLH factors. Fkh binding to the *Kr*MT element is required for the activation of gene expression throughout the MT primordium, while restriction of *Kr* expression involves the proneural bHLH factors in addition, meaning that the proneural bHLH factors cannot activate gene expression on their own. This implies a model in which the restriction of gene expression is achieved by the combined action of Fkh and bHLH in the singled-out cell.

Cell-specific Kr expression by Fkh and proneural bHLH proteins

The above results suggest that the combination of Fkh and proneural bHLH activities is necessary to maintain *Kr* expression in the singled-out tip cell. To test whether Fkh and proneural bHLH proteins are the only components required for the restriction of *Kr* expression and its maintenance in a single cell, we altered the spatial distribution of the bHLH factors by using the *da*-Gal4 driver line that mediates expression in all the tubule cells to activate the expression of various combinations of UAS-bHLH effector genes (Hinz *et al.*, 1994; Giebel *et al.*, 1997),

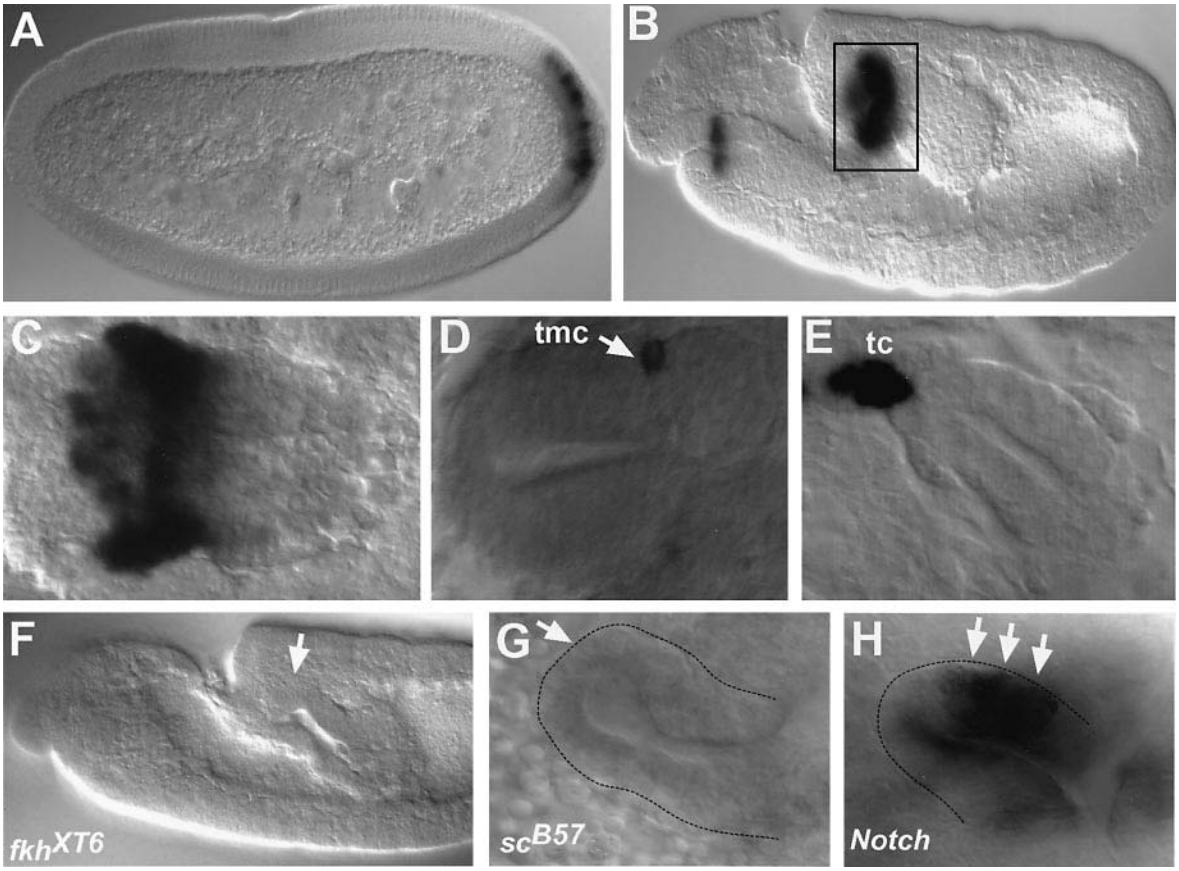

Fig. 3. Reporter gene expression mediated by the *Kr*MT-element. The *Kr*MT element controls reporter gene expression that covers all aspects of *Kr* expression during MT development, including its restriction to the tip cell, as monitored by RNA *in situ* hybridization with a *lacZ* probe (**A–E**). In amorphic fkh^{XT6} embryos (**F**), there is a lack of reporter gene expression (arrow). In proneural sc^{B57} mutants, the reporter gene under the control of the *Kr*MT element is not expressed (**G**, arrow) whereas in zygotic $Notch^{e55}$ mutants (**H**), there is ectopic expression in the tip region (arrows). Top, dorsal; left, anterior. Abbreviations are as for Figure 1.

preventing the Notch-dependent restriction of the bHLH factors. As a consequence, the ectopically expressed bHLH factors become co-expressed with the ubiquitously distributed Fkh in all tubule cells.

No effect was observed when bHLH factors were ectopically expressed in response to one Gal-4 driver transgene. Two copies of the transgene resulted in a stronger bHLH mis-expression (data not shown) and caused weak *Kr*MT fusion gene (Figure 5A and B) as well as endogenous *Kr* gene expression in MT cells (Figure 5C). The increased levels of proneural factors and the induced *Kr* gene expression in all cells of the equivalence group resulted in the formation of additional tip cells at the distal ending of each of the tubules (Figure 5D; Giebel *et al.*, 1997). In addition, multiple cells along the tubules expressed the neuronal marker 22C10, indicating the adoption of neural characteristics by cells that normally develop into epithelial tubule cells (Figure 5D). However, such cells clearly lack the morphological features of tip cells. This indicates that, in the presence of Fkh, the proneural bHLH proteins are able consistently to drive *Kr*MT-mediated reporter gene and *Kr* gene expression in the equivalence group of cells.

The multiple tip-cell phenotype generated in response to ectopic bHLH expression is reminiscent of the *Notch* mutant phenotype (Hoch *et al.*, 1994). This suggests that the repressing activity of the *Notch* pathway, which normally prevents cells of the equivalence group from differentiating into tip cells (Hoch *et al.*, 1994), can be circumvented either by the experimentally raised levels of bHLHs or by lowering functional *Notch* activity within the equivalence group. However, outside the equivalence group the bHLHs have to overcome Emc activity which is low in the equivalence group of cells and absent from the tip cell. The ectopic expression studies with the bHLH factors therefore provide supporting evidence that the combination of Fkh and proneural bHLH factors is able to activate *Kr* expression, provided that bHLH factors are at high enough levels to overcome the activity of an inhibitory component, probably Emc, which antagonizes the selective activity of the bHLH proteins.

Kr activity is sufficient to induce neural fate in the MT

We next asked whether *Kr* activity is a downstream mediator that induces bHLH-dependent neural cell fate or whether it requires the co-expression of bHLH proteins to exert this effect. We therefore allowed for the ectopic expression of *Kr* in all tubule cells using the ubiquitously expressing *da*-Gal4 driver in combination with an UAS-dependent *Kr* effector gene (Figure 5E). As observed with ectopic expression of bHLH, ectopic *Kr* expression in all the tubule cells caused additional tip cells and misexpression of the neural marker 22C10 along the tubules (see

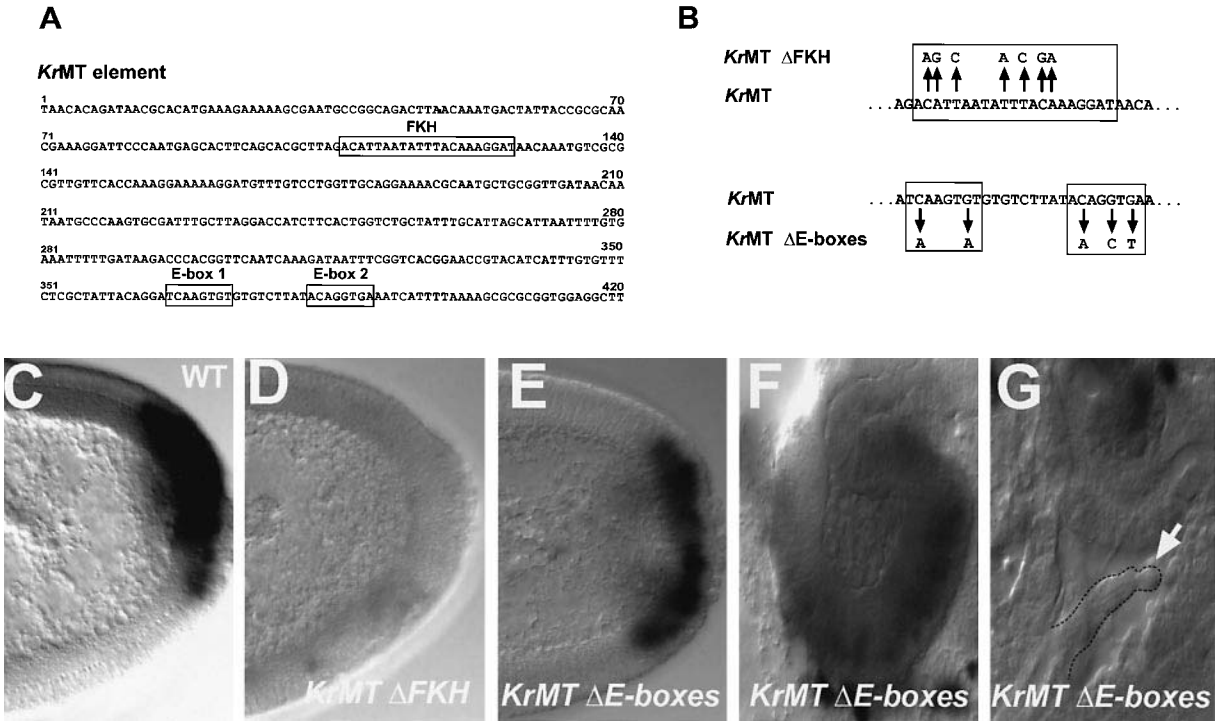

Fig. 4. Fork head and bHLH proteins cooperate within the *Kr*MT element to maintain functional *Kr* expression in the selected cell. (**A**) Sequence of the *Kr*MT element and summary of *in vitro* footprinting experiments (see Materials and methods) showing the location of the Fkh binding site and the two E-box binding sites of the bHLH proteins (open boxes); note that these two sites correspond to sequences shown previously to be bound by bHLH proteins (Murre *et al*., 1989a,b). Fkh binds to one strong site (upper box) in the proximal region of the *Kr*MT element. Homodimers of Asense, Lethal of scute and Achaete (or Scute), and heterodimer combinations between them and Daughterless bind to one strong (E-box 2) and a weaker binding site (E-box 1). (**B**) Site-specific mutagenesis was used to mutate the Fkh binding site (ΔFkh) in the *Kr*MT element (the bases exchanged are shown on top of the wild-type sequence) resulting in the reporter gene constructs *Kr*MT ΔFkh. Similarly, both E-boxes were mutated (the altered bases are shown in the lower part) and the resulting reporter gene construct was designated *Kr*MT ΔE-boxes. (C–G) RNA *in situ* hybridizations with a *lacZ* probe in wild-type embryos carrying reporter genes under the control of the *Kr*MT element (**C**) or mutated versions of it (D–G). (**D**) When Fkh binding is abolished, the reporter gene can no longer be activated in the MT anlage. (**E**) Mutation of the binding sites for the proneural proteins has no effect on early reporter gene expression but during the singling-out process (**F**), the reporter gene cannot be maintained in the selected cell. Consequently, the tip cells (**G**, arrow) do not express the reporter gene.

above; Giebel *et al*., 1997), indicating that *Kr* activity provides neural fate and the capability to induce ectopic tip cells, even in the absence of bHLH proteins which are normally restricted to the single cell of the equivalence group. We next asked whether this neuralizing effect of *Kr* activity is limited to a phenocritical period during MT development. For this, we performed temperature-shift experiments using a heat-shock-inducible *Kr* transgene. Additional tip cells were observed when heat-shock-induced ectopic *Kr* expression occurred between 4–6 h after egg laying (Figure 5F), meaning the time period when the restriction of *Kr* expression occurs.

Kr activity transforms satellite cells into tip cells

The finding of multiple tip cells in response to ectopic *Kr* activity leaves the possibility that *Kr* generates multiple tip mother cells, each giving rise to a satellite and a tip cell. Alternatively, *Kr* could act as a developmental switch gene which transforms satellite into tip cell fate. In order to test this possibility directly, we expressed *Kr* under the control of an *ase*-Gal4 driver in the satellite cell (Figure 1E) which normally loses *Kr* expression and differentiates into an epithelial cell neighbouring the *Kr*-expressing tip cell (Hoch *et al.*, 1994). When *Kr* becomes ectopically expressed in the satellite cell, a second tip cell develops (Figure 6B, compare with A). By morphological means, and based on the expression of the neuronal marker 22C10, this tip cell is indistinguishable from the regular tip cell in each tubule. Thus, in response to *Kr* activity, the satellite cell is transformed into a tip cell. We note, however, that the formation of this second tip cell has no marked effect on the number of tubule cells, indicating that tip cell-dependent control of cell proliferation is not significantly affected by the additional tip cell formed. This suggests then that proliferation control and neural differentiation are not necessarily linked features of tips cells, and that the two functions are under the control of different genetic circuitries that mediate the response to bHLH activity.

Discussion

We used the segregation of the neural tip cell of the MT to study the mechanistics of neural cell fate specification in response to Notch signalling and proneural bHLH activity. The results presented here provide evidence that the *Kr* gene, best known for its function in the early segmentation process of *Drosophila* (Hoch and Jäckle, 1993; Pankratz and Jäckle, 1993), is a direct target of proneural bHLH factors and is required to determine the neural fate of the tip cell within the developing MT. This function involves the restriction of the bHLH to the tip mother cell, a singling-out process through lateral inhibition by *Notch* signalling. The restricted bHLH proteins act in concert with the transcription factor Fkh, present in

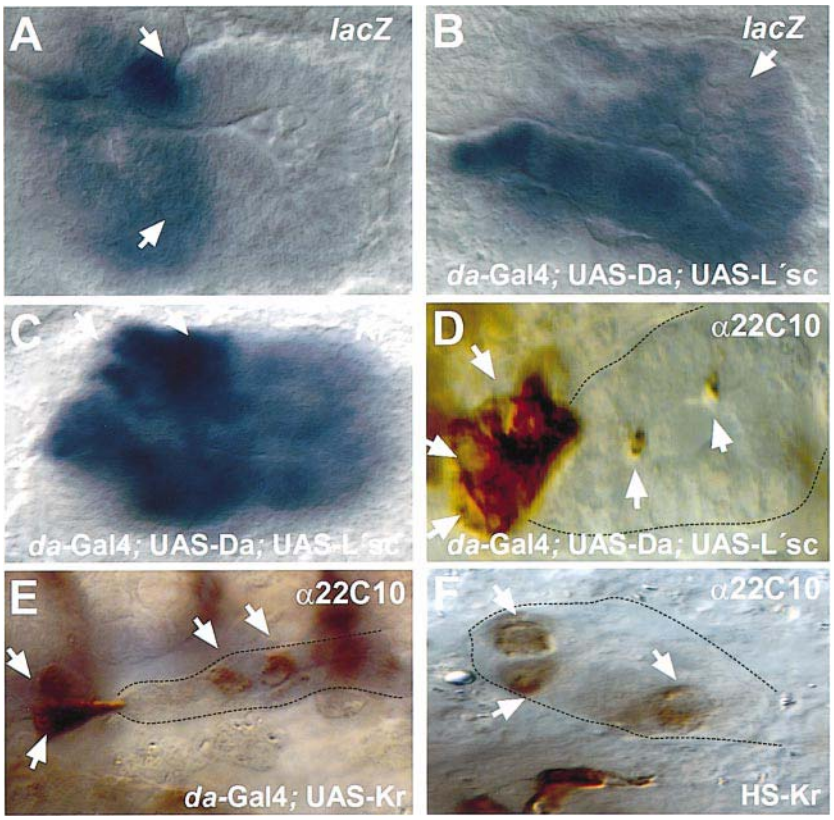

Fig. 5. Ectopic expression of proneural genes or *Kr* by the UAS/Gal4 system (Brand and Perrimon, 1993). (**A**, **B**) Reporter gene expression of the *Kr*MT element during the singling-out process monitored by RNA *in situ* hybridization with a *lacZ* probe. (A) Wild-type. (B–**D**) Embryos bearing two copies of the ubiquitously expressing *da*-Gal4 driver, one copy of UAS-Da, and one copy of UAS-L'sc effector genes. Upon ectopic expression of the bHLH factors, the reporter gene becomes ectopically expressed [compare expression domains marked by arrows in (A) and (B)]. These embryos also show weak ectopic endogenous *Kr* expression which is not properly restricted in the primordium (**C**, arrows). Furthermore, some of these embryos develop multiple tip cells (D; arrows to the left; Giebel *et al*., 1997) and excretory cells along the tubule express the neuronal marker 22C10 ectopically (D; arrows to the right). (**E**) *da*-Gal4; UAS-Kr embryos. Ectopic *Kr* expression in the tubules causes additional tip cells (E; arrows to the left; only one tip cell in focus) and ectopic 22C10 expression in excretory cells (E; arrows to the right). (**F**) Embryos which bear a *Kr* heat-shock transgene (HS-Kr) and which were heat-shocked between 4–6 h develop additional tip cells and strong expression of 22C10 in excretory tubule cells, indicating a neuralization of these cells (arrows).

all MT cells, to either initiate or to maintain *Kr*MT-mediated expression of *Kr* in the tip mother cell. Once this cell has divided asymmetrically, *Kr* continues expression in only one of the daughter cells and acts as a developmental switch gene that causes the neural differentiation of the tip cell as compared with epithelial satellite cell differentiation. It thereby acts upon a cellular state that is predetermined through the expression of proneural bHLH factors.

Kr is a direct target gene of Fkh and bHLH factors

The spatial restriction and the cell fate-determining function of *Kr* depends on a separable 650 bp element within the *cis*-acting control region (Figure 2A). Although this element is sufficient to conduct all spatial and temporal aspects of *Kr* expression *in vivo*, it is not able to provide a full rescue of MT development in otherwise *Kr*-deficient embryos when driving *Kr* expression from two copies of the transgene. The full rescue obtained with four copies (Figure 2C) therefore suggests that the *Kr*MT element lacks sequences for the proper control of the level of *Kr* expression.

The transcription factor Fkh is expressed in the anlagen and subsequently in all the MT cells, thus representing a ubiquitously acting factor during MT development (Weigel *et al.*, 1989a,b; Weigel and Jäckle, 1990; Gaul and Weigel, 1991). Its activity is necessary for the expression of *Kr*. Consistently, *Kr* fails to be properly activated in the MT of Fkh-deficient embryos (Gaul and Weigel, 1991), and a modified *Kr*MT-element which lacks the Fkh-binding site fails to mediate gene activation. However, *Kr* is only initially co-expressed with Fkh in the anlagen and in the primordium of the tubules, whereas during subsequent stages it becomes restricted to the tip mother cell and the tip cell while Fkh remains ubiquitous (Weigel *et al.*, 1989a,b; Hoch *et al.*, 1994). This observation already suggests that the expression of *Kr* depends on at least one additional transcriptional activator during its restriction to the tip cell; this turned out to be the bHLH factors singled out in response to *Notch* activity.

Kr expression in the tip cell is dependent on a direct interaction with proneural bHLH factors. However, these factors are not capable of activating *Kr* on their own and thus fail to function as singular activators of gene expression, as has been described for bHLHs in the yeast system (Cabrera and Alonso, 1991). In mutants for proneural genes (Hoch *et al.*, 1994), or in the absence of binding sites for proneural bHLH proteins on the *Kr*MT-element, no expression is observed at the time when *Kr* expression becomes normally restricted. The combined

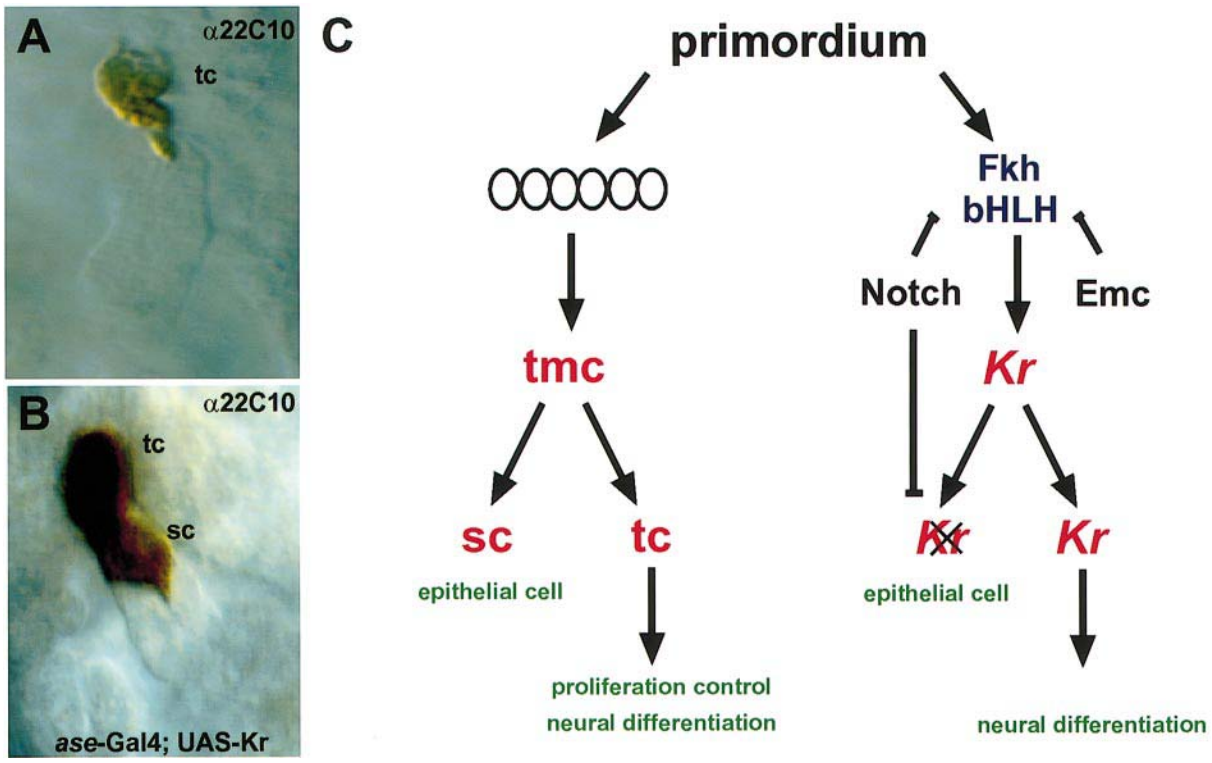

Fig. 6. *Notch*-dependent restriction and maintenance of *Kr* expression in the tip cell in response to Fkh and bHLH factors is required for its neural differentiation. Neural tip cell differentiation, visualized by anti-22C10 staining of (**A**) wild-type tubules or (**B**) tubules in which *Kr* has been ectopically expressed in the satellite cell via the *ase*-Gal4 driver and UAS-Kr effector gene. Upon ectopic *Kr* expression, the satellite cell becomes transformed into a second neural tip cell with a typical stalk projection into the lumen. This second tip cell, however, does not have proliferation control properties (see text). (**C**) Model of how *Notch*-dependent *Kr* restriction and maintenance in the tubule primordium occur in response to combined Fkh and proneural bHLH activities. Fkh which is expressed ubiquitously in MT and proneural bHLH activities are expressed locally in the tip mother cell and the tip cell in response to *Notch* and *Emc*. Note that *Kr* functions as a developmental switch gene distinguishing between neural tip cell and epithelial satellite cell fate and that it promotes neural differentiation.

genetics, the *in vivo* reporter gene expression studies and the results obtained with the ectopic expression of bHLH factors are therefore consistent with a model that predicts that the singling out of *Kr* expression depends on the *Notch*-dependent restriction of one essential component—the bHLH proteins—to continue *Kr* expression in one of the many Fkh-expressing cells, the tip mother cells (Figure 6I). In more general terms, bHLH proteins seem to be required either to reinitiate and/or to maintain the activity of downstream target genes by acting in concert with a more widely distributed transcription factor such as Fkh. These results also suggest the possibility that during peripheral and central nervous system formation, the classical systems where the function of proneural bHLH proteins was identified and found to be restricted by *Notch* signalling (Campos-Ortega, 1993; Artavanis-Tsakonas *et al.*, 1995) may also need a generally distributed factor to activate the target genes required for neural cell fate determination and differentiation. We note that *Kr* might be one of these, as it is expressed in the proneural cluster in the neuroectodermal region and maintained in neuroblasts once they were born (Romani *et al.*, 1996).

The level of *Kr* expression that can be induced outside the equivalence group of MT cells depends on the level of functional bHLH proteins provided. This suggests that these factors have to overcome the antagonizing activity of Emc which is expressed in all non-equivalent cells of the MT (Cubas *et al.*, 1994; Ellis, 1994). The fact that *Kr* could not be induced in all MT cells to the level observed in the singled-out cell might also be due to experimental limitations, meaning that only a subset of these proteins were expressed or that the proper combination of proneural proteins was not provided in these experiments. Alternatively, Emc in combination with bHLH factors other than the proneural bHLHs may negatively regulate *Kr* expression outside the proneural equivalence group of cells.

Krüppel promotes neural fate and transforms satellite into tip cell fate

Ectopic *Kr* expression in all the MT cells causes cells to develop neural characteristics, suggesting a role of *Kr* in the neural cell fate determination. Misexpression experiments in which *Kr* was provided ectopically in all MT cells at various time intervals indicate that *Kr*-dependent promotion of neural cell fate occurs between 4–6 h after egg deposition, at the time when the singling out occurs normally during wild-type development, but the effect was not penetrant in all MT cells. One possible explanation of this phenomenon is that in these cells, Emc is expressed and functions as an antagonist not only of bHLH activity but also as a direct repressor of *Kr* activity. We noted, however, that ectopic Emc does not affect *Kr* expression by itself (M.Hoch, unpublished observation) and thus, it may antagonize *Kr* target gene activities. Although we cannot exclude this possibility, we favour the hypothesis

that *Kr* function requires cells to be in a predetermined state that is provided only during a short phenocritical period. The predetermined state implies that such cells must contain one or several components which facilitate *Kr*-dependent neural development. If these components are limiting or lacking, *Kr* would not be sufficient to induce all aspects of neural differentiation in cells that are normally specified as excretory cells.

In contrast, misexpression of *Kr* in the proneural equivalence group consistently induces neural development and differentiation of these cells into tip cells. In order to demonstrate this transforming capability of *Kr* unambiguously, we have made use of the *ase*-driven Gal4/UAS-*Kr* system specifically to maintain *Kr* expression in the two daughters of the tip mother cell. Instead of developing into an epithelial cell, the satellite cell was found to be transformed into a second tip cell, as indicated by both morphological and molecular criteria. This indicates that *Kr* acts as a developmental switch gene, which distinguishes between satellite and tip cell fates. However, Since ectopic *Kr* expression in other MT cells did not transform them into tip cells, *Kr* does not function as a 'master regulator of tip cell development' but mediates neural development and the binary switch that distinguishes between two alternative cell fates only in cells predetermined by Notch-dependent bHLH factors.

Materials and methods

Fly strains

Oregon R, N^{55e11}, fkh^{XT6}, Kr^{1} (provided by the Tübingen stock centre) and *Df (1) sc*^{B57} (a gift of Juan Modolell, CBM, Madrid) were used in these studies. Flies and embryos were handled according to standard procedures. Mutant chromosomes were scored using blue balancers.

Immunohistochemistry

Antibody staining of whole-mount embryos was done with the Vectastain ABC Elite-horseradish peroxidase system; stained embryos were maintained in capillaries after embedding in Araldite (Hoch *et al.*, 1994). Antibodies were used at the dilutions indicated in parentheses: MAb22C10 (1:20) (Zipursky *et al.*, 1984); MAbfasII (1:20) (Grenningloh *et al*., 1991); MAbachaete (1:50); anti-Krüppel (1:100) (Gaul *et al.*, 1987); anti-β-galactosidase (Cappel; 1:10 000). Antibodies were preabsorbed against wild-type embryos. Whole-mount RNA *in situ* hybridization (*Kr* and *lacZ* probes) was performed as described previously (Tautz and Pfeifle, 1989).

Generation of reporter gene and Kr rescue constructs

To isolate the minimal *cis*-regulatory element that mediates *Kr* expression in the MT domain, various fragments of the *Kr* upstream region were cloned into the polylinker of the pCaSpeR*hs43* vector (Thummel and Pirrotta, 1992). At least three independent transgenic lines were established for each construct; the embryos were assayed for β-galactosidase expression by RNA *in situ* hybridization and antibody stainings. As a result of this analysis, the 650 bp *Bam*HI–*Cla*I fragment, which is located ~14 kb upstream of the *Kr* transcription start site and a subfragment of it, the 415 bp *Hinc*II–*Sna*BI fragment, were found to be the control regions of the *Kr* gene that mediate its expression during all stages of MT development (Figure 2). For the generation of the *Kr* MT rescue construct, the 650 bp *Bam*HI–*Cla*I fragment (Figure 2A) was cloned into the *Kr* SINC vector (Schmucker *et al*., 1992). This vector contains the *Kr* promotor region (up to a *Sal*I site which is located at position 800 from the *Kr* transcription start site), the full open reading frame and 2 kb of downstream genomic sequence. This region is cloned into the Carnegie 20 vector. For P-element-mediated germline transformation, the DNA construct was injected into ry^{506} mutant embryos and several transgenic lines were obtained. For the rescue experiment, a homozygous line in which the P-element was inserted on the third chromosome was crossed with Kr^{1} mutant flies. The progeny flies were crossed between themselves and the resulting embryos were analysed with various markers for MT rescue (Figure 2). With two copies of the 650 bp *Kr* rescue construct, only a partial rescue of the MT phenotype of Kr^{1} mutants could be achieved; full rescue was obtained with four copies. All P-element constructs were integrated into the *Drosophila* germline by P-element-mediated transformation (Rubin and Spradling, 1982). The DNA constructs were injected into *w* sn^{w} or ry^{506} homozygous mutant embryos.

Footprinting experiments and site-specific mutagenesis

DNase I *in vitro* footprinting experiments to localize Fork head and bHLH binding sites in the *Kr*MT DNA were done with bacterially produced proteins using pETFkh (Kaufmann *et al.*, 1994), pETAc, pETL'sc, pETAse (gifts of M.Kunisch and E.Knust) and pETDa (gift of H.Vässin) expression vectors. The preparation of extracts and the footprinting reaction were performed as described previously (Hoch *et al.*, 1991). As a DNA template we used subfragments of the 650 bp *Bam*HI–*Cla*I element (Figure 2A). The various binding sites which were detected for Fork head and the proneural proteins were obtained on both strands in the footprinting reaction. The protected sequences are shown in Figure 4A. Site-specific mutagenesis of the binding sites (as outlined in Figure 3B) in the 415 *Kr* element was done according to standard procedures (Sambrook *et al.*, 1989). Reporter gene expression constructs with the mutated 415 *Kr* elements were generated as described previously.

UAS/Gal4 ectopic expression experiments

For the ectopic expression of the proneural bHLH factors via the Gal4/UAS system, we used the driver line da^{G32}-Gal4 which mediates ubiquitous expression (Wodarz *et al.*, 1995) and the effector lines UAS-Da, UAS-L'sc and UAS-Sc (Hinz *et al.*, 1994). Embryos were collected at 29°C and analysed by RNA *in situ* hybridization or antibody stainings. To generate the UAS-Kr constructs, the *Kr* cDNA was cloned as an RI fragment into the pUAST vector. After P-element-mediated germline transformation, several transformant lines were obtained that were either blue-balanced or made homozygous for the P-element insertion. For the ectopic expression studies with *Kr*, we used da^{G32}-Gal4 or *ase*-Gal4 (a gift of S.Stein) as driver lines. Embryos were collected at 29°C and analysed by RNA *in situ* hybridization or antibody stainings.

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