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RESEARCH ARTICLE

Proneural proteins Achaete and Scute associate with nuclear actin to promote formation of external sensory organs

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ABSTRACT

Basic helix-loop-helix (bHLH) proneural proteins promote neurogenesis through transcriptional regulation. Although much is known about the tissue-specific regulation of proneural gene expression, how proneural proteins interact with transcriptional machinery to activate downstream target genes is less clear. *Drosophila* proneural proteins Achaete (Ac) and Scute (Sc) induce external sensory organ formation by activating neural precursor gene expression. Through co-immunoprecipitation and mass spectrometric analyses, we found that nuclear but not cytoplasmic actin associated with the Ac and Sc proteins in *Drosophila* S2 cells. Daughterless (Da), the common heterodimeric partner of *Drosophila* bHLH proteins, was observed to associate with nuclear actin through proneural proteins. A yeast two-hybrid assay revealed that the binding specificity between actin and Ac or Sc was conserved in yeast nuclei without the presence of additional *Drosophila* factors. We further show that actin is required in external sensory organ formation. Reduction in *actin* gene activity impaired proneural-protein-dependent expression of the neural precursor genes, as well as formation of neural precursors. Furthermore, increased nuclear actin levels, obtained by expression of nucleus-localized actin, elevated Ac–Da-dependent gene transcription as well as Ac-mediated external sensory organ formation. Taken together, our *in vivo* and *in vitro* observations suggest a novel link for actin in proneural-protein-mediated transcriptional activation and neural precursor differentiation.

KEY WORDS: Neural precursor, Nuclear actin, Proneural protein

INTRODUCTION

The highly conserved basic helix-loop-helix (bHLH) proteins regulate key aspects of neurogenesis in invertebrates and vertebrates, including the acquirement of neural competence, neural precursor determination, neuronal subtype specification and differentiation of neural cells (Bertrand et al., 2002; Quan and Hassan, 2005; Pi and Chien, 2007). Most of these functions depend on their transcriptional activity to regulate the expression of downstream target genes (Castro and Guillemot, 2011). Thus, understanding the molecular mechanisms of transcriptional activation by bHLH proneural proteins is pivotal in building the nervous system.

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In *Drosophila*, bHLH proneural proteins encoded by *achaete* (*ac*) and *scute* (*sc*) induce the formation of external sensory (ES) organs that function in mechano- and chemo-sensory reception. As proneural genes, misexpression of *ac* or *sc* in embryonic ectoderm or imaginal discs (the developing larval epithelium) is sufficient to induce ectopic ES organs (Campuzano et al., 1985; Romani et al., 1989; Rodríguez et al., 1990; Cubas et al., 1991; Skeath and Carroll, 1991). Both *ac* and *sc* genes are expressed in the same proneural clusters through shared *cis*-regulatory elements and cross feedback regulation, and function redundantly in the selection and specification of sensory organ precursors (SOPs) from the proneural clusters (Gómez-Skarmeta et al., 1995; Modolell and Campuzano, 1998). Each SOP then divides asymmetrically to give rise to cells that constitute each ES organ (Hartenstein and Posakony, 1989; Usui and Kimura, 1992; Gho et al., 1999; Reddy and Rodrigues, 1999; Fichelson and Gho, 2003).

Ac and Sc initiate the determination and fate specification of SOPs as transcriptional activators that are heterodimers with Daughterless (Da), the *Drosophila* homologue of E12/E47 protein. The heterodimeric complexes bind to a subset of E-box sequences of CAG(C/G)TG and activate transcription of an array of downstream target genes (Cabrera and Alonso, 1991; Singson et al., 1994; Reeves and Posakony, 2005). One of the key target genes is *phyllopod*, which encodes an adaptor protein for the ubiquitin E3 ligase Sina (Li et al., 2002). *phyllopod* is essential for specification and differentiation of SOPs, and its expression in the precursors is mediated through several E-box motifs in its promoter region (Pi et al., 2001; Pi et al., 2004).

Actin is the major cytoskeletal protein in all eukaryotic cells, and it plays fundamental roles in many cellular processes in the cytoplasm. Although the presence of β-actin in nuclei has been identified (Egly et al., 1984; Scheer et al., 1984), the roles of actin as a major component in nuclear structures and functions have only been shown recently (for reviews, see Hofmann, 2009; de Lanerolle and Serebryannyy, 2011). Nuclear β-actin regulates gene expression through association with all three RNA polymerases for efficient transcription (Hofmann et al., 2004; Hu et al., 2004; Philimonenko et al., 2004; Kukalev et al., 2005). β-actin is also a component of the SWI/SNF-like chromatin-remodeling complex, which maximises ATPase activity and allows association of the complex with chromatin (Zhao et al., 1998; Xue et al., 2000; Obrdlik et al., 2008). By binding to the heterogeneous nuclear ribonucleoprotein (hnRNP) complex (Zhang et al., 2002) and small nuclear ribonucleoprotein (snRNP) complexes (Sahlas et al., 1993), β-actin regulates pre-mRNA processing and export (Percipalle et al., 2002; Kukalev et al., 2005; Obrdlik et al., 2008).

Although these studies have clearly established nuclear actin as a key component in transcription and RNA-processing

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complexes, less is known about the interaction between nuclear actin and the tissue-specific transcriptional activators. In addition, the *in vivo* roles of nuclear actin in multicellular organisms have not been reported. Here, we show that proneural proteins Ac and Sc physically associate with nuclear but not cytoplasmic actin. Genetic analyses in *Drosophila* show that *actin* acts synergistically with *ac* and *sc* to promote ES organ formation, and the reduction in the actin levels impairs gene expression in neural precursors in a manner that is dependent on the E-box motif. Furthermore, increases in the levels of nuclear actin but not cytoplasmic actin specifically enhanced Ac–Da-dependent gene expression in *Drosophila* S2 cells and Ac-mediated ES organ formation, suggesting a potential role of nuclear actin as a transcription co-factor of Ac and Sc in mediating neural precursor gene expression.

RESULTS

Ac and Sc associate with nuclear actin

To elucidate the molecular mechanisms of Ac and Sc to activate downstream target gene expression, we performed co-immunoprecipitation experiments to identify nuclear factors associated with Ac and Sc. FLAG-tagged Ac (FLAG-Ac) or Sc (FLAG-Sc) were expressed in *Drosophila* Schneider 2 (S2) cells, and the nuclear and cytosolic extracts were prepared and subjected to co-immunoprecipitation with anti-FLAG antibody. In cells expressing FLAG-Ac or FLAG-Sc, one protein band around 42 kDa was observed in immunoprecipitates from nuclear (N) but not cytosolic (C) fractions (Fig. 1A). This band was also absent in immunoprecipitates from total lysate of the cells transfected with empty vector (lane 1 in Fig. 1A). Subsequent mass spectrometric (MS) analysis of the 42 kDa band associated with FLAG-Ac identified it as actin peptides encoded by *Drosophila Actin 5C* (*Act5C*) and *Actin 42A* (*Act42A*) genes (supplementary material Table S1). Act5C and Act42A are expressed in all cells (Fyrberg et al., 1983), and are almost identical (98% and 97% identity, respectively) to mammalian β-actin. These two actin proteins differ in only two amino acids and function interchangeable during development (Fyrberg et al., 1983; Wagner et al., 2002). To validate the physical interaction between actin and Ac or Sc in nuclei, Myc-tagged Ac or Sc (Myc-Ac or Myc-Sc) was expressed in S2 cells and the nuclear and cytosolic extracts were subjected to co-immunoprecipitation with anti-Myc antibody. Although ectopically expressed Myc-Ac and Myc-Sc were present in both nuclear and cytosolic extracts of S2 cells (Fig. 1B), actin was only precipitated with Myc-Ac and Myc-Sc in nuclear extracts (lanes 1 and 2 in Fig. 1B), not in cytosolic extracts (lanes 4 and 5 in Fig. 1B). Thus, the proneural proteins Ac and Sc associate with actin specifically in nuclei.

Heterodimerization with Da is required for Ac and Sc to activate downstream target gene expression. To examine the physical interaction between Da and actin, S2 cells were either transfected with expression plasmid for Myc-Da, or co-transfected with expression plasmids for Myc-Da and FLAG-Ac. As shown in Fig. 1C, actin was not co-immunoprecipitated with Da in either nuclear or cytosolic extracts (lanes 3 and 7 in Fig. 1C). In the presence of FLAG-Ac, however, a reduced amount of actin and FLAG-Ac were pulled down with Myc-Da in the nuclear, but not the cytosolic extracts (compare lane 4 to lane 8 in Fig. 1C). Thus, Da itself is unable to associate with nuclear actin, but probably forms complexes with nuclear actin through interaction with Ac.

We next asked whether the specific association between Ac and Sc and actin could occur in other eukaryotic cell nuclei. Protein–protein interactions in nuclei of the budding yeast *Saccharomyces cerevisiae* can be detected by the two-hybrid system (Chien et al., 1991). Co-expression of the GAL4 DNA-binding domain fusion protein GBD-Act42A and GAL4 activation domain fusion protein GAD-Ac or GAD-Sc in yeast cells turned on the reporter genes (Fig. 1D), indicating an association between actin and Ac or Sc. Consistent with the immunoprecipitation results, this assay failed to reveal an interaction between actin and Da (Fig. 1D). Thus, our results show that the interaction between actin and proneural proteins Ac or Sc could occur in yeast nuclei without other *Drosophila* nuclear factors present.

Act5C acts cooperatively with *ac* and *sc* to promote ES organ formation

Act5C and *Act42A* are ubiquitously expressed in different types of cells and developmental stages (Fyrberg et al., 1983). To examine more specifically whether actin is involved in Ac- and

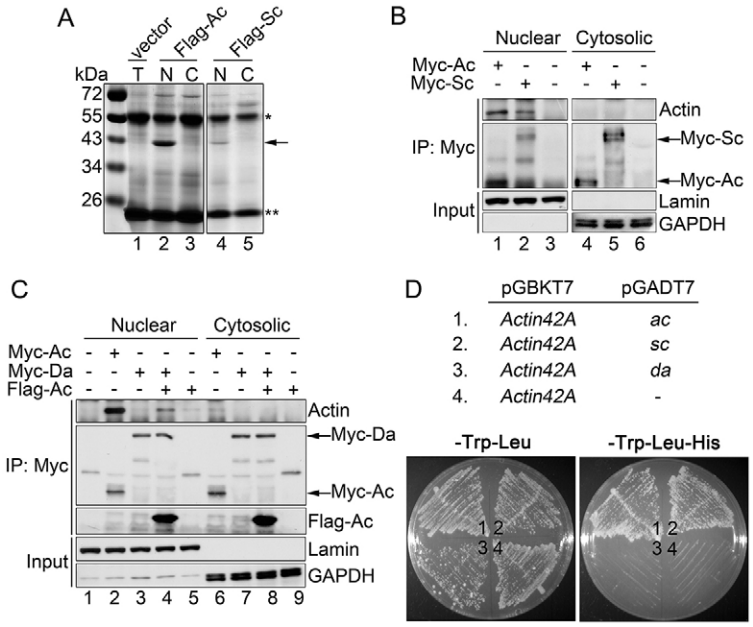

Fig. 1. Nuclear actin associates with proneural proteins Ac and Sc. (A) SDS-PAGE of the co-immunoprecipitates from extracts of cells transfected with empty vector (lane 1), or with expression plasmids for FLAG-Ac (lanes 2 and 3) or FLAG-Sc (lanes 4 and 5) using an antibody against the FLAG epitope. T, total lysate; N, nuclear extract; C, cytosolic extract. Asterisk (*) and double asterisk (**) mark IgG heavy chain and light chain, respectively. The arrow indicates the 42 kDa band specifically present in the nuclear co-immunoprecipitates. (B) Western blot analysis showing co-immunoprecipitation between nuclear actin and proneural proteins Ac and Sc. Co-immunoprecipitation from nuclear and cytosolic extracts of S2 cells transfected with *myc-ac* or *myc-sc* was performed using an antibody against the Myc epitope. Lamin Dm_0 and GAPDH are markers for nuclear and cytosolic extracts, respectively. (C) Western blot analysis showing co-immunoprecipitation between nuclear actin and Da in the presence of Ac. Co-immunoprecipitation from nuclear and cytosolic extracts of S2 cells expressing Myc-Ac, Myc-Da or Myc-Da/FLAG-Ac was performed using antibody against Myc epitope. (D) Interaction between Act42A and proneural proteins Ac and Sc in a yeast two-hybrid assay. Interaction was scored by cell growth on a –Trp –Leu –His plate.

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Sc-promoted ES organ formation, we studied the effect of reducing *Act5C* expression in the sensitized heterozygous $sc^{10\text{-}1}$/+ flies, in which the gene dosage of both *ac* and *sc* is reduced by half. $Act5C^{EY11969}$ is a hypomorphic mutant with reduced expression of *Act5C* isoforms *RA*, *RC* and *RD* (supplementary material Fig. S1B), presumably owing to P-element insertion within the proximal promoter (supplementary material Fig. S1A), which is important for constitutive synthesis of *Act5C* in all cells (Chung and Keller, 1990). We first focused our analyses on the post-vertical (p-v) bristles on the head (arrows in top panel of Fig. 2A) because their formation was very sensitive to the gene activity of *ac*, *sc* and *da*. For example, only 5.5% of $sc^{10\text{-}1}$ heterozygous or 2% of null allele da^{10} heterozygous flies lost their p-v bristles (lanes 2 and 3 in Fig. 2B). However, in the double heterozygous $sc^{10\text{-}1}$/+; da^{10}/+ flies, the percentage of flies missing p-v bristles markedly increased to 95% (bottom panel of Fig. 2A, and lane 4 in Fig. 2B). Although no p-v bristles were lost in $Act5C^{EY11969}$/+ heterozygous flies (lane 5 in Fig. 2B), 45% of the $sc^{10\text{-}1}$ +/+ $Act5C^{EY11969}$ double heterozygous flies lost p-v bristles (lane 8 in Fig. 2B) ($P<0.001$ compared with $Act5C^{EY11969}$/+ or $sc^{10\text{-}1}$/+ by chi-square test). The other two P-element lines $Act5C^{G0010}$ and $Act5C^{G0486}$, inserted in the region near the proximal promoter, expressed relatively normal or slightly reduced levels of *Act5C*, respectively (supplementary material Fig. S1A,B). The percentage of the double heterozygous $sc^{10\text{-}1}$ +/+ $Act5C^{G0010}$ or $sc^{10\text{-}1}$ +/+ $Act5C^{G0486}$ flies missing p-v bristles was not significantly different ($P>0.05$) or only mildly increased ($P<0.05$) compared with $sc^{10\text{-}1}$/+ flies (lanes 9 and 10, respectively, in Fig. 2B). Thus, reduction in one copy of *Act5C* gene activity dramatically enhanced the missing p-v bristle phenotype caused by lower *ac* and *sc* activity.

By targeted expression of *Act5C* RNAi in various parts of the fly body, we also found evidence for the cooperative interaction between *Act5C* and *ac* and *sc* in promoting ES organ formation. There are three campaniform sensilla, the *ac*- and *sc*-dependent strain-detection sensory organs, on the longitudinal vein III of wild-type flies (lanes 1 and 2 in Fig. 2C, and supplementary material Fig. S2A). Removing one copy of *ac* and *sc* resulted in loss of campaniform sensilla in 15% of the $sc^{10\text{-}1}$/+; *dpp-Gal4*/+ flies (lane 3 in Fig. 2C). We used the *Act5C RNAi* line that weakly reduced mRNA levels of *Act5C* (supplementary material Fig. S3), which when knocked down by *dpp-Gal4* did not result in loss of campaniform sensilla (lane 4 in Fig. 2C). Interestingly, the percentage of flies missing campaniform sensilla was dramatically increased to 55% in $sc^{10\text{-}1}$/+; *dpp>Act5C RNAi* flies ($P<0.001$ in comparison with $sc^{10\text{-}1}$/+; *dpp-Gal4*/+) (lane 5 in Fig. 2C, and supplementary material Fig. S2B). Ac and Sc are also required for the formation of anterior and posterior dorsal-central (DC) mechano-sensory macrochaetae (arrows and arrowheads, respectively, in supplementary material Fig. S2C). Likewise, knockdown of *Act5C* by *pnr-Gal4* in the $sc^{10\text{-}1}$/+ fly notum also markedly increased the percentage of flies missing anterior DC macrochaetae from 30% to 87% (lane 3 and 5 in Fig. 2D, $P<0.001$), and missing posterior DC macrochaetae from 0% to 24% (lanes 8 and 10 in Fig. 2D, $P<0.001$). Taken together, these results suggest that formation of two types of Ac- and Sc-dependent ES organs, campaniform sensilla and mechanosensory macrochaetae, is sensitive to the levels of *ac, sc* and *Act5C*.

Act42A is required for neural precursor formation

We also analyzed the role of *Act42A* in *ac*- and *sc*-mediated ES organ formation by driving *Act42A* RNAi that could strongly

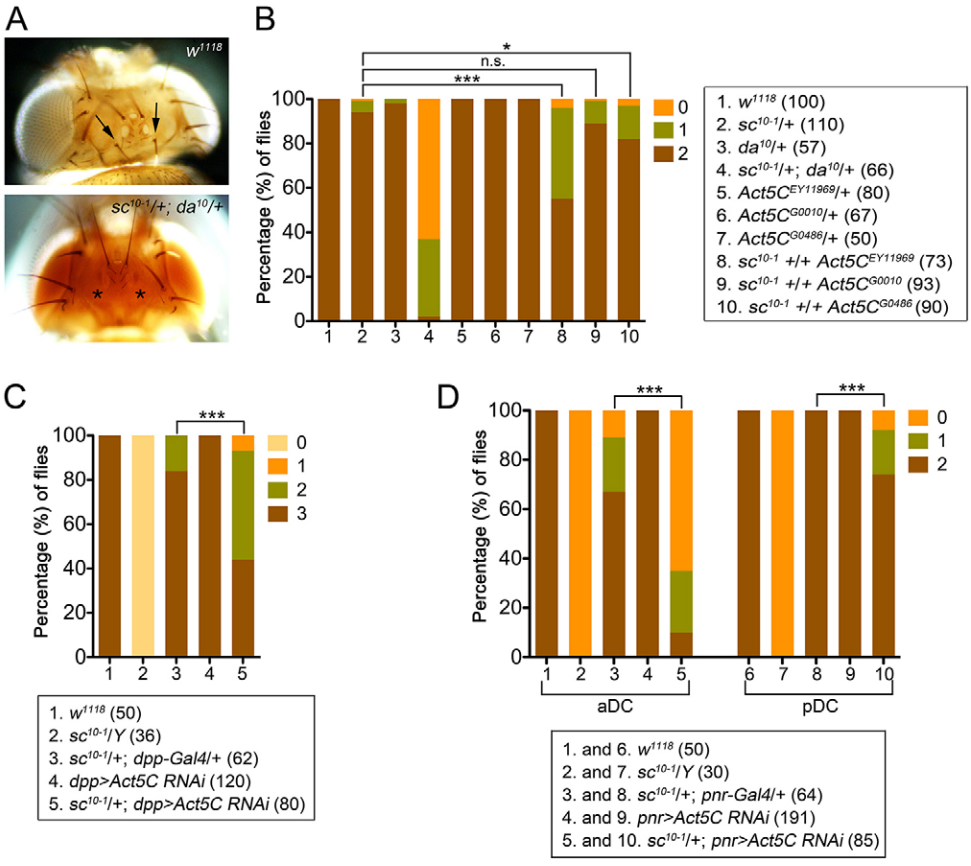

Fig. 2. *Act5C* positively regulates ES organ formation. (A) *Drosophila* adult heads. Arrows in top panel indicate the two p-v bristles present in wild-type w^{1118}. Asterisks in bottom panel mark the location of p-v bristles that were lost in $sc^{10\text{-}1}$/+; da^{10}/+ adult heads. (B) Percentage of flies with 0, 1 or 2 p-v bristles. Missing bristle phenotype in $sc^{10\text{-}1}$/+ flies is significantly enhanced by one copy of $Act5C^{EY11969}$. The number of flies scored is indicated in parentheses. (C) Percentage of flies with 0, 1, 2, or 3 campaniform sensilla. Missing campaniform sensilla phenotype in $sc^{10\text{-}1}$/+ flies is significantly enhanced by RNAi knockdown of *Act5C*. Crosses were incubated at 27°C. (D) Percentage of flies with 0, 1 or 2 DC macrochaetae. Lanes 1–5 and lanes 6–10 show results for anterior and posterior DC macrochaetae, respectively. Significance was evaluated using the chi-square test. ***$P<0.001$; *$P<0.05$; n.s., not significant.

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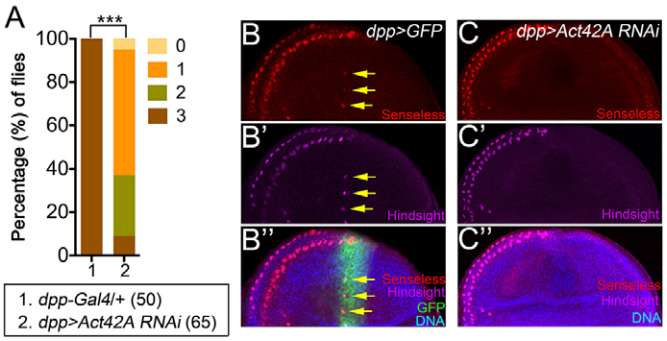

Fig. 3. *Act42A* is required for neural precursor specification. (A) Percentage of flies with 0, 1, 2 or 3 campaniform sensilla. Campaniform sensilla are lost in 89% of the *dpp>Act42A RNAi* wings. (B–C″) Images of 0–4 hour APF wing discs co-immunostained for Senseless (red) and Hindsight (magenta). DNA dye Hoechst 33342 is blue; anterior of the discs is to the left. Arrows indicate the three Senseless (B)- and Hindsight (B′)-positive clusters for developing campaniform sensilla in *dpp>GFP* control discs. (C–C″) SOPs of the campaniform sensilla were lost in *dpp>Act42A RNAi* wing discs.

decrease the mRNA level (supplementary material Fig. S3). Although no campaniform sensilla were lost in the *dpp-Gal4* control wing, knockdown of *Act42A* caused 89% of the wings having fewer campaniform sensilla (Fig. 3A). Loss of other types of ES organs including p-v bristles, macrochaetae and microchaetae, were also observed in flies expressing *Act42A* RNAi (data not shown).

Ac and Sc promote ES organ formation through inducing sensory organ precursors (SOPs). The zinc-finger protein Senseless is specifically expressed in SOPs as well as their progeny, and is required for SOP formation (Nolo et al., 2000). In control *dpp-Gal4>UAS-GFP* wing discs, three Senseless-positive clusters, representing the precursors and the precursor progeny of the three campaniform sensilla, were observed at vein III (arrows in Fig. 3B). By contrast, there were no Senseless-positive cells at vein III in *dpp>Act42A RNAi* wing discs (Fig. 3C). The lack of SOPs in *dpp>Act42A RNAi* wing discs was also confirmed by co-staining with antibody against Hindsight, another marker for SOPs and their progeny (Fig. 3B′,C′) (Pickup et al., 2002). Thus, the lack of campaniform sensilla in *Act42A RNAi* flies could be attributed to the failure to induce SOPs in larval or early pupal stages.

Actin is required for maximal *phyllopod* transcription in neural precursors in an E-box-motif-dependent manner

To further study the roles of actin in Ac- and Sc-mediated transcriptional activation in SOPs, we examined *phyllopod* transcription in these precursors. *phyl*$^{3.4}$*-LacZ*, a transcription reporter gene carrying the 3.4 kb upstream *cis*-regulatory sequences of *phyllopod*, was expressed in two rows of chemosensory SOPs at the anterior wing margin (arrows in left panel of Fig. 4A) in response to Ac- and Sc-mediated transcriptional activation (Pi et al., 2004). In *C96-Gal4*-driven *Act42A RNAi* mutants in which *Act42A* RNAi were expressed along the wing margin, the level of LacZ appeared dramatically reduced (right panel of Fig. 4B). Quantification of anti-β-galactosidase immunofluorescence intensity, which was normalized to the co-stained Engrailed intensity in the posterior compartment, showed that LacZ expression in *C96>Act42A RNAi* wing discs was reduced to 48.3% compared with the *C96-Gal4* control ($P<0.001$, $n=16$ wing discs) (Fig. 4B).

We also examined whether the *phyl*$^{3.4}$*-LacZ* expression is compromised in *Act5C*^{EY11969} wing discs. In *Act5C*^{EY11969} hemizygotes, anti-β-galactosidase intensity was reduced to 67.5% compared with the control ($P<0.001$) (Fig. 4C and supplementary material Fig. S4A). Thus, both *Act5C* and *Act42A* are required for maximal expression of *phyl*$^{3.4}$*-LacZ* in SOPs.

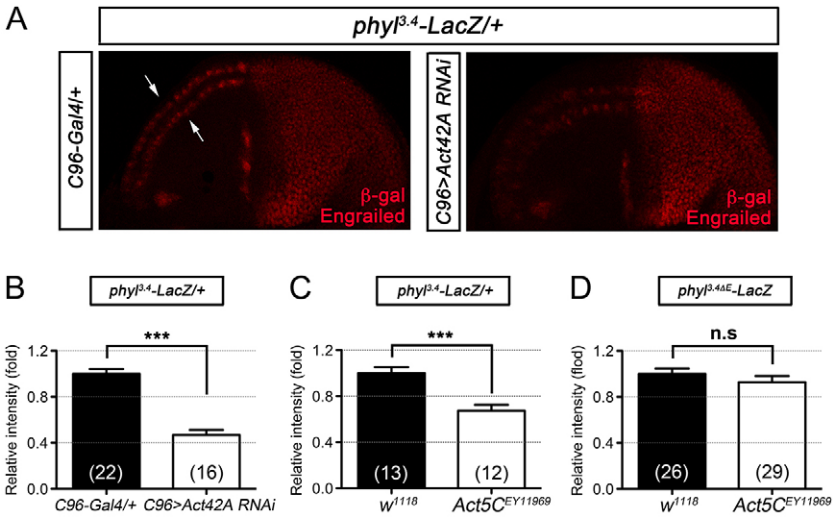

Fig. 4. *actin* positively regulates *phyllopod* transcription in neural precursors. (A) Images of 0–1 hour APF wing discs from *phyl*$^{3.4}$*-LacZ*/+ pupae, immunostained for β-galactosidase (red) and Engrailed (red). Anterior of the discs is to the left. Two rows of anterior wing marginal SOPs, used for quantification of anti-β-galactosidase intensity, are indicated by arrows. Intensity of the anti-Engrailed immunofluorescence detected in the posterior cells was used as an internal control for comparison of anti-β-galactosidase intensity among different discs. (B–D) Bar graphs show mean fold change of the normalized anti-β-galactosidase intensity (anti-β-galactosidase intensity/anti-Engrailed intensity) compared with control discs. Error bars represent s.e.m. Significance was determined using two-tailed Student's *t*-test. The numbers in the parentheses are the total wing discs scored. Significantly decreased *phyl*$^{3.4}$*-LacZ* levels are observed in *C96>Act42A* RNAi wing discs. ***$P<0.001$(B). *phyl*$^{3.4}$*-LacZ* level is significantly reduced in *Act5C*^{EY11969}/*Y* hemizygotes compared with wild-type *w*^{1118}/*Y* (C). ***$P<0.001$. (D) A comparison of β-galactosidase from E-box mutated *phyl*$^{3.4\Delta E}$*-LacZ* in wild-type *w*^{1118}/*Y* and *act5C*^{EY11969}/*Y* hemizygotes. n.s., not significant; $P>0.05$.

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The four putative Ac-Da- and Sc-Da-binding E-box sequences within the 3.4 kb upstream region are essential for *phyllopod* transcription in SOPs (Pi et al., 2004). The *phyl*$^{3.4\Delta E}$*-LacZ* reporter that harbors point mutations in the core sequences of all four E-boxes exhibited an 80% reduction in anti-β-galactosidase intensity compared with *phyl*$^{3.4}$*-LacZ* (data not shown). We asked whether *actin* promotes *phyllopod* transcription through these E-box sequences. In *Act5C*^{EY11969} hemizygotes, the *phyl*$^{3.4\Delta E}$*-LacZ* reporter was expressed at levels comparable with those in the wild-type control ($P>0.05$) (Fig. 4D and supplementary material Fig. S4B). Therefore, these data demonstrate that *Act5C* promotes *phyllopod* expression primarily through Ac-Da- and Sc-Da-bound E-box sequences.

Nucleus-localized actin enhances proneural-protein-mediated gene expression and ES organ formation

Our genetic analyses showed that reduction in total actin levels decreased E-box-dependent *phyllopod* transcription and disrupted SOP differentiation. However, it is unclear whether cytoplasmic or nuclear actin is involved in these processes. To address this, we first expressed nucleus-localized actin in S2 cells and assayed the mRNA levels of *phyllopod* and *asense*, two proneural protein-activated, SOP-specific genes. In S2 cells, *phyllopod* and *asense* mRNA levels, detected by quantitative RT-PCR (qRT-PCR), could be induced by transfected Myc-Ac and Myc-Da (Fig. 5A). As a control, the mRNA levels of *eIF1A* showed insensitivity to transfected Ac and Da (Fig. 5A). When Ac and Da-transfected S2 cells were co-transfected with increasing amounts of *myc-nls-Actin*, dose-dependent increases of *phyllopod* and *asense* mRNA levels were detected (Fig. 5D). qRT-PCR analyses revealed that *phyllopod* mRNA production was increased 2.6±0.8-fold ($P<0.01$) and 3.9±1.4-fold ($P<0.001$), respectively, in cells transfected with 50 and 125 ng of *myc-nls-Actin*, in comparison with cells transfected with GFP control (lanes 1–4 in Fig. 5D). *asense* mRNA levels were also increased 2.7±0.3-fold ($P<0.001$) after transfection with 125 ng *myc-nls-Actin* (lane 8 in Fig. 5D). However, the mRNA levels of *eIF1A* did not respond to increasing levels of Myc-NLS-Actin (lanes 9–12 in Fig. 5D). The enhanced expression of *phyllopod* and *asense* mRNA depend on the nuclear localization of actin, because co-transfection with increasing amounts of *myc-Actin* without the NLS failed to enhance *phyllopod* and *asense* expression in a dose-dependent manner, similar to the control *eIF1A* (lanes 13–24 in Fig. 5D). These analyses suggest that nuclear localization of actin is essential to drive Ac and Da-activated *phyllopod* and *asense* transcription. We confirmed the localization of Myc-NLS-Actin, which was primarily in nuclei, by immunofluorescent staining (bottom panels in Fig. 5B). By contrast, Myc-Actin was mainly localized in cytoplasm (top panels in Fig. 5B). Fractionation of cell lysates also indicated that, whereas Myc-Actin was mostly present in cytosol, the majority of Myc-NLS-Actin existed in nuclei (Fig. 5C).

We next asked whether the increased level of nuclear actin promotes Ac- and Sc-mediated ES organ formation. The formation of four bristles on the scutellum (arrows in left panel of Fig. 6A) requires *ac* and *sc*. In flies expressing GFP from *dpp-Gal4* along the boundary of the scutellum, 2.6% ($n=188$) had ectopic bristles (Fig. 6C). Expression of Myc-NLS-Actin, which also localized primarily in the nucleus of proneural cells (supplementary material Fig. S5A), promoted the formation of ectopic bristles in 27.7% ($n=386$) of the flies (Fig. 6C).

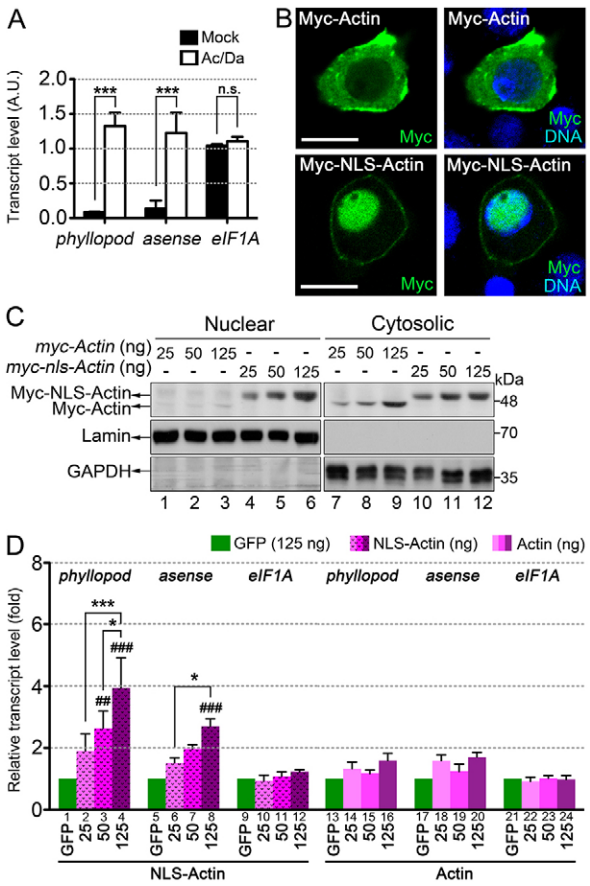

Fig. 5. Nuclear actin enhances proneural-protein-mediated genes expression in S2 cells. (A) qRT-PCR analysis of *phyllopod* and *asense* mRNA in S2 cells. Cells were transfected either with empty vector (mock), or with expression plasmids for Ac (125 ng) and Da (125 ng). Transcript levels were normalized with that of *rpL32*. Average (mean ± s.e.m.) is shown ($n=3$). Significance was determined by two-way ANOVA with Bonferroni's correction (post-tests); ***$P<0.001$; n.s., not significant. (B) Images of S2 cells immunostained with Anti-Myc antibody (green), co-stained with DNA dye Hoechst 33342 (blue). Top panels show Myc-Actin primarily localized in cytoplasm. Low levels of Myc-Actin are also detected in nuclei. Bottom panels show that Myc-NLS-Actin accumulated in nuclei. Scale bars: 10 µm. (C) Western blot analysis showing dose-dependent increases in nuclear Myc-NLS-Actin levels in cells transfected with increasing amounts (25, 50 and 125 ng) of *myc-nls-Actin* expression plasmid. (D) qRT-PCR analysis showing dose-dependent increases in *phyllopod* and *asense* expression in S2 cells transfected with increasing amount of *myc-nls-Actin* expression plasmid. All cells were transfected with expression plasmids for Ac (125 ng) and Da (125 ng), co-transfected with expression plasmids for GFP (125 ng), Myc-NLS-Actin (25, 50 or 125 ng) or Myc-Actin (25, 50 or 125 ng). Results are shown as average (mean ± s.e.m.) fold change compared with that in *GFP*-transfected cells ($n=3$). Significance was determined by two-way ANOVA with Bonferroni's correction (post-tests). ***$P<0.001$; *$P<0.05$; ##$P<0.01$ and ###$P<0.001$ when compared with cells transfected with *GFP* expression plasmid.

Expression of Myc-Actin, which localized mainly in the cytoplasm of proneural cells (supplementary material Fig. S5B), also induced ectopic bristles in 16.3% ($n=692$) of flies (Fig. 6C). These ectopic bristles were always located near the original ones (arrowheads in Fig. 6A), suggesting that they are derived from *ac*- and *sc*-expressing proneural clusters. To further examine the ability of nuclear actin to mediate proneural-protein-induced sensory organ formation, we expressed Myc-Actin or Myc-NLS-Actin together with Ac. Expression of *ac* by *dpp-Gal4* induced numerous ES organs at the boundary of the scutellum and at the

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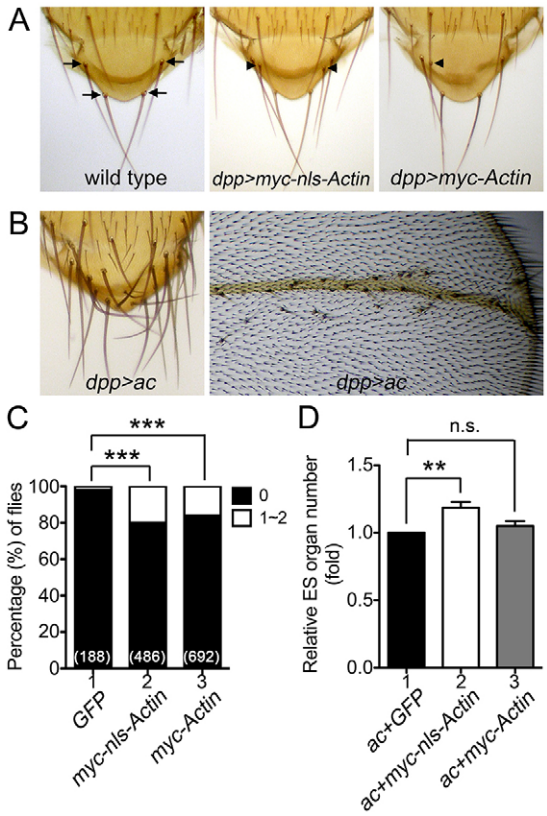

Fig. 6. Nuclear actin enhances proneural protein-mediated ES organ formation. (A) Images of adult scutellum. Four bristles (indicated by arrows) locate at the boundary of scutellum in wild-type fly (left panel). Formation of ectopic bristles (indicated by arrowheads) is observed in the vicinity of the original scutellar bristles in *dpp>myc-nls-Actin* (middle panel) and *dpp>myc-Actin* flies (right panel). (B) Numerous ectopic ES organs including bristles and campaniform sensilla formed at the scutellum (left panel), and on or near LIII vein (right panel) in *dpp>ac* flies. (C) Percentage of flies with ectopic scutellar bristles. Black bar, no ectopic bristles; white bar, one to two ectopic bristles. Significance was determined using chi-square test; ***$P<0.001$. The numbers in parentheses are the totals of adult females scored. Two to four independent transgenic fly lines were used for each genotype (*GFP*, *myc-nls-Actin* and *myc-Actin*). (D) Bar graph showing fold change in comparison to control flies (*dpp>Ac + GFP*) for the mean of the normalized ES organ numbers (ES organ numbers in *dpp*-Gal4-driven co-expression flies/ES organ numbers in sibling *dpp>ac* flies) along LIII vein per wing. Error bars represent s.e.m. Significance was determined using one-way ANOVA; **$P<0.01$; n.s., not significant. Two independent transgenic fly lines were used for each genotype (*GFP*, *myc-nls-Actin* and *myc-Actin*).

longitudinal wing vein LIII (Fig. 6B). Quantification of the number of ectopic bristles and campaniform sensilla on and near the LIII vein showed that co-expression of Myc-Actin did not significantly increase the number of ES organs induced by overexpression of Ac, compared with that in flies co-expressing GFP ($P>0.05$) (Fig. 6D). Co-expression of Myc-NLS-Actin, by contrast, significantly increased the average number of ES organs by 19% compared with GFP ($P=0.005$) (Fig. 6D). Our results suggest that, although both NLS-actin and actin induce bristles, NLS-actin specifically cooperates with Ac to promote ES organ formation.

DISCUSSION

Ac and Sc are master transcriptional activators of ES organ development in *Drosophila*. Nuclear actin is an essential component of the basal transcription machinery and the chromatin remodeling complex. Our study is the first to show a specific association between actin and Ac and Sc in nuclei. Supported by our *in vitro* and *in vivo* data in neural precursor gene expression and ES organ formation, we propose that nuclear actin is required for Ac (and Sc)-Da-mediated transcriptional activation in SOP differentiation.

Our results show that Ac and Sc associates with nuclear but not cytoplasmic actin, suggesting that actin protein alone might not be sufficient to maintain a stable interaction with Ac (and Sc). In the nucleus, actin associates with RNA polymerases and nucleosome-remodeling complexes. Recent studies also show that subfamilies of actin-related proteins, the major binding partner of actin, specifically localize in nuclei and act as crucial components in chromatin function (for reviews, please see Olave et al., 2002; Chen and Shen, 2007). One possibility is that these nuclear-actin-binding factors also contribute to stabilizing the interaction between Ac (and Sc) and actin. Alternatively, Ac (and Sc) might recognize specific modifications or conformations present only in nuclear actin. For example, it has been shown that nuclear actin is SUMOylated (Hofmann et al., 2009). Unlike cytoplasmic actin, nuclear actin can only form short oligomers, indicating that nuclear and cytoplasmic actin might exist in distinct conformations.

Although heterodimerization with Da is required for Ac and Sc to bind to the target E-box and to activate transcription, our results from co-immunoprecipitation and yeast two-hybrid experiments reveal that nuclear actin does not directly associate with Da. Da is ubiquitously expressed in all *Drosophila* cells, and Da-Da homodimers also regulate expression of genes involved in cell survival and growth (Bhattacharya and Baker, 2011). By contrast, *ac* and *sc* mRNA expression is strictly confined to the proneural cells, and Ac and Sc proteins rapidly degrade when SOPs differentiate (Pi et al., 2004; Chang et al., 2008). In addition, Ac-Da and Sc-Da heterodimers only activate neural-specific genes. Thus, association with Ac (and Sc) probably confers the specificity of nuclear actin to neural differentiation without interfering with normal cell growth.

Changes in actin levels in nuclei have been found in several studies to regulate cell differentiation. The dramatic 30-fold increase in nuclear actin levels in HL-60 cells treated with PMA induces gene expression required for differentiation into macrophages (Xu et al., 2010). Depletion of nuclear actin in epithelia cells triggered by treatment with ECM proteins downregulates transcription, leading to growth arrest and cell quiescence (Spencer et al., 2011). Maintenance of high levels of nuclear actin is important for integrity of nuclei in *Xenopus* oocytes (Bohnsack et al., 2006). Our studies in *Drosophila* ES organ development demonstrate that reduction in total actin levels, either in the specific *Act5C* mutant or by *Act5C*/*Act42A* RNAi knockdown, impaired ES organ formation and decreased E-box-mediated $phyl^{3.4}$*-LacZ* expression in neural precursors. Furthermore, increases in the levels of nucleus-localized actin specifically enhanced Ac–Da-mediated gene expression in S2 cells and Ac-induced ES organ formation. The fact that overexpression of actin promoted ectopic ES organs similar to nucleus-localized actin but failed to further increase the numbers of ES organs induced by Ac overexpression is consistent with the observation that normally only a small fraction of actin is localized to the nucleus. In *Drosophila*, an increase in nuclear actin levels has been observed in salivary gland cell nuclei upon overexpression of the nucleoskeleton protein EAST (Wasser and

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Chia, 2000). Owing to the strong non-specificity of anti-actin antibody in immunofluorescence staining of imaginal discs, we have not been able to specifically determine nuclear actin levels in SOPs. It remains to be seen whether nuclear actin levels change during differentiation of neural precursors.

During ES organ development, a cytoplasmic actin-rich structure (ARS) is formed at the apical region of the secondary precursors pIIa and pIIb, and is essential for pIIa/pIIb cell fate determination. The structure and function of ARS requires the Arp2/3 complex and WASp-mediated polymerization of branched actin filaments (Rajan, et al., 2009). Our results show that a reduction in Act42A levels disrupted SOP specification and reduced the number of bristles (Fig. 3; supplementary material Fig. S6A′,B′). In addition, a reduction in Act42A levels also led to decreased phalloidin staining in the apical area of ARS in the remaining two-cell clusters (supplementary material Fig. S6D′,E′). Unlike *Arp2/3* and *WASp* mutants that exhibited defects in pIIa specification, however, a strong reduction in Act42A levels appeared not to affect pIIa/pIIb cell fate determination; single neurons were observed within each developing ES organ (supplementary material Fig. S6C′). In cells with normal Arp2/3 and WASp activity, our results suggest that a reduced but functional ARS can form with decreased actin levels.

Ac and Sc auto-regulation is required for their accumulation in SOPs, an essential step for SOP selection (Culí and Modolell, 1998). In S2 cells, transfected Ac and Da also auto-activated endogenous *ac* expression (data not shown), which was further increased 2.1-fold by co-transfection of 125 ng *myc-nls-Actin* (supplementary material Fig. S7A). However, the levels of total nuclear Ac protein including endogenous Ac and transfected FLAG-Ac, remained similar among cells transfected with *GFP* or different amounts of *myc-nls-Actin* (supplementary material Fig. S7B), indicating that the increased *phyllopod* and *asense* expression by NLS-actin does not result from the increase in total Ac protein levels. For unknown reasons, transfection of Ac and Da was not sufficient to activate *sc* expression in S2 cells (data not shown).

MATERIALS AND METHODS

Cell culture and plasmid construction

Drosophila S2 cells were grown in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% FBS (Gibco BRL). Cells were transiently transfected with UAS-based expression plasmids together with driver *pWA-GAL4* (Kanuka et al., 2005). Transfection was carried out using Cellfectin II reagent (Invitrogen).

The expression plasmids for Ac, Sc and Da were constructed by cloning the ORFs into *Drosophila* gateway vectors pTMW or pTFW. DNA sequences for *myc-Act42A* and *myc-nls-Act42A* were cloned into *pUAST* vectors to generate *pUAS-myc-Actin* and *pUAS-myc-nls-Actin* plasmids, respectively. The sequence for a *Drosophila* nuclear localization signal was amplified from *pStinger* vector (Barolo et al., 2000).

Fractionation of nuclear and cytosolic extracts

Cytosolic and nuclear extracts were prepared from S2 cells 48–96 hours after transfection. All steps were performed on ice. Cells were washed once with PBS, followed by lysis in hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 300 mM sucrose) supplemented with complete protease inhibitors (Roche) for 10 minutes. NP-40 was then added to a final concentration of 0.5% and cells were incubated for 5 minutes to solubilize membrane protein. The efficiency of cell lysis was verified by Trypan Blue staining. After a short centrifugation, supernatant was removed and defined as the cytosolic extract. Pellets were washed twice with hypotonic buffer containing 0.5% NP-40. Nuclei were resuspended in nuclear buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 100 mM KCl, 0.2 mM EDTA, 20% glycerol) supplemented with protease inhibitors and incubated for 15–30 minutes on a nutator, followed by one or two freeze-thaw cycles. After centrifugation, supernatant was removed and defined as the nuclear extract. Cytosolic and nuclear extracts were subjected to a final round of centrifugation at >15,000 ***g*** for 20 minutes at 4℃ to obtain a cleared extract for further analysis.

Co-immunoprecipitation and western blotting

Cell lysate was fractioned into cytosolic and nuclear extracts as described. Protein extracts were diluted in co-immunoprecipitation buffer (20 mM HEPES, pH 7.6, 200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.5% Triton X-100) to a final concentration of 1 µg/µl. For co-immunoprecipitation using anti-FLAG antibody, 20 µl M2 affinity gel (Sigma) was incubated with 500 µl extract for 2 hours. For co-immunoprecipitation using anti-Myc antibody, 0.5 µg anti-Myc 9E10 antibody (Santa Cruz) was incubated with 500 µl extract overnight, followed by incubation with 20 µl Protein-G-agarose beads (Calbiochem) for 1 hour. Beads were collected by centrifugation and washed four times with co-immunoprecipitation buffer and twice with mRIPA buffer. Associated protein complexes were analyzed by SDS-PAGE followed by silver staining, or by western blotting. The following antibodies were used for western blot analyses: mouse anti-Ac (1:100, DSHB), mouse anti-actin (1:10,000, Millipore), mouse anti-FLAG (1:5000, Sigma), mouse anti-Myc (1:5000, Santa Cruz), mouse anti-Lamin Dm_0 ADL67 (1:5000, DSHB) and rabbit anti-GAPDH (1:5000, GeneTex).

Yeast two-hybrid analysis

Act42A ORF was cloned into *pGBKT7* vector to generate the *GAL4DB-Act42A* fusion gene. The *GAL4AD* fusion genes were generated by cloning *ac*, *sc* or *da* ORFs into plasmid *pGADT7* (Clontech). Plasmids were transformed into yeast strain AH109. To determine the interaction, the colonies grown on SD-Trp/-Leu plates were mixed and re-plated on both SD-Trp/-Leu and SD-Trp/-Leu/-His plates.

qRT-PCR and RT-PCR

Total RNA was isolated from 15–20 *Drosophila* adult heads or from S2 cells 48 hours post-transfection using TRIzol (Invitrogen). Quantitative RT-PCR was performed by iQ5 Gradient Real Time SYBR-Green PCR system (Bio-Rad). *rpL32* encoding the ribosomal protein L32 was used as a reference to normalize the qRT-PCR data. RT-PCR was performed using Taq DNA polymerase (NEB) for 25–35 cycles. Amplification products of *Act5C* and *Act42A* were resolved by 1% agarose gel. The relative density ratio of *Act5C* and *Act42A* was measured by ImageJ software with β-tubulin as an internal standard. PCR reactions were performed by using the following pairs of primers: *phyllopod*, 5′-AACCACACTCAGTCGGGAAG-3′ and 5′-CGGTATCGAATTCC-AGCAGT-3′; *asense*, 5′-ACAGCATATAGCCAATCAGAACA-3′ and 5′-GCCTCTTCCGTTGACTGG-3′; *ac*, 5′-CCAAGTTGGAAGCCAGT-TTT-3′ and 5′-TATGGTTGGGTGCGACTAGC-3′; *eIF1A*, 5′-CGCAG-ACAGCAACAACATC-3′ and 5′-TCTTACCACGACGACGATTCT-3′; *Act5C* isoforms, *RA*/*RC*/*RD*: 5′-GCCAGCAGTCGTCTAATCCA-3′ and 5′-GACCATCACACCCTGGTGAC-3′; *Act5C* isoform *RB*, 5′-TCGCC-ACTTGCGTTTACAGT-3′ and 5′-CCCACGTACGAGTCCTTCTG-3′; *rpL32*, 5′-ATCGGTTACGGATCGAACAA-3′ and 5′-GACAATCTCCT-TGCGCTTCT-3′; *Act5C*, 5′-AGCAGTCGTCTAATCCAGAGAC-3′ and 5′-ATACATGGCGGGTGTGTTGAAG-3′; *Act42A*, 5′-GTAGGCGTCG-GTCAATTC-3′ and 5′-CTGAGTCATCTTCTCGCGAT-3′; *βTub60D*, 5′-ATCATTTCCGAGGAGCACGGC-3′ and 5′-GCCCAGCGAGTGCGTC-AATTG-3′.

Fly strains

w^{1118} flies were used as wild type. $sc^{10\text{-}1}$ (Villares and Cabrera, 1987), da^{10} (Caudy et al., 1988), *dpp-GAL4* (Staehling-Hampton et al., 1994) and *pnr-GAL4* (Heitzler et al., 1996) were described previously. $Act5C^{EY11969}$, $Act5C^{G0010}$ and $Act5C^{G0486}$ flies were obtained from

Bloomington *Drosophila* Stock Center. *UAS-Act5C RNAi* (v7140) and *UAS-Act42A RNAi* (v12456) flies were obtained from Vienna *Drosophila* RNAi center (VDRC). $phyl^{3.4}$ and $phyl^{3.4\Delta E}$ promoters (Pi et al., 2004) were cloned into *pPelican* (Barolo et al., 2000) to generate $phyl^{3.4}$*-LacZ* and $phyl^{3.4\Delta E}$ *-LacZ*. Transgenic lines including $phyl^{3.4}$*-LacZ*, $phyl^{3.4\Delta E}$ *–LacZ*, *UAS-myc-Actin*, *UAS-myc-nls-Actin* were generated by standard P-element-mediated transformation. All flies were incubated at 25℃ unless otherwise noted.

Immunostaining and image analysis

Pupal nota and wing discs were dissected in PBS, fixed in 4% paraformaldehyde, and permeabilized with 0.1% and 0.3% TritonX-100 in PBS, respectively. S2 cells were transferred to ConA-treated coverslips (Buster et al., 2010), fixed with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS (PBST) and then blocked with 5% BSA in PBST. Imaginal discs and cells were incubated with primary antibodies overnight at 4℃ and secondary antibodies for 2 hours at room temperature. The following primary antibodies were used: mouse anti-Ac (1:5, DSHB), mouse anti-β-galactosidase (1:500, DSHB), mouse anti-Elav (1:500, DSHB), mouse anti-En (1:500, DSHB), mouse anti-Hnt (1:25, DSHB), mouse anti-Myc (1:1,000, Santa Cruz), rabbit anti-Myc (1:1,000, Santa Cruz) and guinea pig anti-Sens (1:4000, a gift from Dr Bellen, Baylor College of Medicine). Alexa-Fluor-488-conjugated (Molecular Probes) and Cy3-conjugated (Jackson ImmunoResearch) donkey anti-mouse IgG antibodies were used at dilution of 1:500 to 1:1000. Alexa-Fluor-543-conjugated phalloidin was used at 1:25 dilution.

All immunofluorescence images were obtained with a Zeiss LSM 510 Meta microscope at a fixed detection setting. For quantification of anti-β-galactosidase intensity in Fig. 4, pixel intensity in 10 dorsal and 10 ventral anterior wing marginal SOPs in each disc was measured using ImageJ software. The average anti-β-galactosidase intensity for each disc was then subtracted by the average background intensity in the anterior wing pouch cells, normalized with the average anti-En intensity measured from the posterior wing pouch cells.

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Competing interests

The authors declare no competing interests.

Author contributions

Y.-L. H. designed and performed experiments, analyzed data and wrote the manuscript. Y.-J. C. performed co-immunoprecipitation for mass spectromic analysis and fly genetic analysis. H.-F. Y., Y.-J. C. and Y.-C. H. performed fly genetic analysis and immunostaining. H. P. designed the study, analyzed data and wrote the manuscript.

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Supplementary material

Supplementary material available online at
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