phyllopod is a target gene of proneural proteins in *Drosophila* external sensory organ development

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Proneural basic helix–loop–helix (bHLH) proteins initiate neurogenesis in both vertebrates and invertebrates. The *Drosophila* Achaete (Ac) and Scute (Sc) proteins are among the first identified members of the large bHLH proneural protein family. *phyllopod* (*phyl*), encoding an ubiquitin ligase adaptor, is required for *ac*- and *sc*-dependent external sensory (ES) organ development. Expression of *phyl* is directly activated by Ac and Sc. Forced expression of *phyl* rescues ES organ formation in *ac* and *sc* double mutants. *phyl* and *senseless*, encoding a Zn-finger transcriptional factor, depend on each other in ES organ development. Our results provide the first example that bHLH proneural proteins promote neurogenesis through regulation of protein degradation.

E3 ligase | *senseless* | basic helix–loop–helix | neurogenesis

The basic helix–loop–helix (bHLH) proneural proteins promote neurogenesis from flies to mammals (for reviews, see refs. 1 and 2). In *Drosophila*, the proneural proteins Achaete (Ac), Scute (Sc), Atonal (Ato), and Amos are bHLH transcriptional factors that are essential for the generation of neural precursors in the central and peripheral nervous systems (3–5). In mammals, the bHLH proteins Mash1, homolog of Ac and Sc, and Neurogenins, homologs of Ato and Amos, are essential for the initiation of neurogenesis (6, 7). Proneural genes are expressed in small clusters of cells, called proneural clusters, and they endow cells the potential to adopt neural fate, such as sensory organ precursors (SOPs) in the *Drosophila* peripheral nervous system. However, lateral inhibition mediated by the ligand Delta and the receptor Notch restricts the expression of proneural genes to only one or a few cells that differentiate into neural precursors, and prevents neighboring cells of the selected neural precursors from adapting the same fate (8).

The *Drosophila* proneural genes *ac* and *sc* function redundantly in the formation of external sensory (ES) organs; in *ac* and *sc* double mutants, formation of ES organs is disrupted, and misexpression of either *ac* or *sc* induces ectopic ES organs (9–12). The Ac and Sc proteins share 70% identity in their bHLH domains (3), and form heterodimers with the ubiquitously expressed bHLH protein Daughterless (Da) to activate transcription of downstream target genes (13, 14). One target gene of Ac and Sc, *asense* (*ase*), also encodes a bHLH protein that is specifically expressed in SOPs and involved in SOP differentiation (15–17). Likewise, NeuroD, the mammalian homolog of Ase, also plays an important role in neuronal differentiation (18). In addition to the bHLH genes, a number of Ac and Sc target genes have been identified. For example, *senseless* (*sens*) is expressed in SOPs and is required to maintain high levels of proneural proteins in SOPs (19, 20). Genes involved in lateral inhibition to select SOPs are also targets for Ac and Sc, including *scabrous* (*sca*), *Delta* (*Dl*), and those in the *Enhancer of split* [*E(spl)*] and *Bearded* (*Brd*) complexes (21, 22). However, target genes essential for SOP differentiation and the mechanism(s) by which they promote the differentiation process are relatively unknown.

Phyl is an adaptor protein that functions to link the ubiquitin ligase Seven in absentia (Sina) to the transcriptional repressor Tramtrack (Ttk) (23), leading to Ttk degradation. Phyl is required in the specification of SOPs and a subset of photoreceptors (24, 25). In this report, we show that *phyl* promotes SOP differentiation; in *phyl* hypomorphic mutants, expression of genes in SOP differentiation and lateral inhibition are affected. *phyl* is directly activated by Ac and Sc through their cognate binding sites in the *phyl* promoter region. *phyl* misexpression restores efficiently ES organ formation in the *ac* and *sc* double mutant. Taken together, our results suggest that Phyl executes the program of SOP differentiation directed by Ac and Sc proneural proteins. Lastly, we examine the relationship between *phyl* and *sens* in SOP differentiation.

Materials and Methods

Flies. *phyl* mutants ($phyl^1$, $phyl^2$, $phyl^{2245}$, and $phyl^4$) were described (26). $sc^{10\text{-}1}$ is a compound mutation that inactivates both *ac* and *sc* function (3). sc^{B57} is a small deletion in which *ac*, *sc*, *l'sc*, and *ase* genes are removed, and sc^{B57} clones were generated by x-ray-induced recombination. $sens^{E2}$ $FRT^{80B}/TM6B$ and FRT^{42d} *pwn* $phyl^2$ *Bc*/*CyO* were used to generate *sens* and *phyl* mutant clones, respectively. For misexpression experiment, *Eq-GAL4* (26, 27), *dpp-GAL4* (28), *UAS-myc-phyl* (26), *UAS-sc* (29), and *UAS-sens* (19) were used.

Plasmid Construction. The 4.1-, 3.4-, and 2.2-kb *phyl* promoter fragments were cloned into pStinger (30) to generate $phyl^{4.1}$-*GFP*, $phyl^{3.4}$-*GFP*, and $phyl^{2.2}$-*GFP*, respectively, and 4.1- and 3.4-kb fragments were fused to *phyl* ORF to generate $phyl^{4.1}$-*ORF* and $phyl^{3.4}$-*ORF* rescue constructs.

For site-specific mutagenesis, the Ac/Da and Sc/Da binding consensus C$\underline{\text{A}}$NNT$\underline{\text{G}}$ was mutated to C$\underline{\text{C}}$NNT$\underline{\text{T}}$, and the Sens binding consensus AAAT$\underline{\text{C}}$A was mutated to AAAT$\underline{\text{G}}$A (19).

Results

***phyl* in SOP Development.** In $phyl^2$-null mutant clones, adult ES organs are absent, and this defect is caused by a failure in SOP specification (26). In $phyl^2/phyl^4$ hypomorphic mutants, most ES organs are also absent, and expression of two SOP markers, *ase-lacZ* and the *A101* enhancer trap line, are strongly compromised (26). However, Sens is expressed in single, selected SOPs at 12–14 h after puparium formation (APF) (Fig. 1*B*), suggesting a defect in SOP differentiation, but not in SOP selection in *phyl* hypomorphic mutants.

We then examined Ac expression, which is initially in proneural clusters and restricted in SOPs at 12–14 APF in wild type (Fig. 1*C*). However, in $phyl^2/phyl^4$ mutants, Ac expression was not only detected in SOPs (indicated by red arrowheads in Fig. 1*D Inset*), but also weakly in SOP-neighboring cells. Ac expression in SOP-neighboring cells is later diminished at 16–18 APF (data not shown). This result suggests that lateral inhibition is

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Abbreviations: bHLH, basic helix–loop–helix; SOP, sensory organ precursor; ES, external sensory; APF, after puparium formation; CH, chordotonal.

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Fig. 1. *phyl* is required for SOP differentiation. (*A*) Wild-type notum at 12–14 h APF stained with anti-Sens antibodies. (*B*) In *phyl*^{2}/*phyl*^{4} mutants at 12–14 h APF, Sens is still expressed in single SOPs. (*C*) Ac protein is specifically expressed in single SOPs at 12–14 APF in wild type. (*D*) Ac protein is also expressed at a lower level in cells surrounding SOPs in *phyl*^{2}/*phyl*^{4} mutants at 12–14 h APF. Red arrowheads in *Inset* indicate the Sens-positive cells. (*E*) In wild type, *E(spl)m8-LacZ* is expressed strongly in cells of proneural clusters at 12–16 h APF. (*F*) In *phyl*^{2}/*phyl*^{4} mutants, *E(spl)m8-LacZ* expression is abolished at 12–16 h APF. (*G*) In wild type at 16–18 h APF, Sens is expressed in two cells in each ES organ. (*H*) In *phyl*^{2}/*phyl*^{4} mutants even at 24–26 h APF, many Sens-positive cells are still at one-cell stage. (*I*) At 12–16 h APF, CycE is expressed at a elevated level in Sens-positive SOPs (arrowheads, Sens expression is not shown). (*J*) In *phyl*^{2}/*phyl*^{4} mutants at 12–16 h APF, only the uniform, low-level CycE expression is present in all cells. (*K*) Cut is expressed in single SOPs or SOP progenies (IIa and IIb cells) in wild-type ES organs at 14–16 h APF. (*L*) Only residual Cut expression is present in *phyl*^{2}/*phyl*^{4} mutants at 14–16 h APF.

partially affected. To test this, *E(spl)m8-lacZ* was used as a reporter to monitor *Notch* signaling (31, 32). Although *E(spl)m8-lacZ* is strongly expressed in a proneural pattern in wild type (Fig. 1*E*), the expression is abolished in *phyl*^{2}/*phyl*^{4} mutants (Fig. 1*F*), suggesting that activation of the *Notch* pathway in the SOP-neighboring cells is compromised in *phyl* mutants.

In wild-type ES organ development, Sens staining appears in two SOP-daughter cells at 16–18 h APF (Fig. 1*G*) and in four daughter cells at 24–28 h APF (data not shown). In *phyl*^{2}/*phyl*^{4} mutants, Sens is still maintained mostly in single cells even at 24–28 h APF (Fig. 1*H*). In wild-type animals, SOPs express elevated levels of the cell-cycle regulator Cyclin E (CycE) (Fig. 1*I*) (33). In *phyl*^{2}/*phyl*^{4} mutants, SOPs fail to express a higher level of CycE (Fig. 1*F*), suggesting a failure in cell cycle progression. The SOPs and SOP daughter cells of ES organs express *cut*, a selector gene in the determination of ES organ identity (Fig. 1*K* and refs. 34–36). In *phyl*^{2}/*phyl*^{4} mutants when SOP differentiation has been arrested, Cut expression is absent (Fig. 1*L*). Taken together, these data indicate that Phyl is required for gene expression in SOP differentiation and lateral inhibition, for SOP cell cycle progression and for ES organ identity.

***phyl* Is a Direct Target Gene of Ac and Sc.** Ac and Sc are bHLH transcriptional activators, and Ac/Da and Sc/Da heterodimers bind specifically to the E boxes CAG(G/C)TG with high affinity and CACGTG with low affinity (21). Within the 4.1-kb *phyl* promoter region, there are four such E boxes (E1–E3, CAGCTG; E4, CACGTG; Fig. 2*A*). We constructed three *phyl* reporter genes by fusing 4.1-, 3.4-, and 2.2-kb promoter regions of *phyl* to *GFP*, and all three reporters show similar expression patterns with difference in the GFP signal intensities (the 4.1-kb promoter being the strongest and 2.2-kb being the weakest). For example, the 3.4-kb region is sufficient to drive GFP expression in embryonic SOPs (Fig. 2*B*), SOPs of the late third-instar larval wing and leg disks (Fig. 2 *C* and *D*), and SOPs in early pupal nota (Fig. 2*E*). These *phyl-GFP* reporter genes are also expressed in the proneural clusters at earlier stages in both wing disks and pupal nota (Fig. 2*C* and data not shown).

To test whether these promoter regions are sufficient for *phyl in vivo* function, we made *phyl*$^{4.1}$*-ORF* and *phyl*$^{3.4}$*-ORF* rescue constructs by fusing the 4.1- and 3.4-kb promoter regions, respectively, to the *phyl* ORF. The *phyl*^{1}/*phyl*^{2} mutants die at late embryonic or first-instar larval stages. However, both *phyl*$^{4.1}$*-ORF* and *phyl*$^{3.4}$*-ORF* are sufficient to rescue the viability of *phyl*^{1}/*phyl*^{2} animals to the adult stage ($33 \pm 3\%$ and $8 \pm 6\%$, respectively, Fig. 3*A*), with well developed ES organs on the notum (50 ± 6 and 37 ± 4, respectively, Fig. 3*B*). The inabilities to fully rescue the viability and ES organ number of *phyl*^{1}/*phyl*^{2} are caused by insufficient expression levels of the transgenes, as suggested by the fact that two copies of *phyl*$^{3.4}$*-ORF* further improve the viability of the *phyl*^{1}/*phyl*^{2} mutants to 77% (Fig. 3*A*) and increase the bristle number to 110 ± 7 (Fig. 3*B*). Hypomorphic *phyl*^{4}/*phyl*^{2245} mutants, which display a greatly reduced number of ES organs on the notum (30 ± 3), are completely rescued by two copies of *phyl*$^{3.4}$*-ORF* (184 ± 7) (Fig. 3*B*). Therefore, all of these results show that both 4.1- and 3.4-kb regions of the *phyl* promoter contain sufficient temporal and spatial information in regulating *phyl* expression.

We then tested whether activity of the 3.4-kb promoter region is regulated by *ac* and *sc*. *sc*$^{10\text{-}1}$ is a compound mutation in which both *ac* and *sc* are inactivated (3). Expressions of *phyl*$^{3.4}$*-GFP* in *sc*$^{10\text{-}1}$ wing disks and pupal nota are abolished (Fig. 2 *F* and *G*). In contrast, when *sc* is misexpressed by *dpp-GAL4* at the anterior/posterior boundary of the wing disk, *phyl*$^{3.4}$*-GFP* is strongly activated in this region (Fig. 2*H*). Similar results are also observed for *phyl*$^{4.1}$*-GFP* (data not shown). Therefore, these

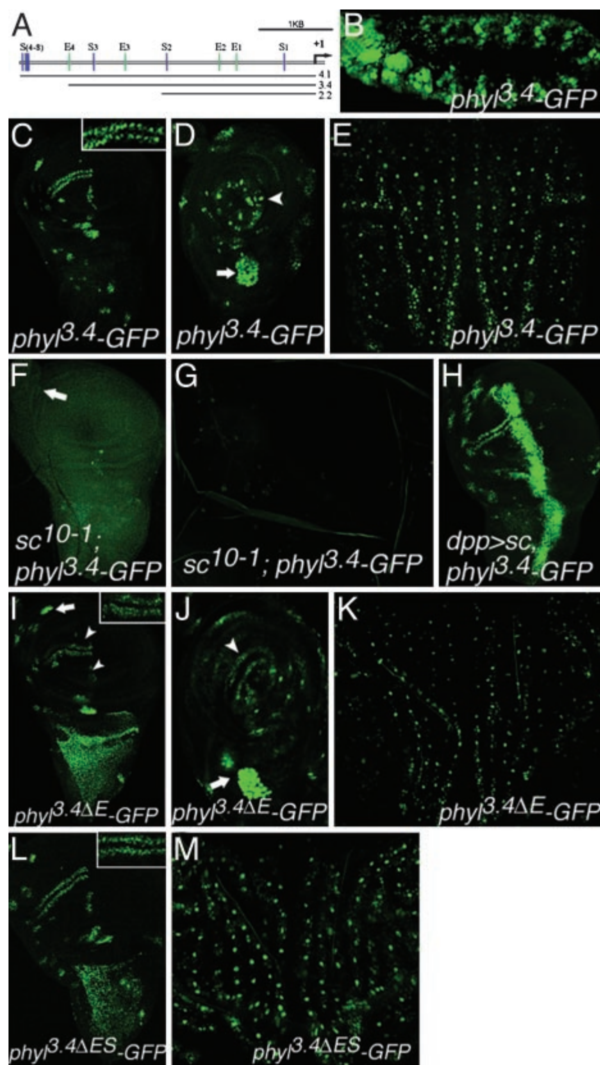

Fig. 2. *phyl* transcription depends on *ac* and *sc* activity. (*A*) Schematic diagram of the 4.1-kb upstream region of *phyl*. E1–E4 represent the Ac/Da and Sc/Da binding sites (E1–E3: CAGCTG, E4: CACGTG). S1–S8 represent the Sens-binding sites (S box, AAATGA). (*B*) *phyl*$^{3.4}$-*GFP* is expressed in SOPs of the embryonic peripheral nervous system at stage 11. (*C*) *phyl*$^{3.4}$-*GFP* is expressed in SOPs and proneural clusters in late third-instar larval wing disks. (*C Inset*) Magnified picture of wing margin SOPs. (*D*) *phyl*$^{3.4}$-*GFP* is expressed in the SOPs of ES (arrowhead) and CH (arrow) organs in the late third-instar leg disks. (*E*) *phyl*$^{3.4}$-*GFP* expression in the SOPs of pupal nota at 14 h APF. (*F*) In late third-instar wing disks of *sc*$^{10-1}$, all *phyl*$^{3.4}$-*GFP* expression in SOPs is abolished except for the ones for the CH organs (arrow) at the ventral radius. (*G*) *phyl*$^{3.4}$-*GFP* expression is abolished in *sc*$^{10-1}$ pupal nota at 14 h APF. (*H*) *phyl*$^{3.4}$-*GFP* expression is strongly activated by *sc* misexpression driven by *dpp-GAL4* at the anterior–posterior boundary. (*I* and *J*) *phyl*$^{3.4\Delta E}$-*GFP* with four E boxes mutated is expressed weakly in SOPs of ES organs (arrowheads) in the late third-instar wing (*I*) and leg (*J*) disks, but maintains strong expression in CH SOPs (arrows). (*I Inset*) Magnified picture of GFP expression in wing margin SOPs, which is reduced by 50% compared to *C Inset*. For unknown reasons, *phyl*$^{3.4\Delta E}$-*GFP* shows uniform expression in the perspective notal region (*I*). (*K*) *phyl*$^{3.4\Delta E}$-*GFP* expression in pupal nota at 14 h APF is reduced compared to *phyl*$^{3.4}$-*GFP* in *E*. (*L*) Expression of *phyl*$^{3.4\Delta ES}$-*GFP* in late third-instar wing disk. GFP intensity in wing margin SOPs (*Inset*) is increased by 20% compared to *phyl*$^{3.4\Delta E}$-*GFP* (*I Inset*). (*M*) *phyl*$^{3.4\Delta ES}$-*GFP* in pupal nota at 14 h APF.

results clearly show that proneural genes *ac* and *sc* are necessary and sufficient to activate *phyl* promoter activity.

To test whether Ac and Sc directly regulate *phyl* expression, we mutated all four E boxes in the 3.4-kb promoter region (Fig. 2*A*) to make the *phyl*$^{3.4\Delta E}$-*GFP*. The expression of *phyl*$^{3.4\Delta E}$-*GFP* in the SOPs of ES organs in late third-instar wing and leg disks (Fig. 2 *I* and *J*, arrowheads) and in pupal nota (Fig. 2*K*) is strongly reduced when compared to the expression of *phyl*$^{3.4}$-*GFP*. When the GFP intensity was quantified in the anterior wing margin SOPs, E box mutations in the 3.4-kb promoter region contribute to a 50% reduction (see *Insets* in Fig. 2 *C* and *I*). In contrast, the expression level of *phyl*$^{3.4\Delta E}$-*GFP* in the SOPs of chordotonal (CH) organs promoted by the proneural gene *ato* is comparable to that of *phyl*$^{3.4}$-*GFP* (Fig. 2 *I* and *J*, arrows). These results indicate that the *phyl* promoter is activated by Ac and Sc through these four E boxes. To test the *in vivo* significance of the four E boxes, we compare the rescue abilities between *phyl*$^{3.4}$-*ORF* and *phyl*$^{3.4\Delta E}$-*ORF*. Although *phyl*$^{3.4}$-*ORF* can rescue *phyl*^{1}/*phyl*^{2} to the adult stage, *phyl*$^{3.4\Delta E}$-*ORF*-rescued animals only survive to the third-instar larval stage (Fig. 3*A*). The abilities of *phyl*$^{3.4\Delta E}$-*ORF* to rescue the viability and the notal ES organ of *phyl*^{4}/*phyl*^{2245} mutants are strongly reduced to 36 ± 11% and 67 ± 12, respectively (Fig. 3 *A* and *B*). Many of the rescued ES organs show abnormal configuration such as double hair/double socket (Fig. 3*E*, arrow and *Inset*), which is a phenotype frequently observed in hypomorphic *phyl* mutants (Fig. 3*C*, arrow and *Inset*) (22). Therefore, these results suggest that these four E boxes are required for full *phyl* promoter activity in SOPs.

***phyl* Is Sufficient to Induce ES Organ Development in the Absence of Proneural Genes *ac* and *sc*.** In *sc*$^{10-1}$ flies, *phyl* expression is diminished and ES organ development is disrupted (Fig. 4*B*). We then asked whether forced expression of *phyl* can functionally substitute for the absence of *ac* and *sc* activities. Misexpression of *phyl* by *Eq-GAL4* in *sc*$^{10-1}$ flies efficiently rescues ES organ formation (Fig. 4*D*), to a level similar to that rescued by misexpression of the proneural gene *sc* (Fig. 4*F*). The rescued ES organs by *phyl* are arranged in a pattern similar to that of the wild-type flies; the ES organs are aligned in rows and well separated. SOP-specific expressions of *neu-LacZ* (*A101*), *ase-LacZ*, Sens, and Cut, as well as expression of *E(spl)m8-LacZ*, are restored (Fig. 4 *I–K* and data not shown). As a comparison, we misexpressed *sens,* whose expression also depends on *ac* and *sc* (19), by *Eq-GAL4* and found that it poorly rescues *sc*$^{10-1}$ in ES organ formation (Fig. 4*H*), although *sens* is more effective than *phyl* and *sc* in inducing ES organs in wild-type background (Fig. 4 *C*, *E*, and *G*). Therefore, these results suggest that *phyl* is able to execute the developmental program of ES organs in the absence of proneural genes *ac* and *sc*.

Ac and Sc activate the bHLH gene *ase* in SOPs to promote SOP differentiation. Misexpression of *ase* or another bHLH gene *lethal of scute* (*l'sc*) (37) is capable to generate ES organs independent of *ac* and *sc* (15). We then tested whether *phyl* can rescue ES organ formation in the absence of all four bHLH genes, *ac, sc, ase*, and *l'sc*, in *sc*^{B57} mutant clones. Although, in a control experiment, misexpression of *sc* can rescue the ES organ formation in *sc*^{B57} mutant clones (data not shown), misexpression of *phyl* fails to rescue (Fig. 4*L*). From this result, we infer that *phyl* requires *ase* (and/or *l'sc*) in inducing ES organ formation.

Relationship Between *phyl* and *sens* in ES Organ Development. Our promoter analysis suggests that *phyl* expression in SOPs might be activated by factors other than Ac and Sc. Within the 4.1-kb promoter region, eight putative Sens-binding sites (AAATCA, S box) were identified, with three sites distributed within the 3.4-kb proximal region and five sites in a cluster located in a very distal region (see Fig. 2*A*). We then tested whether Sens plays a role in *phyl* activation in SOPs, using *phyl*$^{4.1}$-*GFP* as a reporter. At 10–12 h APF, *phyl*$^{4.1}$-*GFP* is expressed in dorsoventral stripes along the notum in a pattern analogous to early Ac and Sc expression patterns (data not shown). At 15 h APF, *phyl*$^{4.1}$-*GFP* expression is restricted in SOPs (Fig. 5*A*, LacZ-positive tissue).

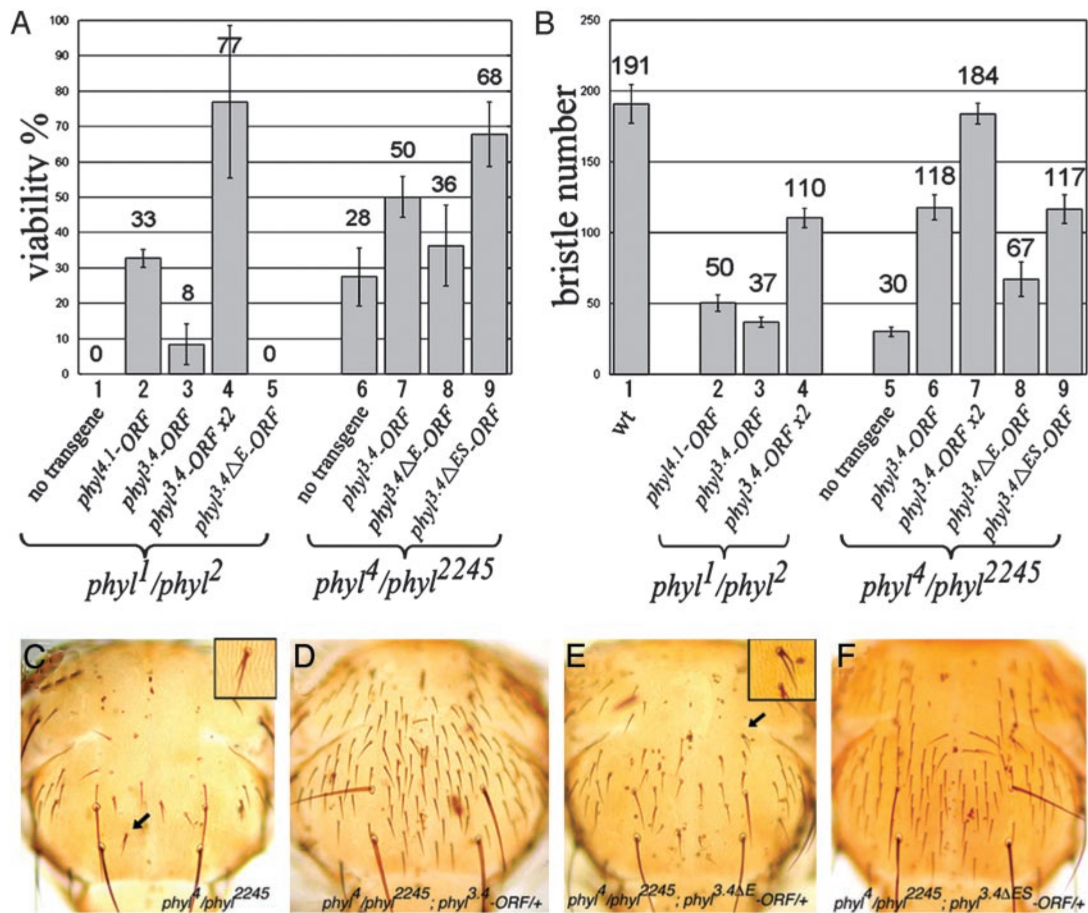

Fig. 3. Mutations in E1–4 sites reduce *phyl* promoter activity. (*A* and *B*) Abilities of the *phyl* transgenes to rescue the viability (*A*) and ES organ number (*B*) of *phyl* mutants. At least three independent lines were used for each transgene. (*A*) The percentage of viability is calculated as the number of adult flies with indicated genotypes divided by the number of $phyl^{2}/+$; transgene/+ flies or $phyl^{2245}/+$; transgene/+ flies. (*B*) The ES organ numbers are averaged from six male nota for each independent line. (*C–F*) Adult nota of $phyl^{4}/phyl^{2245}$ (*C*), $phyl^{4}/phyl^{2245}$ with one copy of $phyl^{3.4}$-*ORF* (*D*), one copy of $phyl^{3.4\Delta E}$-*ORF* (*E*), or one copy of $phyl^{3.4\Delta ES}$-*ORF* (*F*). Double hair/socket phenotype indicated by arrows in *C* and *E* is magnified in *Insets*.

In $sens^{E2}$-null clones (LacZ-negative tissue), $phyl^{4.1}$-*GFP* is expressed in dorsoventral stripes (indicated by a bracket in Fig. 5*A*), and this expression is quickly restricted to single SOPs at 16 h APF, identical to that in wild-type tissue (Fig. 5*B*). At 20 h APF, when wild-type SOPs have divided to two daughter cells, $phyl^{4.1}$-*GFP* expression in $sens^{E2}$ clones is still maintained in

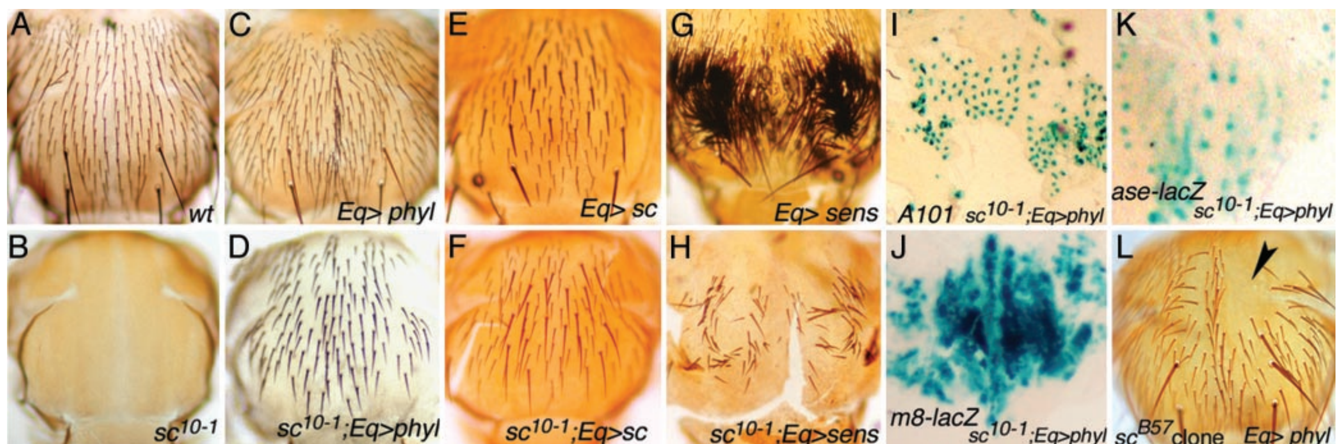

Fig. 4. *phyl* rescues *ac* and *sc* mutants in ES organ development. (*A*) Wild-type notum. (*B*) In the $sc^{10\text{-}1}$ notum, all ES organs are missing. (*C*) Expression of *phyl* by *Eq-GAL4* (indicated by *Eq*>*phyl*) induces ectopic ES organs. (*D*) Misexpression of *phyl* restores ES organs in $sc^{10\text{-}1}$. (*E*) Expression of *sc* by *Eq-GAL4* in a *Sb* background. (*F*) Expression of *sc* by *Eq-GAL4* restores ES organs in $sc^{10\text{-}1}$. (*G*) Expression of *sens* by *Eq-GAL4* induces numerous ES organs. (*H*) In $sc^{10\text{-}1}$, only a few ES organs are restored by *sens* misexpression. (*I–K*) Expression of *phyl* by *Eq-GAL4* restores *A101* (*I*), *E(spl)m8-lacZ* (*J*), and *ase-LacZ* (*K*) expression in $sc^{10\text{-}1}$. (*L*) Misexpression of *phyl* is unable to rescue the ES organ-missing phenotype in sc^{B57} mutant clone (the balding region is indicated by the arrow).

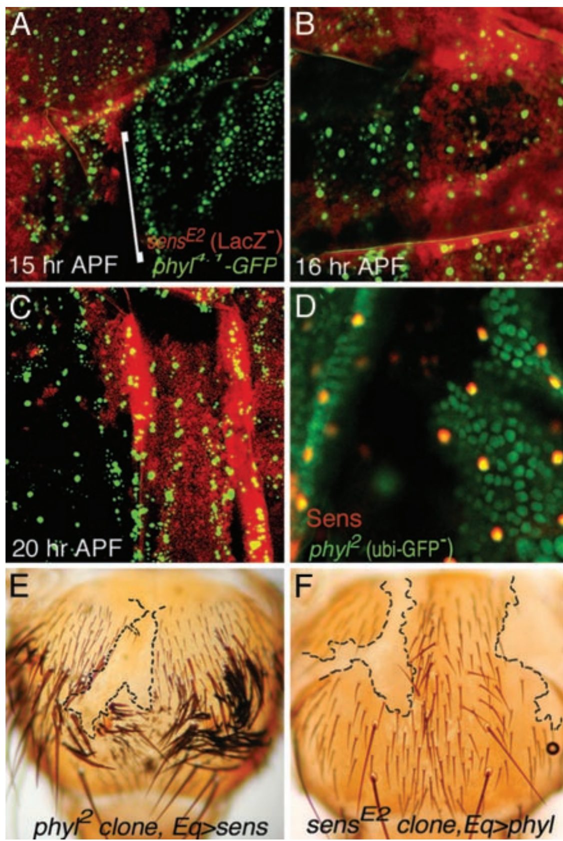

Fig. 5. Relationship between *phyl* and *sens* in expression and function in ES organ development. (*A*) The $phyl^{4.1}$*-GFP* expression pattern at 15-h APF. When GFP expression is restricted to single SOPs in wild-type tissue (LacZ-positive in red), the $phyl^{4.1}$*-GFP* expression in the $sens^{E2}$ clone (lack of LacZ staining) remains in stripes. (*B*) $phyl^{4.1}$*-GFP* is expressed in SOPs in a similar pattern and level in both wild-type and $sens^{E2}$ mutant tissues at 16 h APF. (*C*) When GFP is expressed in two SOP daughter cells in wild-type tissue at 20 h APF, the $phyl^{4.1}$*-GFP* is still expressed in single cells in $sens^{E2}$ mutant tissue. (*D*) Sens expression in SOPs is strongly reduced in $phyl^2$ mutant clones. Anti-Sens staining is shown in red, and the mutant clone is marked by the lack of *ubi-nGFP* expression (green). (*E*) Expression of *sens* by *Eq-GAL4* fails to rescue the ES organ-missing defect in $phyl^2$ mutant clones. Clone is identified by the presence of trichome phenotype (pwn^-), and clone boundary is marked by black dashed lines. (*F*) Similarly, expression of *phyl* by *Eq-GAL4* fails to rescue the ES organ-missing defect in $sens^{E2}$ mutant clones, which are identified by the absence of y^+ expression in epidermis.

single SOPs (Fig. 5*C*), and mostly in two cells at 23 h APF when wild-type cells are in GFP-positive clusters containing three or four cells (data not shown). Therefore, these results suggest that, in the absence of *sens* activity, SOP development is delayed, but $phyl^{4.1}$*-GFP* expression is minimally affected.

To determine the contribution of Sens binding sites to *phyl* expression, we used the 3.4-kb *phyl* promoter region whose expression pattern is analogous to the 4.1-kb promoter in both wild-type and *sens* mutant background (Fig. 2 and data not shown). The $phyl^{3.4\Delta S}$*-GFP* reporter with all three S boxes mutated expresses little difference in the GFP pattern and intensity when compared to $phyl^{3.4}$*-GFP* (data not shown). However, the reporter with mutations in all four E boxes and three S boxes ($phyl^{3.4\Delta ES}$*-GFP*) enhances GFP intensity by 20% when compared to $phyl^{3.4\Delta E}$*-GFP* with mutations only in four E boxes (Fig. 2 *L* and *M*). This 20% increase in GFP intensity reflects an increase in the *phyl* activity *in vivo* because $phyl^{3.4\Delta ES}$*-ORF* shows stronger abilities than $phyl^{3.4\Delta E}$*-ORF* in rescuing both the viability and the ES organ number of $phyl^4/phyl^{2245}$ flies (Fig. 4 *A*, *B*, *E*, and *F*). Therefore, these data suggest that these S boxes play a negative role in regulation of *phyl* activity.

To test whether *phyl* regulates *sens* expression, we examined Sens protein expression in *phyl* mutants. In $phyl^2$-null clones, Sens expression is almost diminished in all stages examined, including the single-SOP stage (Fig. 5*D*), the two-cell stage and the four-cell stage (data not shown), suggesting that *phyl* is required for Sens expression in ES organ development.

To analyze the functional relationship between *phyl* and *sens* further, we performed rescue experiments. Misexpression of *sens* by *Eq-GAL4* fails to induce ES organ formation in $phyl^2$ mutant clones (Fig. 5*E*). Similarly, ES organ formation induced by *phyl* misexpression is blocked in $sens^{E2}$ mutant clones (Fig. 5*F*). This result suggests that although Sens expression depends on *phyl* activity, they function in parallel to promote ES organ development.

Discussion

It is generally thought that neurogenesis in both vertebrates and invertebrates is regulated by a cascade of bHLH proteins for the specification and differentiation of neural precursors (38, 39); however, *phyl* is a non-bHLH gene that can functionally substitute for proneural bHLH genes to execute neural developmental program. This ability of *phyl* is also manifested from the analysis of *phyl* loss-of-function phenotypes: *sens* and *ase* required for SOP differentiation are inactivated, and *neuralized* (*A101* insertion locus) in the activation and *E(spl)-m8* in the transduction of the *Notch* pathway are not expressed. Furthermore, SOP cell division, a prerequisite step to generate distinct daughter cells for constructing a complete ES organ, is blocked in *phyl* mutants. This defect likely reflects a role for *phyl* in controlling cell cycle progression, because CycE expression in SOPs maintains at a basal level. Therefore, although SOPs have been selected from proneural clusters in *phyl* hypomorphs, they are associated with several defects as described.

Studies of proneural genes have shown that *ac* and *sc* promote ES organ identity, whereas *ato* promotes CH organ identity (29). *cut* is the selector gene to specify the ES organ identity; in its absence ES organs are transformed into CH organs and misexpression of *cut* transforms CH organs into ES organs (34, 35). The absence of Cut expression in *phyl* mutants suggests that specification of ES organ identity may be through a regulation of *cut* expression by Phyl. Although *phyl* is expressed in SOPs for both ES and CH organs, we found that, in $phyl^2/phyl^4$ and $phyl^1/phyl^4$ mutants, *A101* expression in leg CH organ precursors remained normal. Also, misexpression of *phyl* fails to rescue *ato* mutants in CH organ formation (H.P. and C.-T.C., unpublished data). These results suggest that *phyl* only mediates functions of *ac* and *sc* in ES organ development.

In the rescue experiment for the lack of proneural activity in sc^{10-1}, expression driven by *Eq-GAL4* gave a uniform expression of Phyl on the developing notum, as visualized by the expression of a Myc-tagged Phyl (data not shown). However, the global expression of Phyl leads to patterned ES organ formation in the adult (Fig. 4*D*). SOP-specific expression of *neu-LacZ* (*A101*), *ase-LacZ*, and Sens was observed in the developing notum, suggesting that it is the activity of the Phyl protein being subjected to further regulation to activate SOP formation, but not the activity of Sens or Ase. This patterning activity was also observed when global expression of the proneural gene *sc* in sc^{10-1} mutants, although these spaced ES organs are less organized (Fig. 4*F*). We think that lateral inhibition in the developing tissue, in this case the developing notum, operates under the global expression of Sc and Phyl, even in the wild-type background (Fig. 4 *C* and *E*), to generate spaced SOPs. However, when Sens is ubiquitously expressed by *Eq-GAL4*, this lateral inhibition process is inhibited, leading to the formation of tufted ES organs on the notum (Fig. 4*G*). One mechanism can be

mediated through antagonizing the activity of members of *E(spl)-C* by Sens (19, 20).

Both *sens* and *phyl* are expressed specifically in SOPs, and essential for ES organ formation. However, *phyl* and *sens* should play some distinct roles in ES organ development, as suggested by our rescue experiment (Fig. 5). *sens* is required for the augment of proneural protein expression, antagonism of lateral inhibition, and maintenance of cell survival (19). The result that lose of *phyl* activity could not be rescued by *sens* misexpression suggests that, although *sens* misexpression may activate *ac* and *sc* expression, the activity of *ac* and *sc* to promote ES organ development relies on some specific functions of *phyl* that cannot be substituted by *sens*. *phyl* is involved in controlling gene expression, including Sens and Ase, in SOP differentiation. Misexpression of *phyl* rescues ES organ development in *ac* and *sc* but not *sens* mutants indicates that, in addition to proneural gene enhancement, *sens* plays additional roles downstream of *phyl*. Therefore, *phyl* and *sens* have different functions, and they depend on each other in promoting ES organ formation.

One well characterized function of Phyl is to bring the Ttk protein to the ubiquitin–protein ligase Sina for degradation (40, 41). During SOP development, *phyl* is expressed in SOPs, and Ttk is expressed ubiquitously except in the SOPs and the proneural clusters (42). Our genetic studies among *phyl*, *sina*, and *ttk* suggest that *phyl* and *sina* promote ES organ development by antagonizing *ttk* activity (26). Ttk contains a BTB/POZ domain and functions as a transcriptional repressor (43). Therefore, degradation of Ttk can lead to the derepression of SOP-specific genes. Our studies suggest that degradation of a general transcriptional repressor play a crucial role in regulating gene expression in different aspects of neural precursor differentiation.

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