Development 128, 2699-2710 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV8780

A dual function of *phyllopod* in *Drosophila* external sensory organ development: cell fate specification of sensory organ precursor and its progeny

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Accepted 23 April 2001

SUMMARY

During *Drosophila* external sensory organ development, one sensory organ precursor (SOP) arises from a proneural cluster, and undergoes asymmetrical cell divisions to produce an external sensory (es) organ made up of different types of daughter cells. We show that *phyllopod* (*phyl*), previously identified to be essential for R7 photoreceptor differentiation, is required in two stages of es organ development: the formation of SOP cells and cell fate specification of SOP progeny. Loss-of-function mutations in *phyl* result in failure of SOP formation, which leads to missing bristles in adult flies. At a later stage of es organ development, *phyl* mutations cause the first cell division of the SOP lineage to generate two identical daughters, leading to the fate transformation of neurons and sheath cells to hair cells and socket cells. Conversely, misexpression of *phyl* promotes ectopic SOP formation, and causes opposite fate transformation in SOP daughter cells. Thus, *phyl* functions as a genetic switch in specifying the fate of the SOP cells and their progeny. We further show that *seven in absentia* (*sina*), another gene required for R7 cell fate differentiation, is also involved in es organ development. Genetic interactions among *phyl*, *sina* and *tramtrack* (*ttk*) suggest that *phyl* and *sina* function in bristle development by antagonizing *ttk* activity, and *ttk* acts downstream of *phyl*. It has been shown previously that *Notch* (*N*) mutations induce formation of supernumerary SOP cells, and transformation from hair and socket cells to neurons. We further demonstrate that *phyl* acts epistatically to *N*. *phyl* is expressed specifically in SOP cells and other neural precursors, and its mRNA level is negatively regulated by *N* signaling. Thus, these analyses demonstrate that *phyl* acts downstream of *N* signaling in controlling cell fates in es organ development.

Key words: *phyllopod*, SOP, Cell fate, *Notch*, *seven in absentia*, *tramtrack*, *Drosophila melanogaster*

INTRODUCTION

The *Drosophila* external sensory (es) organ is an excellent model system to study cell fate specification. Its development involves multiple steps of cell fate determination, which are governed by combined effects of intrinsic and extrinsic signals. Eventually, these signals are transduced to the nucleus and integrated to change the state of gene expression that promotes a particular fate (Jan and Jan, 1994). In the formation of an es organ, the first step is expression of proneural genes in a small group of ectodermal cells, known as 'proneural clusters'. *achaete* (*ac*) and *scute* (*sc*) are the proneural genes that promote es organ formation, and both genes encode basic helix-loop-helix (bHLH) transcriptional activators (for reviews, see Ghysen and Dambly-Chaudiere, 1988; Campuzano and Modollel, 1992). Although each cell in the proneural cluster is competent to form a neural precursor, these proneural-cluster cells compete with each other so that a single cell is selected to develop into a sensory organ precursor (SOP). The remaining cells are prevented from adopting the SOP neural fate by the process of lateral inhibition that involves cell-cell interaction (for reviews, see Greenwald, 1998; Artavanis-Tsakonas et al., 1999) mediated by the receptor Notch (N) and its ligand Delta. Upon ligand binding, the membrane receptor N is processed, and the intracellular domain (N^{ICD}) (Schroeter et al., 1998) is released from the membrane to the nucleus (Lecourtois and Schweisguth, 1998; Struhl and Adachi, 1998). N^{ICD} forms a complex with the DNA-binding protein Suppressor of Hairless [Su(H)] to activate transcription of the *Enhancer of split* complex [*E(spl)*-C] (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995). Members of the E(spl)-C down-regulate *ac* and *sc* expression in cooperation with the transcriptional repressor Groucho (Heitzler et al., 1996), thus leading to suppression of the SOP cell fate. In neurogenic mutants that disrupt this lateral inhibition process, supernumerary cells adopt SOP rather than alternative epidermal cell fate.

An adult es organ (bristle) consists of four different daughter cells (neuron, sheath cell, hair cell and socket cell) that are generated through asymmetric division of a SOP cell (Gho and Schweisguth, 1998; Gho et al., 1999; Reddy and Rodrigues, 1999). After the first asymmetric division, the SOP cell produces two secondary precursors, IIa and IIb (see schematic drawing in Fig. 7A). The IIb cell again divides asymmetrically

to generate a precursor IIIb and a glial cell that migrates away soon after, and the IIa cell divides to generate a hair cell and a socket cell (the two outer support cells). Finally, the IIIb precursor divides to produce a neuron and a sheath cell. Proteins encoded by *numb* and *N* play a key role in the binary fate determination. Numb, a membrane-associated protein, localizes to a crescent during mitosis and segregates preferentially to one daughter cell after cell division (Rhyu et al., 1994; Knoblich et al., 1995). In *numb* loss-of-function mutants, cell fate transformation from IIb cells to IIa cells, hair cells to socket cells, and neurons to sheath cells occur (Uemura et al., 1989; Rhyu et al., 1994; Wang et al., 1997). By contrast, misexpression of *numb* results in cell fate transformation that is opposite to the phenotype observed in *numb* mutants (Rhyu et al., 1994). Biochemical and genetic analyses suggest that *numb* controls cell fate at least in part by antagonizing *N* activity (Guo et al., 1996). The asymmetrically segregated Numb protein prevents N activation in the daughter cell that inherits the Numb protein. Activation of *N* in the other daughter cell leads to activation of downstream signaling events that specify a cell fate distinct from that of its sibling. The zinc-finger protein Tramtrack (Ttk) is one of the nuclear targets to receive such N signaling (Guo et al., 1995; Guo et al., 1996). The *ttk* gene encodes two alternatively spliced forms of protein, Ttk69 and Ttk88 (Read and Manley, 1992), and both forms act as transcriptional repressors (Read et al., 1992; Xiong and Montell, 1993). The roles of *ttk* in cell fate determination have been closely examined in the developing eye and peripheral nervous system (PNS). In the eye, loss of *ttk88* function results in formation of supernumerary R7 cells (Xiong and Montell, 1993; Li et al., 1997). In es organ development, *ttk* is expressed in the outer support cells and sheath cells, and loss of *ttk* function results in transformation from IIa to IIb cells and from sheath cells to neurons (Guo et al., 1995). Misexpression of *ttk* causes a phenotype opposite to that of *ttk* mutants in the es organ lineage (Guo et al., 1995), indicating that *ttk* acts as a binary cell fate switch in these asymmetric cell divisions. Genetic analyses show that *ttk* functions downstream of *numb* and *N*, and that Ttk protein levels are positively regulated by *N* (Guo et al., 1996)

phyllopod (*phyl*) was identified as being required for the cell fate determination of photoreceptor cells (Chang et al., 1995; Dickson et al., 1995). In the absence of *phyl*, R1, R6 and R7 cells are transformed into additional cone cells, and misexpression of *phyl* in cone cell precursors transforms these cells into additional R7 cells. During eye development, *phyl* is specifically expressed in R1, R6 and R7 cells, and its transcription is up-regulated by RAS1/MAPK signaling. Yeast two-hybrid and in vitro binding assays revealed that Phyl, Ttk and Seven in absentia (Sina), which is another protein required for fate determination of R7 cells (Carthew and Rubin, 1990), interact directly with each other (Li et al., 1997; Tang et al., 1997). During eye development, misexpression of *phyl* down-regulates the Ttk protein level in a *sina*-dependent manner (Tang et al., 1997). Cell culture experiments also showed that Ttk protein degradation is enhanced when both Phyl and Sina are present, and is dependent on the ubiquitin/proteasome pathway (Li et al., 1997; Tang et al., 1997). Together, these results suggest that Phyl and Sina function together to promote cell fate determination of photoreceptors by targeting Ttk for protein degradation.

In this report, we describe the roles of *phyl* in cell fate specification of SOP and SOP daughter cells in es organ development. We present our characterization of *phyl* loss-of-function and *phyl* misexpression phenotypes in both embryonic and adult es organ development. Also, we show that *phyl* interacts genetically with *sina*, and promotes es organ development by antagonizing *ttk* activity. *phyl* acts epistatically to *N* in cell fate specification of both SOP and its daughter cells. *phyl* is expressed in cells with neural potential (neuroblast, SOP and IIb cells), and its expression is negatively regulated by *N* signaling.

MATERIALS AND METHODS

Drosophila genetics and stocks

The following mutant alleles were used: $phyl^{1}$, $phyl^{2}$, $phyl^{4}$ (Dickson et al., 1995), $phyl^{2245}$ (Chang et al., 1995), $sina^{2}$, $sina^{3}$ (Carthew and Rubin, 1990), ttk^{lell} (Xiong and Montell, 1993), ttk^{osn} (Guo et al., 1995), N^{55e11} and $E(spl)^{b32.2\ gro+}$ (Heitzler et al., 1996). *lacZ* reporter genes, *A101* (Usui and Kimura, 1993) and *ase-lacZ* (Jarman et al., 1993), were used for labeling the SOP cells; *A1(2)29* (Bier et al., 1989) for outer support cells of es organs, and *ac-lacZ* (Gomez-Skarmeta et al., 1995) for the proneural clusters. The *GAL4* lines used were: *sca-GAL4* (Hinz et al., 1994), $GAL4^{109\text{-}68}$ (Frise et al., 1996), *dpp-GAL4* (Masucci et al., 1990; Staehling-Hampton et al., 1994) and *Eq-GAL4* (this study).

To produce *phyl* mutant clones, *yw hs-FLP;* FRT^{42d} $phyl^{2245}$/ FRT^{42d} *y+*, FRT^{42d} *pwn* $phyl^{2}$/FRT^{42d}*; hs-FLP Sb/+* and *yw UAS-FLP; sca-GAL4* FRT^{42d} $phyl^{2245}$/FRT^{42d} *y+* flies were generated. To *generate phyl* mutant clones in N^{ts} flies, females of N^{ts} omb^{p1}/+; FRT^{42d} *p[conD]/+* were crossed with males of FRT^{42d} *pwn* $phyl^{2}$*; hs-FLP Sb/SM5-TM6B*. Larvae at 24-48 hours after egg laying were heat treated at 37°C for 15 minutes twice to induce the mutant clones. Supernumerary microchaetes were induced by incubating the N^{ts} pupae (0-6 hours after puparium formation; APF) at 30°C for 6 hours.

For misexpression of *phyl*, five independent *UAS-phyl* transgenic lines were generated. All lines gave similar phenotypes. *UAS-phyl 1b* and *2a* gave stronger phenotypes and were used for further experiments. *UAS-*N^{ACT} (Doherty et al., 1996) and *UAS-ttk69* (Lai and Li, 1999) were also used in this study.

For heat shock treatment of N^{ts} embryos, embryos collected between 10-15 hours at 17°C were incubated at 32°C for another 5 hours to eliminate the *N* activity, and were then placed at 25°C for another 2.5 hours prior to fixation.

Immunohistology

Embryos were fixed and stained as described previously (Chien et al., 1998). The primary antibodies used were mouse anti-Elav antibody (1:200), rabbit anti-Prospero antibody (1:1000) (Vaessin et al., 1991), rat anti-Cut antibody (1:2000) (Blochlinger et al., 1990), rat anti-Su(H) antibody (1:1000) (Gho et al., 1996), and mouse and rabbit anti-β-galactosidase antibodies (1:250 and 1:1000, respectively). For antibody staining of pupal nota, nota were dissected in 1× PBS and fixed in 4% paraformaldehyde. After washing with 1× PBS, nota were then incubated with primary antibodies: mouse anti-Elav antibody (1:50), rabbit anti-Prospero antibody (1:200) and rat anti-Cut antibody (1:1000), followed by Cy3, Cy5, Alexa-488, or FITC-conjugated secondary antibodies.

X-gal staining

Imaginal disks and pupal nota were dissected in 1× PBS, and fixed in 0.3% glutaraldehyde solution (in 1× PBS) for 10 minutes. Disks and nota were washed in PBS for 15-30 minutes before staining in 0.3% X-gal in Fe/NaP solution.

In situ RNA hybridization

The procedure used for in situ hybridization has been described by Tautz and Pfeifle (Tautz and Pfeifle, 1989). Digoxigenin-labelled RNA probes of *phyl* and *lacZ* were prepared as described previously (Lehmann and Tautz, 1994).

RESULTS

Bristle phenotypes in *phyl* mutants

Previous reports (Chang et al., 1995; Dickson et al., 1995) have described *phyl* mutations causing defective sensory bristles in adult flies. However, no detailed analyses have been performed. We examined the phenotypes in adults and embryos homozygous or transheterozygous for several loss-of-function *phyl* alleles, $phyl^{1}$, $phyl^{2}$, $phyl^{4}$ (Dickson et al., 1995) and $phyl^{2245}$ (Chang et al., 1995). According to their mutant phenotypes in adult bristles and the embryonic PNS (see below), $phyl^{1}$, $phyl^{2}$ have been classified as strong loss-of-function alleles, $phyl^{2245}$ as a slightly weaker allele, and $phyl^{4}$ as a weak hypomorphic allele. $phyl^{1}$, $phyl^{2}$, and $phyl^{2245}$ alleles were found to be lethal as homozygotes or in heteroallelic combinations. However, the $phyl^{1}/phyl^{4}$, $phyl^{2}/phyl^{4}$ and $phyl^{2245}/phyl^{4}$ animals were semiviable, and the surviving adults exhibited two distinct classes of defects on sensory bristles. The first class of defects was missing bristles. For example, approximately 78% of the notal and 87% of the abdominal microchaetes were missing in $phyl^{1}/phyl^{4}$ flies (Fig. 1B,D; Table 1). The second class of defect associated with *phyl* mutations was abnormal configuration of the bristles (arrows in Fig. 1B,D; Table 1). The most prominent phenotype of this class on the notum was duplicated bristles (e.g., 45% in $phyl^{1}/phyl^{4}$ animals), with two shafts emerging from either two sockets or a fused socket. Other phenotypes included abnormal bristles with 1 shaft surrounded by 2-3 sockets, and 2- or 4-socket clusters without shafts. In a weaker *phyl* mutant ($phyl^{2245}/phyl^{4}$), the missing bristle phenotype was less prominent, but the number of abnormal bristles was increased (data not shown). Similar phenotypes (missing and abnormal bristles) were also observed in $phyl^{2245}$ and $phyl^{2}$ mutant clones (data not shown and Fig. 5D, respectively).

phyl is required for specification of SOP cell fate

The missing bristle phenotype in *phyl* mutants might result from failure of SOP formation. During SOP formation, the first step is the expression of proneural genes in the proneural clusters. Thus, we examined *achaete-lacZ* (*ac-lacZ*) expression in wild-type and *phyl* mutant nota at 14-18 hours APF. In the wild-type notum, *ac-lacZ* was expressed in a regular array of cells, which were the precursors of adult microchaetes (Fig. 1E). In the *phyl* mutant, the *ac-lacZ* transgene was also expressed in a pattern analogous to that of the wild type (Fig. 1F). These data indicate that the missing bristle phenotype caused by *phyl* mutations does not result from a failure of proneural gene expression.

Closer examination of *phyl* mutants indicated that at 14-18 hours APF, *ac-lacZ* expression remained in clusters of cells (Fig. 1F, inset), while in the wild type animal at the same stage, *lacZ* expression was restricted to single cells (Fig. 1E, inset). This observation suggests that *phyl* may function at a later stage than proneural genes to promote SOP formation. We tested this possibility by examining the SOP markers. The enhancer trap line *A101* drives *lacZ* expression in SOP cells and is an early marker for SOP cell fate (Huang et al., 1991) (Fig. 1G). In *phyl* mutants, most of the *A101* expression was eliminated (Fig. 1H). We also examined the expression of another SOP marker, *asense-lacZ* (*ase-lacZ*). *asense* encodes a bHLH protein that is specifically expressed in SOP cells (Brand et al., 1993), and the *ase-lacZ* transgene was detected

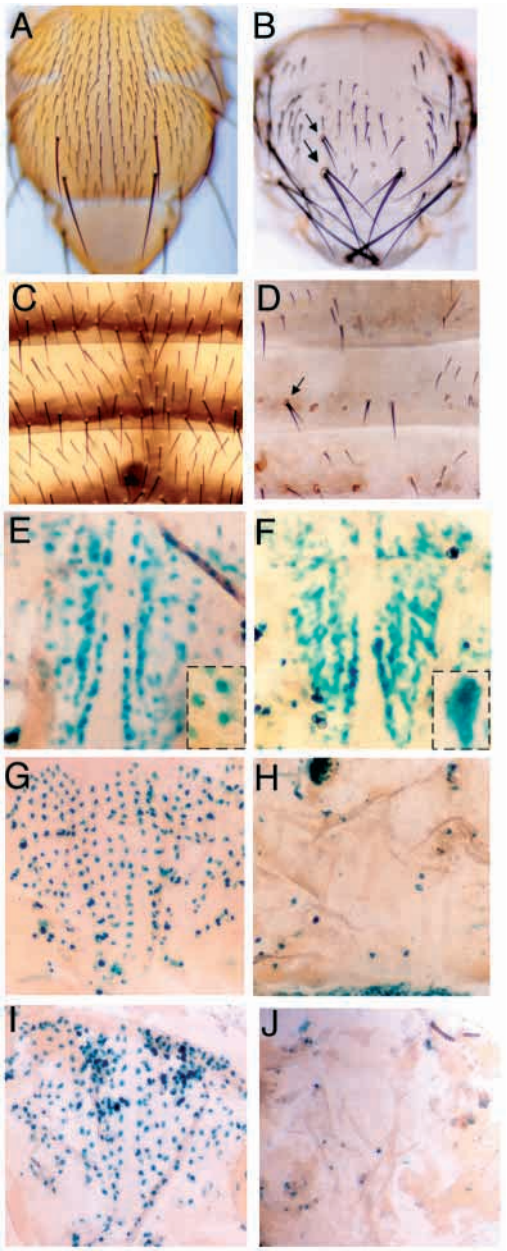

Fig. 1. Two classes of bristle phenotypes in *phyl* mutant adults. (A,C,E,G,I) Wild types. (B,D) $phyl^{1}/phyl^{4}$ and (F,H,J) $phyl^{2245}/phyl^{4}$ mutants. (A,B) Adult nota. (C,D) Adult abdominal segment 2-4. Arrows in B and D mark the abnormal bristles. (E-J) X-gal staining of nota dissected from *ac-lacZ* (E,F), *A101* (G,H), and *ase-lacZ* (I,J) pupae at 14-18 hour APF (for *ac-lacZ* and *A101*) or 18-22 hour APF (for *ase-lacZ*).

Table 1. Adult phenotypes of *phyl* and *sina* mutants, and their genetic interactions with *ttk* in bristle development

		+/+ (*n*=8)	$sina^2/sina^3$ (*n*=16)	$phyl^{2245}/+$; $sina^2/sina^3$ (*n*=14)	$phyl^2/+$; $sina^2/sina^3$ (*n*=19)	$sina^2$ ttk^{osn}/ $sina^3/+$ (*n*=7)	$phyl^1/phyl^4$ (*n*=16)	$phyl^2/phyl^4$ (*n*=11)	$phyl^2/phyl^4$; $ttk^{lell}/+$ (*n*=15)	$phyl^2/phyl^4$; $ttk^{osn}/+$ (*n*=14)
Number of bristles	notum*	260±22	154±14	123±12	134±11	156±16	56±8.7	63±9	157±15	117±21
	abdomen‡	145±11	56±10	39±8	27±7	163±21	19±7	31±13	59±6	99±10
1 hair/ 1 socket	notum*	100%	82.5±5.2%	61.9±5.6%	58.9±5.9%	99.7±0.4%	25±8.7%	30.7±9%	83.3±7.4%	75.4±9.5%
	abdomen‡	100%	33.0±6.1%	31.4±10.9%	28.8±14%	51.8±5.7%	79±18%	81±11%	43.1±9.9%	55.8±11.1%
2 hairs/ 2 sockets	notum*	0%	17.2±5.1%	37.5±5.4%	40.7±6.0%	0.3±0.4%	45±9%	58±8.5%	16.5±7.3%	24.5±9.5%
	abdomen‡	0%	44.5±5.4%	41.8±8.0%	34.6±12.5%	44.1±6.5%	1±2%	6.7±7%	29.0±8.0%	27.2±8.2%
Others§	notum*	0%	0%	0%	0%	0%	28±8.6%	9.2±4%	0%	0%
	abdomen‡	0%	20.9±7.8%	23.0±10.7%	34.6±8.7%	3.6±2.0%	20±17.5%	11.6±9%	26.9±7.8%	15.1±11.1%

*Only the microchaetes on female flies were scored.
‡Only the microchaetes on female abdominal segments 3 and 4 were scored.
§Including bristles with 1 hair/2-3 sockets, 2 sockets and 4 sockets.

at a later stage than *A101* (see Fig. 1I and Figure legends). In *phyl* mutants, most of the X-gal staining was absent (Fig. 1J). We observed similar results for macrochaetal SOP cells in the wing disks in which staining for *A101* disappeared in *phyl* mutants (data not shown). Anti-Cut antibody can recognize all daughter cells of the es organ (Blochlinger et al., 1990). Consistently, only a few Cut-positive clusters were seen in pupal nota of *phyl* mutants (data not shown). These results indicate that *phyl* is essential for the fate specification of SOP cells.

phyl is required for binary cell fate specification in the es organ lineage

In *phyl* mutants, some SOP cells formed and developed into bristles with abnormal compositions (Fig. 1B,D; Table 1), which was likely due to fate transformation of the sibling cells within the es organ lineage. To determine whether *phyl* is required for the binary cell fate decisions in the es organ lineage, we examined the composition of es organs in *phyl* mutants during embryonic development. In *phyl* mutant embryos, the numbers of the sensory neurons and the sheath cells, recognized by anti-Elav and anti-Prospero antibodies, respectively, were significantly reduced (compare Fig. 2B with Fig. 2A). In strong *phyl* mutants ($phyl^1/phyl^2$), 75% of the neurons and 50% of the sheath cells were missing. We focused subsequent studies on two es organs in the dorsal-most (dm) region of abdominal hemisegments (Fig. 2C,E) since they are well separated from the rest of the sensory organs, thus making it easier to identify their constituent cells. In strong loss-of-function $phyl^{2245}/phyl^{2245}$ and $phyl^1/phyl^2$ mutant embryos, more than 80% of the neurons and sheath cells in the dm region were missing (Fig. 2D). However, anti-Cut antibody staining revealed that only 12% of the es organs at the dm region were absent (Cut-negative) (Fig. 2F), which might have resulted from failure in SOP formation. These results suggest that in *phyl* mutant embryos, most of the SOP cells of the dm es

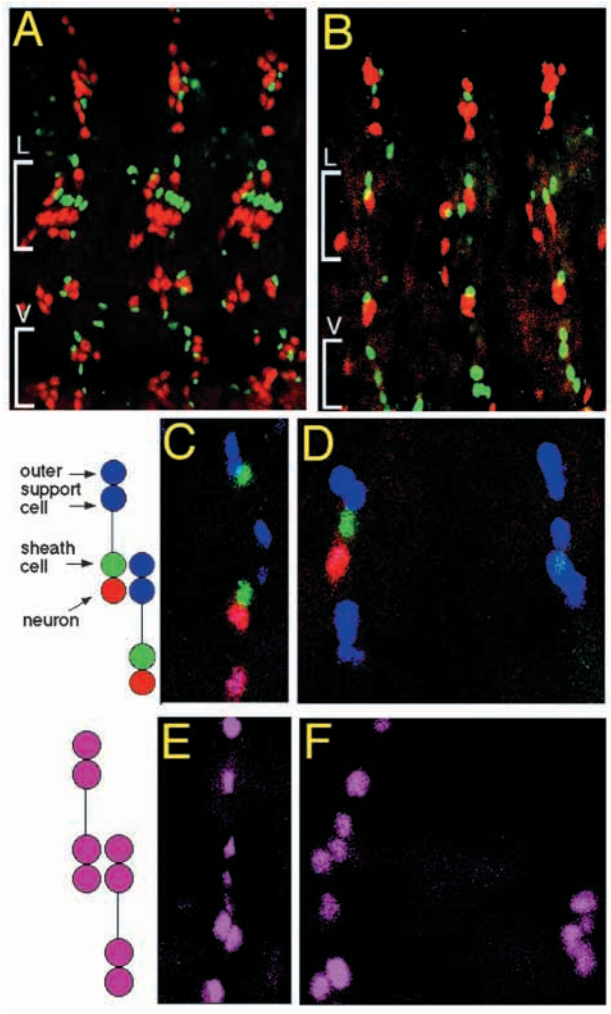

Fig. 2. Transformation from neurons and sheath cells to outer support cells in embryos lacking *phyl*. In this and all the following figures, neurons are stained with anti-Elav antibody (red), sheath cells are stained with anti-Prospero antibody (green), and cells of es organs are stained with anti-Cut antibody (purple). (A,B) Abdominal hemisegments of the PNS in wild type (A), and $phyl^1/phyl^2$ (B) embryos. L, lateral region and V, ventral region of the PNS. (C) In the *A1(2)29* embryo, the dm region of each abdominal hemisegment contains four outer support cells (blue), two neurons (red), and two sheath cells (green). In total, there are eight Cut-positive cells for these two es organs (E). (D) In $phyl^{2245}/phyl^{2245}$ embryos, six outer support cells with one neuron and one sheath cell (left), or eight outer support cells (right) were most frequently observed. (F) In most cases, eight Cut-positive cells are still present (left hemisegment) in $phyl^{2245}/phyl^{2245}$. Occasionally, four Cut-positive cells were observed (right). Schematic drawings of the two dm es organs are on the left.

organs form and divide to produce four Cut-positive cells, and that the large number of missing neurons and sheath cells is not due to failure of SOP formation.

We next examined whether the absence of neurons and sheath cells in *phyl* mutants is the result of changes in cell identity. An enhancer trap line, *A1(2)29*, was used to label the outer support cells (hair cells and socket cells). In wild-type embryos, the two es organs in the dm region contained four outer support cells (Fig. 2C). In *phyl* mutants, most hemisegments contained more than four outer support cells; as shown in Fig. 2D, there were six outer support cells accompanied by one neuron and one sheath cell in the left hemisegment, and eight outer support cells in the right hemisegment. These results suggest that the neurons and the sheath cells were transformed into outer support cells in *phyl* mutants. We further examined whether *phyl* was required for binary cell fate specification between neurons and sheath cells, and between hair cells and socket cells. For the dm es organs, cell fate transformation from hair cells to socket cells [recognized by the anti-Su(H) antibody] occurred in less than 5%, and transformation from neurons to sheath cells was rarely seen (data not shown).

In *phyl* mutant adults, most SOP cells of notal bristles were missing, while in *phyl* mutant embryos, the majority of SOP cells formed in the dm es organs. One likely reason for this difference is the masking of the embryonic SOP phenotype by maternally deposited *phyl* transcripts. When both maternal and zygotic *phyl* mRNA were removed, however, the defects were similar to the zygotic mutant phenotypes; 75% of the neurons and 60% of the sheath cells in the abdominal hemisegments, and 10% of the dm es organs were missing (data not shown). Furthermore, in $phyl^{1}/phyl^{2}$ mutants, the staining pattern with anti-Cut antibody showed that, in addition to the dm es organs, most es organs of the embryonic PNS formed (data not shown). These results suggest that *phyl* is partly required for the fate specification of SOP cells in embryonic es organ development.

Misexpression of *phyl* promotes ectopic SOP formation and transforms outer support cells to neurons and sheath cells

In *phyl* mutant adults, the phenotype of missing bristles is due to failure in SOP formation. To test whether misexpression of *phyl* can promote SOP formation, we used *Eq-GAL4* to drive *phyl* expression in the notum. *Eq-GAL4* was expressed in the anterior of the presumptive notum in the wing disk (Fig. 3A), and of the pupal notum (6-8 hours APF) with stronger expression in the anterior midline region (indicated by arrows).

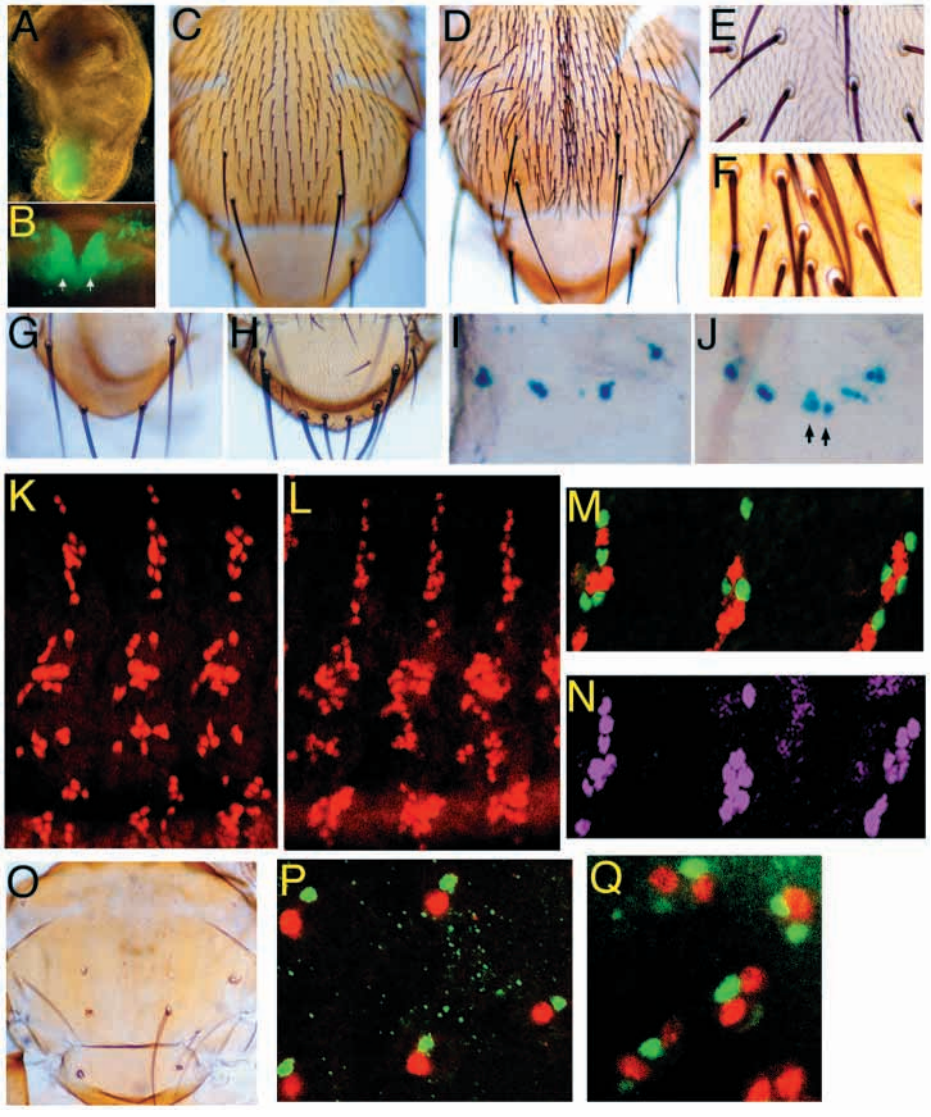

Fig. 3. Misexpression phenotypes of *phyl*. (A,B) Expression pattern of *UAS-GFP/+; Eq-GAL4/+*. (A) The wing disk at 3 hour APF. Anterior is to the left. (B) The developing notum at 7 hour APF. At this stage, the notal regions of the two wing disks are attaching to each other to form a complete notum. The arrows indicate the future midline region and anterior is to the top. (C) Wild type notum, and (E) its midline region. (D,F) In the *UAS-phyl/+; Eq-GAL4/+* notum, the density of microchaetes increases (D), and is highest in the midline region (F). (G) Wild-type and (H) *UAS-phyl/+; dpp-GAL4/+* adult scutella. (I,J) X-gal staining of *A101* in wild-type (I) and *UAS-phyl/+; dpp-GAL4/+* (J) pupal scutella. Ectopic SOP cells are indicated by arrows in (J). (K,L) Confocal images of the abdominal hemisegments in embryos stained with anti-Elav antibody. (K) Wild-type and (L) *sca-GAL4/UAS-phyl*. (M,N) Confocal images of the abdominal dm region of a *sca-GAL4/ UAS-phyl* embryo to reveal the numbers of neurons (red) and sheath cells (green) (M), and the total numbers of es organ progeny (N). (O,Q) In $GAL4^{109-68}$/*UAS-phyl* adults, the hair cells and socket cells of the microchaetes are missing (O), and are transformed into neurons (red) and sheath cells (green) (Q). (P) Clusters of one neuron and one sheath cell were seen in wild-type pupal notum.

When the *phyl* expression was driven by *Eq-GAL4*, we observed ectopic bristles in the notum (Fig. 3D). In general, there was a 30%-70% increase in the number of notal microchaetes, with a highest density in the midline region (Fig. 3F). When *dpp-GAL4* was used to misexpress *phyl*, ectopic bristles were also observed on the scutellum (Fig. 3H) and the third wing vein (data not shown). These ectopic bristles were derived from ectopic SOP cells, as was evident from the appearance of ectopic β-gal-positive cells in the *A101* scutellum (Fig. 3J, arrows). These data indicate that misexpression of *phyl* can promote ectopic SOP formation.

To determine whether *phyl* indeed functions as a genetic switch for the binary cell fate specification of SOP daughters, we examined the phenotypes of *phyl* misexpression in sensory organ lineage by two *GAL4* lines, $GAL4^{109-68}$ (Frise et al., 1996) and *sca-GAL4* (Hinz et al., 1994). When *phyl* was misexpressed by *sca-GAL4* in embryonic PNS, supernumerary sensory neurons were generated (Fig. 3L). This increase in the number of neurons (approximately 50% increase) was due to fate transformation of outer support cells. As shown in Fig. 3M, in the dm region, two out of the three hemisegments had four neurons and four sheath cells, and the third hemisegment had 5 neurons and three sheath cells. Since only eight Cut-positive cells were observed in each dm region (Fig. 3N), these results indicate that the extra neurons and sheath cells are derived from transformation of IIa cells to IIb cells in both es organs.

A similar phenotype of cell fate transformation was also observed in adult bristles. As shown in Fig. 3O, the outer hair and socket structures of microchaetes were all missing when *phyl* was misexpressed by $GAL4^{109-68}$. Pupal nota stained with anti-Elav and anti-Prospero antibodies showed clusters of two neurons and two sheath cells (Fig. 3Q), in contrast to wild-type es organs with clusters of one neuron and one sheath cell (Fig. 3P). Clusters of 3-4 neurons with 0-2 sheath cells were occasionally observed (data not shown). Therefore, misexpression of *phyl* in both embryonic and pupal es organ lineages most often resulted in transformation of outer support cells (hair cells and socket cells) to internal cells (neurons and sheath cells). Together with the *phyl* loss-of-function mutant phenotypes, these data indicate that *phyl* functions as a binary cell fate determinant in the formation of IIa and IIb cells in es organ development.

sina is involved in es organ development

During R7 photoreceptor differentiation, Sina forms a complex with Phyl to promote R7 cell fate by targeting the transcriptional repressor Ttk for degradation (Li et al., 1997; Tang et al., 1997). We found that *sina* is also involved in es organ development. Similar to *phyl* mutants, two distinct bristle phenotypes were observed in *sina* loss-of-function mutants (Fig. 4B,H and Table 1). In $sina^2/sina^3$ adults, 41% of the notal and 62% of the abdominal microchaetes were missing. Also, some of the remaining microchaetes showed abnormal phenotypes such as duplicated bristles (17% in the notum and 65% in the abdomen). To determine whether formation of SOP

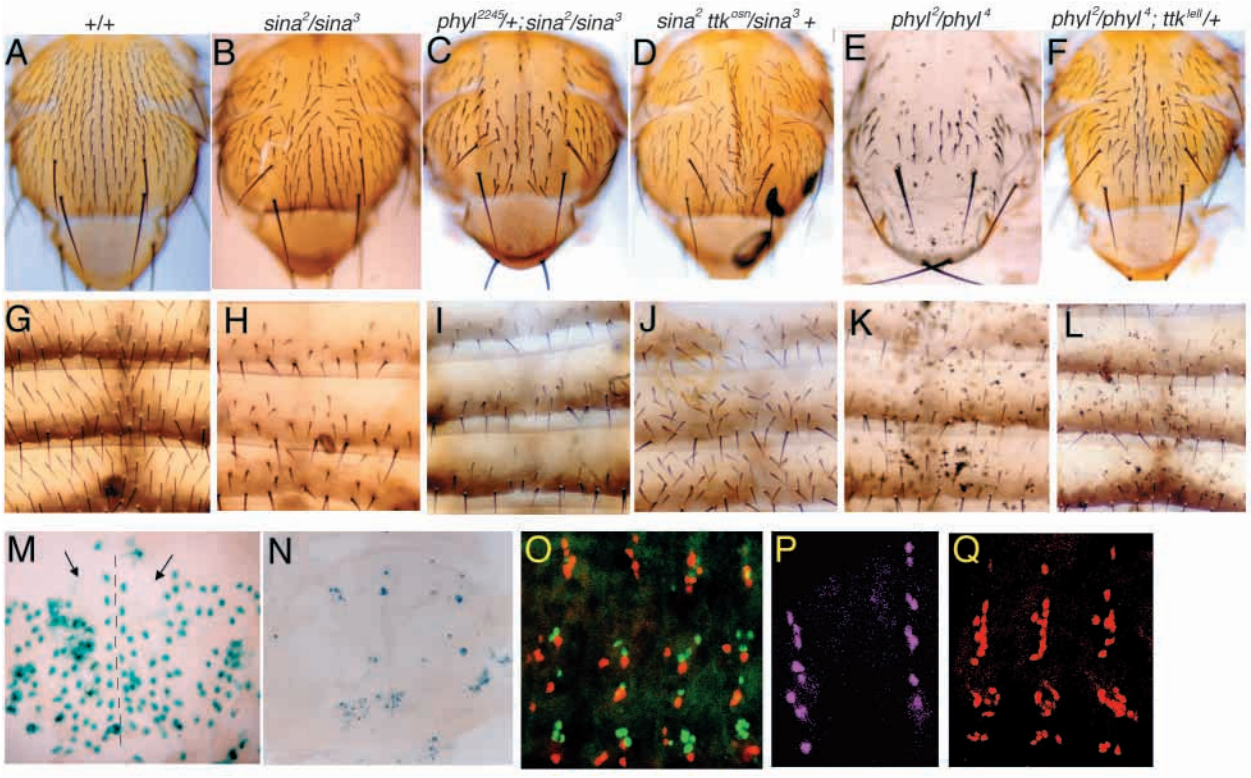

Fig. 4. *phyl*, *sina* and *ttk* interact genetically in es organ development. (A-F) Adult nota. (G-L) Adult abdominal segment 2-4. The genotypes of A-L are indicated above. (M,N) X-gal staining to show expression of *A101* (M) and *ase-lacZ* (N) in $sina^2/sina^3$ pupal nota. In M, the dashed line marks the midline, and arrows mark the region where *A101*-positive cells were missing. (O,P) *sina* mutant embryos that lack both maternal and zygotic *sina* transcripts show strong reduction in the number of neurons (red) and sheath cells (green) (O), but most of the dm region still contains eight Cut-positive cells (purple) (P). (Q) Anti-Elav staining of a $phyl^1/phyl^2$; $ttk^{osn}/+$ embryo. The ventral region was out of focus.

cells is affected in *sina* mutants, we examined the expression pattern of *A101* and *ase-lacZ* in $sina^2/sina^3$ pupae. Compared to wild-type pattern (see Fig. 1G), in $sina^2/sina^3$ mutants, some *A101*-positive cells were missing near the anterior midline region (arrows in Fig. 4M) where the microchaetes were affected most in mutant adults (Fig. 4B). This result demonstrates that *sina* is required for the formation of some microchaetal SOP cells. To our surprise, *ase-lacZ* expression in *sina* mutants was mostly eliminated (Fig. 4N), despite the fact that only a fraction of bristles in $sina^2/sina^3$ adults are missing. Since *ase* is not essential for bristle formation (Jarman et al., 1993), the missing *ase* expression does not necessarily correlate with the missing bristles. Nevertheless, these data indicate that *sina* is important for *ase* expression in most SOP cells.

We next examined the functions of *sina* in embryonic es organ development. In $sina^2/sina^3$ mutants, embryonic development (including the development of es organs) was normal (data not shown), and the mutants survived to adulthood. However, when both the maternal and zygotic *sina* transcripts were removed, embryonic development was affected to different degrees. While some embryos showed obvious defects in their overall morphology, others exhibited no defects. In embryos with normal morphology but severe defects in the PNS, approximately 75% of the neurons and 60% of the sheath cells in the abdominal hemisegments were missing (Fig. 4O). In the dm region, 90% of the es organs were still present (Fig. 4P), suggesting that *sina* was only partly required for the formation of embryonic dm SOP cells, and the reduction in the number of neurons and sheath cells might result from cell fate transformation. Indeed, we found an increase in the number of outer support cells in the *A1(2)29* enhancer trap line (data not shown) in these *sina* mutant embryos. These data indicate that *sina* is required for the cell fate determination of IIb cells in the embryonic es organ lineage.

phyl, *sina* and *ttk* interact genetically in es organ development

Because *phyl* and *sina* mutations cause similar phenotypes, and *ttk* is also essential for the binary cell fate decision of SOP progeny, we explored the possibility that *phyl*, *sina* and *ttk* function in the same genetic pathway in es organ development. We first examined their genetic interactions in adult bristle development. When one copy of $phyl^{2245}$ or $phyl^2$ alleles was introduced into the $sina^2/sina^3$ mutants, the number of microchaetes was reduced on both nota and abdomens. Also, the percentage of abnormal notal microchaetes was increased more than twofold (Fig. 4C,I; Table 1), indicating that *phyl* mutations dominantly enhanced the *sina* mutant phenotypes. We then tested whether *ttk* mutations suppress *sina* and *phyl* mutant phenotypes. We used two *ttk* mutant alleles: ttk^{lell}, a null allele (Xiong and Montell, 1993), and ttk^{osn}, in which the 69 kDa protein expression is absent and the level of the 88 kDa Ttk protein is reduced (Guo et al., 1995). When one copy of ttk^{lell} or ttk^{osn} was introduced into $phyl^2/phyl^4$ mutants, the numbers of both notal and abdominal bristles were significantly increased, and the percentage of abnormal notal bristles was largely reduced (Fig. 4F,L; Table 1). A similar suppression effect was also observed when one copy of the ttk^{osn} allele was introduced into $sina^2/sina^3$ mutants (Fig. 4D,J; Table 1). Therefore, our genetic analyses are most consistent with the notion that *phyl*, *sina* and *ttk* function in the same genetic pathway, and *phyl* and *sina* promote bristle development by antagonizing *ttk* activity. One exception is that removing one copy of *ttk* enhanced the abnormal bristle phenotype in *phyl* mutant abdomen (Fig. 4L and Table 1).

In embryonic PNS, *phyl* also showed strong genetic interaction with *ttk*. In $phyl^1/phyl^2$ mutant embryos, 75% of the neurons in abdominal hemisegment and all of the dm neurons were missing (see Fig. 2B). However, in $phyl^1/phyl^2$; $ttk^{osn}/+$ embryos, only 10% of the PNS neurons were absent and each dm region contained at least one neuron (Fig. 4Q). This result shows that the *ttk* mutation strongly suppresses the *phyl* mutant phenotypes in embryonic PNS, and indicates that *phyl* promotes cell fate specification of embryonic PNS neurons by antagonizing *ttk* activity.

phyl acts upstream of *ttk* and downstream of *N* in es organ development

Our genetic analyses suggest that *phyl* and *ttk* function in the same genetic pathway in es organ development. In embryonic development, lack of *ttk* activity causes fate transformation in sensory organ lineages that leads to overproduction of sensory neurons (Fig. 5H). This phenotype is in contrast to that observed in *phyl* mutant embryos (see Fig. 2B). In embryos lacking both *phyl* and *ttk* activity, we observed the formation of supernumerary sensory neurons (Fig. 5I) that was indistinguishable from that of the *ttk* mutant embryos. This result suggests that *ttk* functions epistatically to *phyl*.

In adults, most microchaetes were missing when *ttk* was misexpressed by *Eq-GAL4* (Fig. 5A). The lack of microchaetes was the result of failure of SOP formation as most *A101*-positive cells disappeared (Fig. 5C). When *phyl* and *ttk* were coexpressed by *Eq-GAL4*, most notal microchaetes were missing (Fig. 5B), suggesting that misexpression of *ttk* can suppress the ectopic bristle phenotype caused by misexpression of *phyl*. Therefore, these results are consistent with the model that *ttk* acts downstream of *phyl* in the formation of SOP cells.

In es organ development, *N* is required to single out the SOP cells and to specify the cell fate of hair cells and socket cells. In *N* mutants, many supernumerary SOP cells are formed and hair cells and socket cells are transformed into neurons (Hartenstein and Posakony, 1990). Since *phyl* mutant phenotypes are opposite to those of *N* mutants, and both genes acts upstream of *ttk* in es organ development (Fig. 5 and Guo et al., 1996), we were interested to examine their epistatic relationship. In the strong loss-of-function $phyl^2$ mutant clones, most bristles were missing and the few remaining ones had a four-socket phenotype (Fig. 5D). In contrast, when the activity of *N* was eliminated during the stage of SOP emergence (6-12 hours APF) by using the N^{ts} allele, the number of microchaetes was significantly increased (Fig. 5E). When a $phyl^2$ mutant clone was generated in the N^{ts} fly with *N* activity inactivated, most of the bristles were lost within the clone (Fig. 5F). This result shows that *phyl* is epistatic to *N* in SOP cell fate specification. We also tested whether *phyl* acts epistatically to *N* in cell fate specification of SOP progeny in embryos. In the embryos from the cross of N^{ts}/N^{ts}; $phyl^2/+$ females with N^{ts}/Y; $phyl^1/+$ males, a quarter of them were N^{ts} and *phyl* double mutants and the rest were N^{ts} mutants. When

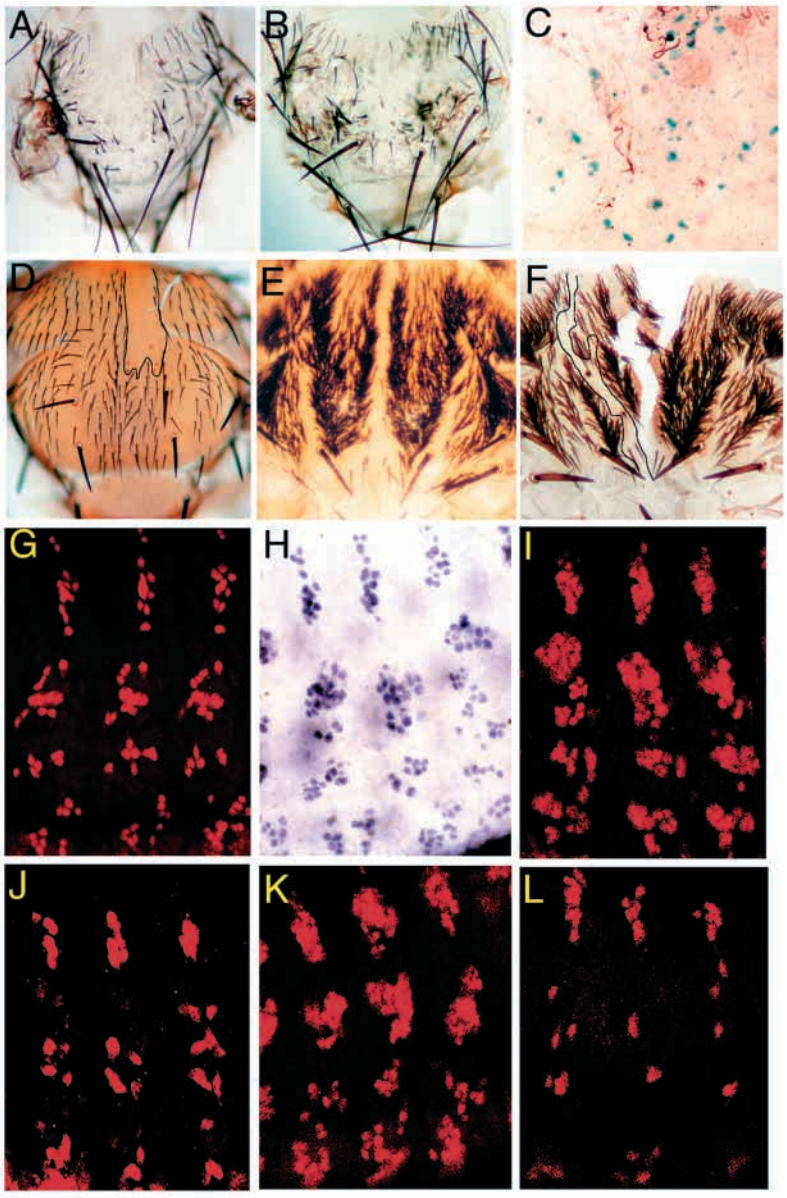

Fig. 5. *phyl* acts upstream of *ttk* and downstream of *N*. (A,B) Adult nota of *Eq-GAL4/UAS-ttk69* (A) and *UAS-phyl/+*; *Eq-GAL4/UAS-ttk69* (B). (C) *A101* expression in *Eq-GAL4/UAS-ttk69* pupal notum. Most of *A101*-positive cells were missing. (D) Adult notum with a $phyl^2$ mutant clone (clone boundary is indicated by black lines). (E) Notum of N^{ts} flies. (F) Adult notum of N^{ts} with a $phyl^2$ mutant clone (clone boundary is marked with black lines). Most bristles in the mutant clone were missing. (G-L) Anti-Elav staining of the embryonic abdominal hemisegments. (G) Wild type, (H) ttk^{osn}/ttk^{osn} mutant, (I) $phyl^1/phyl^2$; ttk^{osn}/ttk^{osn} double mutant. Neuron overproduction was observed in both mutant embryos (H,I). (J) N^{ts}; $phyl^1/phyl^2$ double mutant at 17°C. (K) N^{ts} mutant at 32°C. (L) N^{ts}; $phyl^1/phyl^2$ double mutant at 32°C.

these embryos were incubated at the permissive temperature (17°C), 24% of them (*n*=40) showed a strong reduction in the number of sensory neurons (<20 neurons per abdominal hemisegement; Fig. 5J). The rest of the embryos (76%, *n*=127) had a normal number of neurons (42 in each abdominal hemisegment). This result indicates that about one quarter of the embryos in this cross were indeed *phyl* mutants. To inactivate *N* activity during embryonic development, the embryos were incubated at the non-permissive temperature (32°C) when SOP cells and their progeny were undergoing asymmetric divisions (Guo et al., 1996). We found that 21% of the embryos (*n*=39) had fewer than 20 neurons in each abdominal hemisegment (Fig. 5L), and a small fraction of embryos (2.6%, *n*=5) exhibited an intermediate phenotype with 20-40 neurons (data not shown). Overproduction of neurons was seen in 76% of the embryos (*n*=143; Fig. 5K). In a control experiment, we confirmed that this phenotype of neuron overproduction was indistinguishable from that of N^{ts} embryos at 32°C (data not shown). Therefore, this data showed that *phyl* acts epistatically to *N* in cell fate specification of SOP daughter cells in embryos.

phyl is expressed in SOP cells and IIb lineage and its expression is negatively regulated by *N* signaling

The distribution of *phyl* mRNA was examined by whole-mount in situ hybridization. Although weak, the expression of *phyl* in wing imaginal disks was detected in the SOP cells of wing margin bristles (Fig. 6A,B), notal macrochaetes (Fig. 6A,C), and other sensory organs (Fig. 6A). In leg disks, *phyl* mRNA was also detected in the precursors of the femoral chordotonal organs, as well as in external sensory SOP cells (data not shown). During embryogenesis, the maternally contributed *phyl* transcripts were ubiquitously present before cellularization (data not shown). During stages 9-11, *phyl* was expressed in neuroblasts (Fig. 6E) and the SOP cells (Fig. 6F). From stage 12 onward, *phyl* transcripts diminished gradually, but remained detectable in a subset of PNS cells at stages 12-14 (Fig. 6G). These cells were probably the IIb cells and their progeny (neurons and/or sheath cells), since at the same stage, *phyl* mRNA was no longer detected in *numb* mutants (Fig. 6H), in which the IIb cells were transformed to the IIa cells (Uemura et al., 1989).

Our genetic analyses suggest that *phyl* is acting downstream of *N* in es organ development. We were interested in examining whether *N* signaling regulates *phyl* expression. In wild type, *phyl* was expressed in SOP cells in both wing disks and embryos. In N^{ts} mutants at restrictive temperature, *phyl* mRNA was detected strongly in clusters of cells in wing disks (Fig. 6D). *phyl* was also expressed in many more cells in *N* (Fig. 6K) and *E(spl)-C* (Fig. 6L) mutant embryos at stage 11, demonstrating that *N* signaling negatively regulates *phyl* expression. Consistently, when the constitutively active form of *N* (N^{ACT}) in which the extracellular domain is deleted (Doherty et al., 1996) was expressed in the embryonic sensory organ lineage by *sca-GAL4*, the levels of *phyl* mRNA at stage 12 were also strongly reduced (Fig. 6I).

To further confirm that *N* negatively regulates *phyl* activity by repressing its expression, we tested whether misexpression

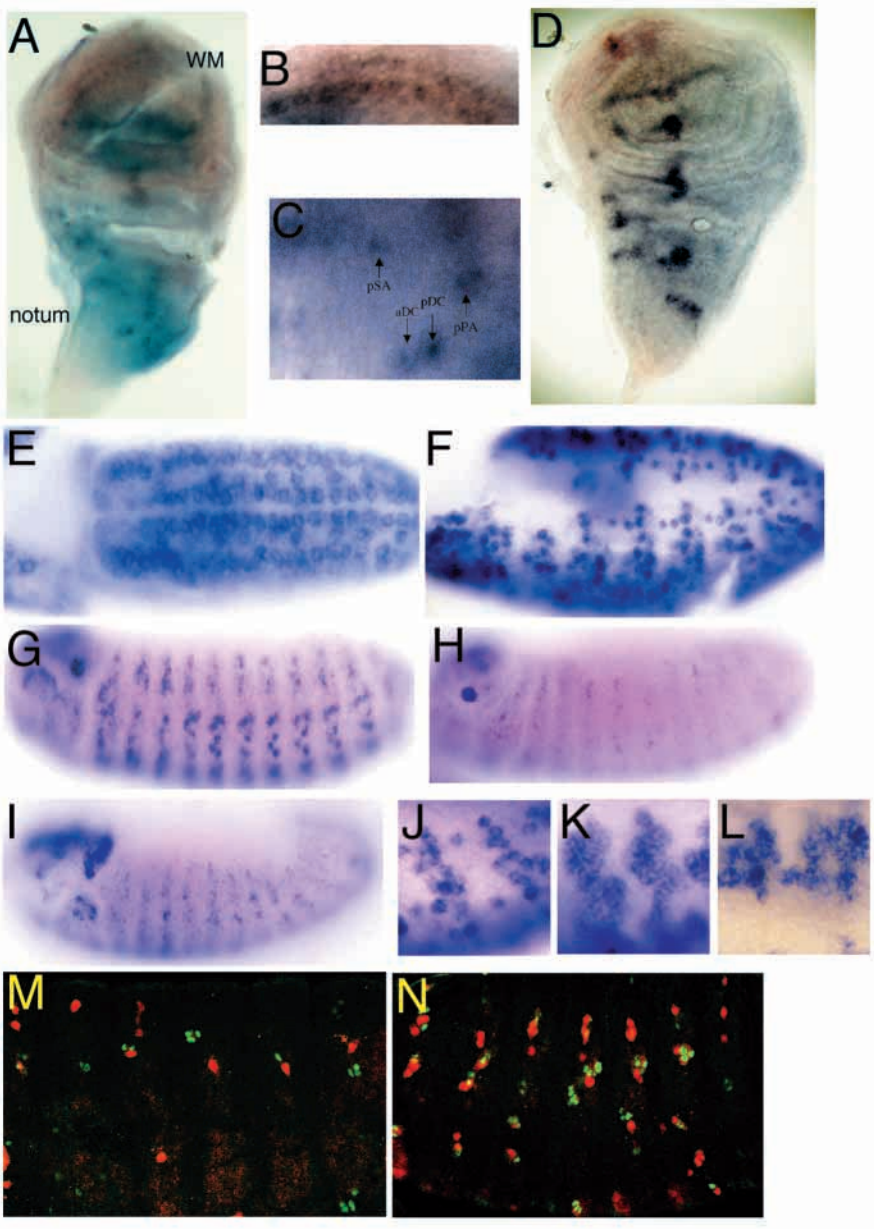

Fig. 6. *phyl* expression is negatively regulated by *N* signaling. Whole-mount in situ hybridization of *phyl* mRNA in wing disks (A-D) and embryos (E-L). (A) *phyl* expression in wild-type wing disk. (B,C) Higher magnification of the *phyl* expression in SOP cells of wing margin (B) and macrochaetes (C). (D) *phyl* expression in N^{ts} mutant wing disk. (E-G) Late third instar larvae of N^{ts} were incubated at 30°C for 5 hours before dissecting. In the embryos, *phyl* mRNA is expressed in (E) neuroblasts at stage 9 (ventral view), (F) SOP cells at stage 11 (lateral view), and (G) a subset of PNS cells at stage 13 (lateral view). (H) *phyl* mRNA in PNS is no longer detected in the $numb^1$ mutant, and (I) is reduced in the *sca-GAL4/+;UAS-*N^{ACT}/+ embryo. (J) In wild-type embryos, *phyl* is expressed in approximately 20-30 SOP cells in each hemisegment at stage 11. (K,L) *phyl* is expressed in many more cells in the N^{55e11} (K) and $E(spl)^{b32.2\ gro+}$ (L) mutant embryos at stage 11. (M,N) Confocal images of the embryonic abdominal hemisegments stained with anti-Elav antibody (red) and anti-prospero antibody (green). (M) *sca-GAL4/+; UAS-*N^{ACT}/+ embryo. (N) *sca-GAL4/+; UAS-*N^{ACT} *UAS-phyl* embryo.

of *phyl* rescues the defects caused by misexpression of N^{ACT}. *phyl* is essential for fate specification of IIb cells in the embryonic sensory organ lineage. When *UAS-phyl* and *UAS-*N^{ACT} were coexpressed in embryos by *sca-GAL4*, the total numbers of PNS neurons and sheath cells (both are progeny of IIb cells) on average were double that in *sca-GAL4/+; UAS-*N^{ACT}/+ embryos (Fig. 6M,N). This result shows that misexpression of *phyl* rescues the defects caused by N^{ACT} in the embryonic sensory organ development.

DISCUSSION

We found that *phyl* is essential for two steps in es organ development: in *phyl* mutants, SOP cells of the adult es organs fail to form (Fig. 7B), and IIb cells are transformed into IIa cells in both adult and embryonic es organs (Fig. 7C). Also, our genetic analyses are consistent with the model in which Phyl, in collaborating with Sina, promotes fate specification of SOP and IIb cells by degrading Ttk protein (Fig. 7D,E). Furthermore, we showed that *phyl* is expressed in SOP and IIb cells, and its mRNA level is negatively regulated by *N* signaling (Fig. 7D,E).

The functions and regulations of *phyl* in es organ development

Our genetic analyses show that *phyl* functions together with *sina* to promote SOP formation by antagonizing *ttk* activity. These results suggest that degradation of the Ttk protein is a major function of Phyl in the cell fate specification of SOP cells. Consistent with this idea, we found that misexpression of *ttk* can inhibit the formation of SOP cells and suppress the ectopic bristle phenotype caused by misexpression of *phyl*. Several lines of evidence also indicate that Ttk functions as a repressor to inhibit SOP cell fate. (1) Ttk is expressed ubiquitously in the pupal notum except in SOP cells (Ramaekers et al., 1997). (2) In embryos, overexpression of *ttk* inhibits the formation of es organs (Guo et al., 1995). (3) Injection of *ttk* dsRNA results in extensive increase of neurons in embryonic PNS, a phenotype observed in neurogenic mutants (Kennerdell and Carthew, 1998). All of these results suggest that *ttk* might play a negative role in the fate specification of SOP cells, and *phyl* promotes SOP fate specification by degrading Ttk.

phyl is essential for formation of pupal SOP cells, but is partly required for embryonic SOP cells. In *senseless* mutants, the larval SOP cells fail to form, but the embryonic SOP cells form and divide to generate daughter cells that fail to differentiate (Nolo et al., 2000). These results suggest that there

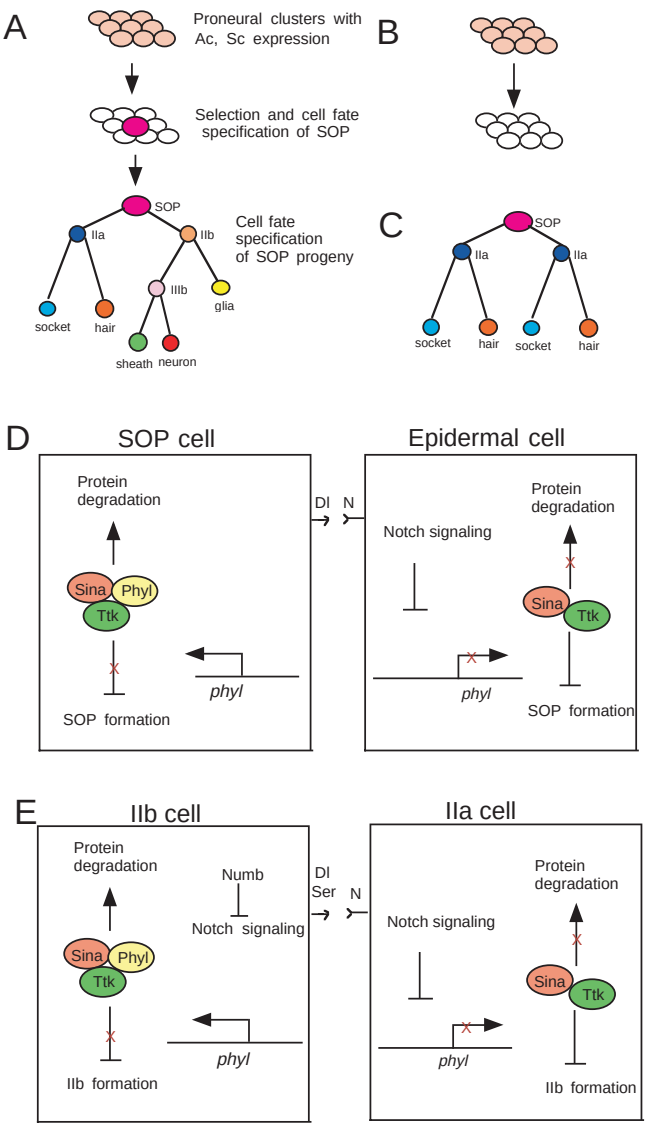

Fig. 7. Summary and models of *phyl* in es organ development. (A) The process of es organ development. (B) In *phyl* mutants, SOP fails to form although the expression of proneural genes is not affected. Also, *phyl* is required for cell fate specification of IIb cells; IIb cells are transformed to IIa cells in *phyl* mutants (C). (D,E) Our data suggests that Phyl is expressed in SOP and IIb cells, and functions together with Sina to degrade Ttk. In non-SOP cells (which ultimately become epidermal cells) and IIa cells, the levels of *phyl* mRNA are down-regulated by *N* signaling.

are some distinctions between the SOP cell fate specification of embryos and larvae.

Our studies showed that *phyl* is required for IIb cell fate specification. In *phyl* mutants, more than half of the adult notal microchaetes and more than 80% of the embryonic dm es organs exhibited IIb to IIa cell fate transformation. Cell fate transformation from hair cells to socket cells also occurs in adult bristle (four-socket phenotype seen in $phyl^{2}$ clone), but much less frequent in embryonic dm es organs (less than 5%). Transformation from neurons to sheath cells in es organs was rarely seen in *phyl* embryos. These results suggest that unlike *numb* and *N*, *phyl* is mainly required for the binary cell fate determination between IIa and IIb cells in both adult and embryonic es organs. In support of this conclusion, misexpression of *phyl* in both adult and embryonic sensory organ lineages most often resulted in a two-neuron/two-sheath cell phenotype.

In $phyl^{1}/phyl^{2}$ mutant embryos, most of the neurons and sheath cells in chordotonal organs were also lost (Fig. 2B, lateral and ventral region), indicating that *phyl* is also required for the formation of neurons and sheath cells in chordotonal organ lineage. In weaker mutant embryos ($phyl^{2245}/phyl^{2245}$), we often observed more sheath cells than neurons in the regions where chordotonal organs lch5 and vchA and B are located. Therefore, *phyl* may be required for cell fate determination between neurons and sheath cells in the development of chordotonal organs.

During lateral inhibition, the *N* pathway is essential to single out the SOP cells. Our in situ analyses indicate that *phyl* expression is negatively regulated by *N*. Also, the supernumerary bristles in *N* mutants were suppressed by *phyl* mutation. These results strongly suggest that down-regulation of *phyl* expression is a major function of *N* signaling to suppress SOP cell fate.

In the SOP lineage, *N* also plays an important role in the cell fate specification of SOP progeny. Several components of the *N* pathway, such as Delta, Serrate (Zeng et al., 1998), Su(H) (Schweisguth and Posakony, 1994), Hairless (Bang and Posakony, 1992), and proteins of the Bearded (Leviten and Posakony, 1996; Lai et al., 2000) and E(spl) (Tata and Hartley, 1995) families, have been shown to be involved in the cell fate specification of all or subsets of progeny. We found that *phyl* acts epistatically to *N* in the cell fate specification of SOP daughter cells and is expressed in IIb cells. Also, misexpression of *phyl* rescues the defects caused by N^{ACT}, indicating that *N* regulates the *phyl* activity in sensory organ lineage at the transcriptional level.

Functions of *sina* in es organ development

Our genetic analyses of *phyl*, *sina* and *ttk* are mostly consistent with the model that Phyl functions together with Sina to promote es organ development by degrading Ttk. In embryos, strong defects are only detected when both maternal and zygotic *sina* transcripts are removed, suggesting that maternally contributed *sina* transcript play an essential role in the development of embryonic es organs. Consistently, no genetic interaction between zygotic *sina* and *phyl*, and between zygotic *sina* and *ttk* was detected in embryonic PNS development (data not shown). In adults, the bristle phenotypes in *sina* mutants are weaker than in *phyl* mutants. One possible reason is that the perdurance of *sina* gene products from maternal transcripts might supply activity for some adult bristles to develop normally. Another possibility is that *phyl* is able to down-regulate *ttk* activity in a *sina*-independent manner. In the *Drosophila* genome, a sequence (CG 13030) is located next to *sina* in the genome and encodes a putative protein with 50% identity and 70% similarity with Sina. It might be possible that *sina* functions redundantly with this gene in bristle development.

Comparison of *phyl* in cell fate specification of photoreceptors and es organs

Our studies of *phyl/sina/ttk* in es organ development and

previous studies in photoreceptor differentiation indicate that the *Drosophila* eye and es organs depend on the same protein complex to specify their cell fate. In both cases, *phyl* mutations transform neural cells to non-neural cells. Both studies also show that *phyl* expression is tightly regulated by the upstream signaling pathways. The expression of *phyl* is activated by the *Ras* pathway in photoreceptor cells. In SOP cells, the transcription of *phyl* is likely activated by the proneural genes *ac* and *sc*, and is repressed by *N* signaling. Interestingly, it has been shown that the Egrf/Ras/Raf pathway acts antagonistically with the *N* pathway in SOP formation of adult macrochaetes (Culi et al., 2001) and chordotonal organs (zur Lage and Jarman, 1999). Whether these two pathways converge on *phyl* expression to regulate sensory organ formation remains to be examined.

We thank E. Hafen, P. Heitzler, J.-c. Hsu, Y. N. Jan, T. Laverty, V. Rodrigues, F. Schweisguth, Y. H. Sun, Bloomington Stock Center and the Developmental Studies Hybridoma Bank (University of Iowa) for antibodies and flies. We thank E. Hafen for phyl cDNA. We also thank Y.-H. Chou, M.-C. Li and J.-M. Bai for their help with whole-mount in situ hybridization and A.-C. Tien for his help with microinjection of the UAS-phyl construct. We are grateful to J.-c. Hsu, G.-j. Liaw and Y. H. Sun for critical reading of the manuscript and D. Platt for English editing. This work was supported by grants from Academia Sinica and the National Science Council of Taiwan to C.-T. Chien (NSC 89-2311-B-001-225). H. Pi is supported by a postdoctoral fellowship from Academia Sinica, Taiwan.

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