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The kinase Sgg modulates temporal development of macrochaetes in *Drosophila* by phosphorylation of Scute and Pannier

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SUMMARY

Evolution of novel structures is often made possible by changes in the timing or spatial expression of genes regulating development. Macrochaetes, large sensory bristles arranged into species-specific stereotypical patterns, are an evolutionary novelty of cyclorraphous flies and are associated with changes in both the temporal and spatial expression of the proneural genes *achaete* (*ac*) and *scute* (*sc*). Changes in spatial expression are associated with the evolution of cis-regulatory sequences, but it is not known how temporal regulation is achieved. One factor required for *ac-sc* expression, the expression of which coincides temporally with that of *ac-sc* in the notum, is Wingless (Wg; also known as Wnt). Wingless downregulates the activity of the serine/threonine kinase Shaggy (Sgg; also known as GSK-3). We demonstrate that Scute is phosphorylated by Sgg on a serine residue and that mutation of this residue results in a form of Sc with heightened proneural activity that can rescue the loss of bristles characteristic of *wg* mutants. We suggest that the phosphorylated form of Sc has reduced transcriptional activity such that *sc* is unable to autoregulate, an essential function for the segregation of bristle precursors. Sgg also phosphorylates Pannier, a transcriptional activator of *ac-sc*, the activity of which is similarly dampened when in the phosphorylated state. Furthermore, we show that Wg signalling does not act directly via a cis-regulatory element of the *ac-sc* genes. We suggest that temporal control of *ac-sc* activity in cyclorraphous flies is likely to be regulated by permissive factors and might therefore not be encoded at the level of *ac-sc* gene sequences.

KEY WORDS: *Drosophila*, Scute, Phosphorylation, Sgg, GSK-3, Bristle, Wingless

INTRODUCTION

Evolution of novel structures can result from changes in either temporal or spatial expression of genes during development. Both mechanisms appear to underlie evolution of bristle patterns in Diptera. The dorsal thorax of many dipteran flies is covered with evenly spaced mechanosensory bristles. In species of the Nematocera, a basal group, the bristles are of similar size and are not patterned, such that their number and position varies from one individual to another (McAlpine, 1981). The thorax of many species of the more recently evolved Cyclorrapha bear evenly spaced small bristles of uniform size, called microchaetes, that also vary in position between individuals (Simpson et al., 1999). However, in the lineage leading to the Cyclorrapha an evolutionary novelty, the macrochaetes, has arisen (Simpson et al., 1999). Macrochaetes are large bristles that are not evenly spaced but are arranged into stereotypical species-specific patterns (McAlpine, 1981). Their development is associated with two derived features. First, in species of Nematocera, such as *Anopheles gambiae*, duration of development of the imaginal thorax is very short and sensory organ precursors arise from a single phase of expression of the proneural genes that regulate bristle development (Wulbeck and Simpson, 2002; Simpson and Marcellini, 2006). In cyclorraphous flies there are two temporally separate phases of proneural gene expression during the protracted development of the thoracic imaginal epithelium, an early one for macrochaetes and a later one for microchaetes (Simpson et al., 1999; Simpson and Marcellini, 2006). Second, microchaete precursors arise from ubiquitous or very broad domains of proneural gene expression, as do the sensory organ precursors in *Anopheles gambiae* (Sato et al., 1999; Wülbeck and Simpson, 2000; Pistillo et al., 2002; Wulbeck and Simpson, 2002). Proneural gene expression for macrochaete development, however, is not uniform but spatially patterned such that it prefigures the sites at which macrochaete precursors arise (Cubas et al., 1991; Skeath and Carroll, 1991; Wülbeck and Simpson, 2000; Pistillo et al., 2002).

In *Drosophila* the proneural genes *achaete* (*ac*) and *scute* (*sc*), which encode basic helix-loop-helix transcription factors, underlie bristle development (Villares and Cabrera, 1987; Gonzalez et al., 1989; Modolell and Campuzano, 1998). Spatially restricted expression of *ac-sc* in discrete proneural clusters at the sites of origin of macrochaete precursors on the thorax has been investigated. On the one hand, a number of antagonists prevent activity of *ac-sc* at locations outside the proneural clusters, by interfering with the accumulation of *ac-sc* products arising from activity of the basal promoters (Usui et al., 2008). On the other hand, a prepattern of transcription factors activates expression through an array of discrete cis-regulatory elements scattered throughout the *ac-sc* complex (*AS-C*) (Gomez-Skarmeta et al., 1995; Garcia-Garcia et al., 1999; Calleja et al., 2002). Gene duplication events at the *ac-sc* locus are thought to have provided material for the acquisition of the regulatory elements that have presumably evolved successively over an extended period of time (Skaer et al., 2002b; Negre and Simpson, 2009). It is not known how *ac-sc* expression is

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temporally regulated or whether the heterochronic shift from a single to two phases of expression is also linked to evolution of regulatory sequences at the *ac-sc* locus.

Factors responsible for transcriptional activation of *ac-sc* in proneural clusters are present in the imaginal disc for a considerable time before *ac-sc* expression and so do not adequately account for timing (Calleja et al., 2000; Klein, 2001; Cavodeassi et al., 2002). We have started to examine temporal control by investigating the mode of action of *wingless* (*wg*), a gene for which expression coincides temporally with the initiation of expression for macrochaetes and that is essential for development of macrochaetes on the medial notum (Phillips and Whittle, 1993). The product of *wg* is a diffusible Wnt signalling factor that has been shown to have a permissive, rather than instructive, role with respect to the patterning of *ac-sc* expression (Garcia-Garcia et al., 1999). The Wg signal acts by downregulating the activity of the serine/threonine kinase Sgg (Logan and Nusse, 2004). We show that Scute and Pannier (a transcriptional activator of *ac-sc*) (Haenlin et al., 1997; Garcia-Garcia et al., 1999) are phosphorylated by Shaggy (Sgg) and that phosphorylation-resistant versions of these proteins are hyperactive. We suggest that the Wg signal allows nonphosphorylated Sc to accumulate sufficiently to allow autoregulation and segregation of bristle precursors at mid third larval instar. Temporal control of *ac-sc* activity in cyclorraphous flies could therefore be due to permissive factors and not encoded at the level of *ac-sc* gene sequences.

MATERIALS AND METHODS

Microscopy

Confocal images were taken with a Leica SP1 or SP5. Brightfield images were taken with a Leica DMRA microscope fitted with a QImaging camera and QCapture Pro software. Images were processed in Adobe Photoshop CS and Adobe Illustrator CS. Thorax images were assembled by taking images at sequential focal planes (approximately 20 μm intervals), and the image stacks were then merged with the Stack Focuser plugin (Michael Umorin) for ImageJ (http://imagej.nih.gov/ij).

Fixation and immunohistochemistry

Wandering third instar larvae were fixed according to standard protocols (Sullivan et al., 2000). Primary antibodies used were: anti-GFP (Goat) 1:500 Abcam ab6673; anti-Sc (Rb) 1:1000 Y-N Jan; anti-Ac (M) 1:10 DSHB; anti-β-Gal (Rb) 1:10,000 Cappel; anti-Hnt (M) 1:100 DSHB; anti-Wg (M) 1:200 DSHB. Fluorescence-conjugated secondary antibodies were obtained from Invitrogen and Jackson Laboratories. Wing discs were mounted in Vectashield (Vector Laboratories).

Thorax preparations

Adult flies were collected and stored in 70% ethanol. Thoraxes were dissected and incubated in 0.3 M NaOH at 70°C until cleared. After washing, thoraxes were mounted in Euparal (Fisher Scientific). Staples were used to raise the coverslip and prevent cuticle deformation. Bristle measurements were made with QCapture Pro software.

Fly stocks

The following fly stocks were used: *pnr^{MD237}-GAL4/TM6B* (Bloomington); *c765-GAL4/TM6B* (Gomez-Skarmeta et al., 1996); *pnr^{VX6}* (Ramain et al., 1993); *DC1.4-lacZ* (Garcia-Garcia et al., 1999); *P[GFP], P[FRT]101; MKRS, P[hs-FLP]86E/TM6B* (Bloomington); *P[GFP], P[FRT]18A; MKRS, P[hs-FLP]86E/TM6B* (Bloomington); *sgg/GSK-3^{M11}, P[FRT]101/FM7* (Bloomington); *sgg/GSK-3^{D127}, P[FRT]18A/FM7* (Ruel et al., 1993); *wg^{CX4}/CyO, P[act-GFP]* (Bloomington); *wg^{Sp-1}/CyO, P[act-GFP]* (Bloomington); *DC-GAL4*; *UAS-sgg/GSK-3^{WT}* (wild-type Sgg/GSK-3, Bloomington); *UAS-sgg/GSK-3^{S9A}* (activated Sgg, Bloomington); Oregon R (wild type, Bloomington).

Crosses were carried out at 25°C. Clones mutant for *sgg* were induced in mid first to mid second instar larvae using the FLP/FRT method (Xu and Rubin, 1993). Discs were harvested from wandering third instar larvae, or adult flies were collected for bristle analysis.

Site-directed mutagenesis and generation of transgenic flies

Mutagenesis was performed using the QuikChangeII kit (Stratagene), according to the manufacturer's instructions. See supplementary material Table S1 for primer sequences. Note that Pnr exists in two isoforms (Fromental-Ramain et al., 2008), with conserved Sgg phosphorylation sites: in this work, the *pnrβ* isoform was used.

To produce phosphorylation-resistant forms of Dm-pnrβ and Dm-Sc, the appropriate sequences were cloned into the pGEM-T Easy vector (Promega) for mutagenesis. *Dm-pnrβ* was derived from pGEX-2T Pannier (Biryukova and Heitzler, 2008), whereas *Dm-sc* was PCR amplified. Mutated sequences were then subcloned into pUAST (Brand and Perrimon, 1993).

For the T123 reporter construct, putative dTCF binding sites were identified using MatInspector (Genomatix) (Cartharius et al., 2005). The DC enhancer sequence (GenBank accession no. AF132808.1) was cloned into the pGEM-T Easy vector (Promega) for mutagenesis, and then subcloned into the pStinger-GFP (DGRC) reporter construct.

In all cases, mutations were verified by sequencing. Flies were transformed by Genetic Services, MA and Department of Genetics, University of Cambridge.

Protein expression

Dm-pnrβ and *Dm-sc* were cloned into the pGEM-T Easy vector as described above, with the addition of *Eco*RI and *Hin*dIII restriction sites. They were then subcloned into the expression vector pET-40b(+) with His-Tag (Novagen) and expressed in the BL21(DE3) *Escherichia coli* strain (Agilent Technologies). To solubilize His-tagged fusion proteins from inclusion bodies, harvested cells were suspended in ST buffer with 5% sarkosyl (Tao et al., 2010). Recombinant proteins were affinity purified using Ni-NTA Agarose beads (Qiagen), and checked by western blots with appropriate antibodies.

Immunoprecipitation

For Dm-Pnr and Dm-Scute, protein extracts were made from wild-type *Drosophila* larvae, and immunoprecipitation performed using the Pierce Direct IP kit (Thermo Scientific) or the protein-G-agarose kit (Roche). Immunoprecipitated Sgg and immunodepleted Sgg were prepared from extracts of *Drosophila* wing discs according to the method of Gogel (Gogel et al., 2006). Antibodies used were pAb-Pnr (Haenlin et al., 1997), pAb-Sc (Vaessin et al., 1994) and mAb-Sgg 2G2C5 (Ruel et al., 1993).

In vitro kinase assays

Purified recombinant His-tagged Pnr attached to Ni-NTA agarose beads (4 μg) was incubated with either immunoprecipitated Sgg kinase or immunodepleted Sgg (Sgg–) in 40 μl kinase buffer (40 mM HEPES, pH 7.4, 2 mM ATP, 5 mM EGTA, 2 mM $MgCl_2$, 2 mM DTT, 1× protease inhibitor and phosphatase inhibitor cocktail (Roche) overnight at 37°C. The Sgg inhibitor LiCl (30 mM) was added to some reactions. The phospho-specific antibody pAb BUGS (Trivedi et al., 2005) was used to detect phosphorylation of the His-pnr substrate. Protein-loading levels were monitored by probing the stripped blot with a rabbit His-tag antibody (GenScript).

Sc protein does not react with the BUGS antibody in vitro, so in this case kinase assays were performed using radiolabelled λ-P^{33}ATP. Reactions with immunoprecipitated Sgg were carried out in 20 μl kinase buffer supplemented with 200 μM cold ATP plus 2 μCi of [λ-P^{33}] ATP (PerkinElmer), and 1 μg of purified recombinant Sc. After incubation at 30°C for 2 hours, the reactions were terminated with SDS sample buffer followed by 12% SDS-PAGE. The gel was dried and the phosphorylated protein was visualized by autoradiography.

RESULTS

Temporal expression of *wingless* coincides with that of *achaete-scute*

Cells for the future wing disc are set aside in the embryo of *Drosophila* and separation of the notum and wing pouch takes place during the first half of the second larval instar, mediated by the restriction of expression of *wg* to the presumptive wing blade

and of the genes of the *Iroquois* complex (*Iro-C*), which encode homeodomain-bearing transcription factors, to the presumptive notum (reviewed by Klein, 2001). At late second instar, *pannier* (*pnr*), a gene encoding a GATA factor, is expressed in the medial half of the notum where, together with U-shaped (Ush), it represses the *Iro-C* genes restricting their activity to the lateral notum (Sato and Saigo, 2000; Tomoyasu et al., 2000; Cavodeassi et al., 2002) (supplementary material Fig. S1A). The products of *pnr* and the *Iro-C* genes directly activate *ac-sc* in proneural clusters at specific sites in the epithelium through discrete cis-regulatory elements (Cubas et al., 1991; Skeath and Carroll, 1991; Gomez-Skarmeta et al., 1995; Gomez-Skarmeta et al., 1996; Garcia-Garcia et al., 1999). However, *ac-sc* products become detectable in discs only at mid third larval instar, in spite of the fact that Pnr and Iro-C proteins are present much earlier.

Another factor that is required for *ac-sc* expression is Wg. *wingless* is activated by Pnr in a longitudinal stripe in the Ush-free region of the notum (Couso et al., 1993; Phillips and Whittle, 1993; Sato and Saigo, 2000; Tomoyasu et al., 2000) (supplementary material Fig. S1A,B). However, Wg does not appear to be involved in determining spatial expression of *ac-sc*; rather, it has been shown to have a permissive role (Garcia-Garcia et al., 1999). Unlike that of *pnr* and *Iro-C*, expression of *wg* does coincide temporally with that of *ac-sc*. *wingless* protein is first detectable at early third larval instar and accumulates substantially by mid third larval instar when Sc first becomes visible (supplementary material Fig. S1C,D).

Loss or gain of function of *wingless* and *shaggy/GSK-3* affect the time of birth of bristle precursors

Binding of Wg to its receptor leads to inactivation of Sgg, and it has previously been shown that, although loss of function of *wg* leads to a loss of macrochaetes in the medial notum, loss of function of *sgg* causes the opposite phenotype of additional macrochaetes (Simpson and Carteret, 1989; Couso et al., 1993; Phillips and Whittle, 1993; Neumann and Cohen, 1996). Correspondingly, expression of a constitutively active form of Sgg (SGG10S9A) mimics *wg* mutants and results in a loss of macrochaetes on both the medial and lateral notum (Fig. 1) (Bourouis, 2002).

In addition to bristle loss it was noted that the bristles that do form after expression of constitutively active Sgg appeared smaller (Papadopoulou et al., 2004). Similarly, we find that hypomorphic mutants of *wg* display some macrochaetes that have shortened shafts (Fig. 1D). For these studies we focussed on $wg^{Sp\text{-}1}/wg^{CX4}$, a viable allelic combination. In flies of this genotype, expression of *wg* on the notum is strongly reduced, being restricted to the posterior notum (supplementary material Fig. S1F). We have quantified the number and size of dorsocentral (DC) bristles after loss of function of *wg* or gain of function of *sgg*. The anterior DC bristle (aDC) is missing in 61% of $wg^{Sp\text{-}1}/wg^{CX4}$ male flies. When present, the aDC in flies of this genotype is 14% shorter (212 µm±4.3, n=15) than in control siblings (246 µm±4.8, n=37; P<0.01) (Fig. 1D,E).

We used the driver *c765-Gal4*, which gives a generalized expression over most of the thorax (Gomez-Skarmeta et al., 1996), to overexpress wild-type Sgg (*UAS-sgg/GSK-3*^{WT}) and constitutively active Sgg (*UAS-sgg/GSK-3*^{act}) (Bourouis, 2002). Overexpression of Sgg/GSK-3^{WT} does not cause loss of bristles but the length of the posterior DC (pDC) bristle shaft is decreased by about 15% (281.9 µm±4.8, n=58) when compared with wild type (333 µm±2.37, n=39) (Fig. 1B,E). Nearly 20% of flies

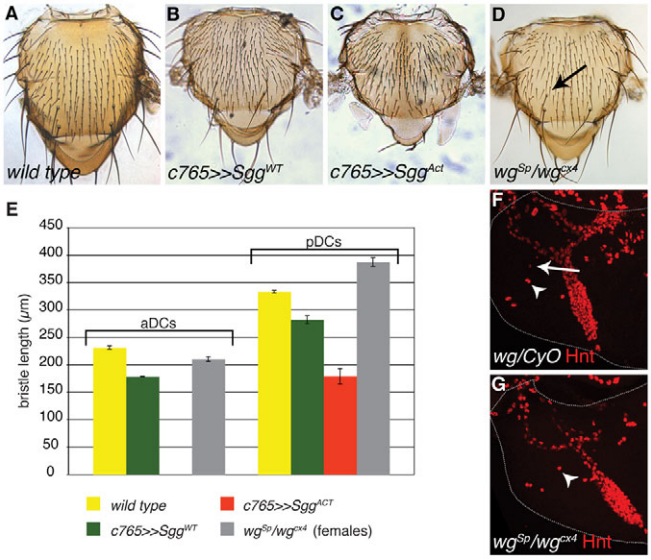

Fig. 1. Bristles are shorter in *wingless* loss-of-function and *shaggy/GSK-3* gain-of-function mutants and formation of bristle precursors is delayed. (**A-E**) The thoraces of wild type (A), *c765-Gal4 >UAS-Sgg*^{WT} (B), *c765-Gal4>UAS-Sgg*^{act} (C) and wg^{Sp}/wg^{CX4} (D) are shown. Arrows indicate the aDC bristle. The lengths of the DC bristles are given in E. Error bars represent mean±s.e. (**F**,**G**) Wing discs from white prepupae of wg^{Sp}/wg^{CX4} (G) and heterozygous *wg*/+ control larvae (F) are stained with an anti-Hindsight antibody to reveal bristle precursors. Arrows point to the DC precursors, which are visible in the control disc but have not yet formed in the mutant disc. Arrowheads point to pDC precursors present in both wild-type and mutant discs.

overexpressing Sgg/GSK-3^{act} are missing all DC bristles and the remainder display a single tiny pDC that is 46% shorter in length (178.7±5.9, n=38) than seen in wild type (Fig. 1C,E).

The results show that loss of function of *wg* or gain of function of *sgg* results in a loss of bristles, as well as the development of smaller bristles. Bristle length correlates with the time of precursor formation (Skaer et al., 2002a; Usui-Ishihara and Simpson, 2005) because the size of the bristle is proportional to the number of endocycles undergone by the socket and shaft cells (Szuplewski et al., 2009). Smaller bristles are therefore likely to be due to delayed segregation of bristle precursors. A delay in precursor formation could also lead to bristle loss because precursors need to form within a defined period of competence (Rodriguez et al., 1990). This is consistent with the preferential loss of the aDC, as the precursor of the aDC is known to form later than that of the pDC (Huang et al., 1991). To determine whether precursor development is delayed in *wg* mutants, we examined the time of precursor formation in $wg^{Sp\text{-}1}/wg^{CX4}$ flies using an anti-Hindsight (Hnt) antibody (Lai, 2003). In white prepupae, 10.5% of discs displayed two Hnt-expressing DC precursors, 60.5% displayed a single DC precursor and 29% had no DC precursors at all (n=38). Nearly 40% of adults of this genotype bear two DCs and the remainder have a single one, so the segregation of about 40% of the expected precursors must occur after pupariation. In control discs from white prepupae of *wg/CyO* siblings, 80% of discs displayed two DC precursors and the remainder had a single one (n=15), so only 10% of precursors segregate after pupariation (Fig. 1F,G).

We conclude that Wg acts through Sgg to regulate the time of birth of bristle precursors. Loss of function of *wg* leads to a delay in the formation of DC bristle precursors that can lead to the

development of smaller bristles and might also cause an absence of bristles. A gain of function of *sgg* is likely to have a similar effect. The additional bristles that develop after loss of function of *sgg* could therefore result from an extended period of precursor segregation provided by an earlier start.

The dorsocentral enhancer of *achaete-scute* does not contain functional dTCF binding sites

Expression of *ac-sc* for DC bristle development depends on the activity of a cis-regulatory element called the *DC enhancer* (*DCE*) (Gomez-Skarmeta et al., 1995; Garcia-Garcia et al., 1999). In wg^{cx3}/wg^{cx4} mutants, a transheterozygous combination in which Wg protein levels are significantly reduced in the notum region of third instar wing discs (Klein et al., 1998), expression of the *DCE-lacZ* enhancer gene is notably reduced (Garcia-Garcia et al., 1999). Here we have examined the activity of *DCE-lacZ* in clones of cells mutant for null alleles of *sgg*, sgg^{D127} or sgg^{M11}. The *DCE*-reporter transgene is ectopically expressed in large *sgg* clones (Fig. 2A). Thus, Wg signalling is required for activation of the *DCE* in its normal spatial domain.

If Wg acts via the canonical Wnt signalling pathway, then the *DCE* should contain a functional dTCF binding site(s). A search for the dTCF consensus sequence (van de Wetering et al., 1997), using MatInspector (Cartharius et al., 2005) (see Materials and methods), identified three potential binding sites (supplementary material Fig. S2). Two of the sites are conserved in the *D. virilis DCE* (not shown). To determine whether the dTCF sites are functionally relevant, all three sites in a *DCE-GFP* enhancer construct were mutated. At least two bases of the core TTGA motif were altered for each site and the modified construct was named DCE^{T123}*-GFP* (supplementary material Fig. S2). The expression pattern of DCE^{T123}*-GFP* was compared with the unmodified *DCE-lacZ* reporter in transheterozygous flies. The expression patterns correspond almost perfectly, demonstrating that the predicted dTCF sites are not required for normal *DCE* activity (Fig. 2B). This suggests that the sites are not functional and that Wg acts on the *DCE* via a noncanonical Wnt signalling pathway.

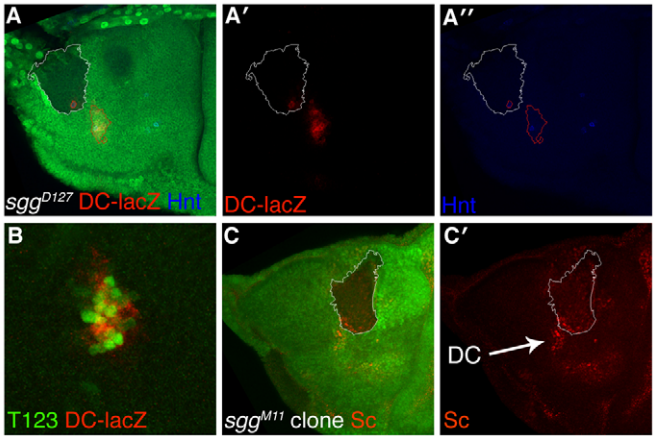

Fig. 2. Expression of *scute* and a *DCE* reporter gene in *sgg* mutants and after mutation of putative dTCF binding sites. (**A-A″**) Expression of the *DCE-lacZ* reporter (red) is visualized in a third-larval-instar wing disc bearing a clone of cells mutant for $sgg/GSK\text{-}3^{D127}$ (outlined). Staining for Hindsight (blue) indicates the dorsocentral bristle precursors. An additional domain of *DCE-lacZ* reporter expression is present in the $sgg/GSK\text{-}3^{D127}$ clone and includes an ectopic precursor. (**B**) Co-expression of the *DCE-lacZ* (red) and the DCE^{T123}*-GFP* (green) reporters, in which the three putative dTCF binding sites are mutated, is shown in a third-larval-instar wing disc. The two coincide at the position of the dorsocentral proneural cluster. (**C**,**C′**) Ectopic expression of *scute* (red) in a clone of cells mutant for $sgg/GSK\text{-}3^{M11}$ (outlined) is shown.

Pannier is a target for phosphorylation by Sgg

Activation of *ac-sc* transcription via the *DCE* is through the GATA factor Pnr (Garcia-Garcia et al., 1999). The phosphorylation predication network NetPhosK 1.0 Server (Blom et al., 2004), reveals two potential sites that fit the consensus for phosphorylation by Sgg (at amino acids at 101 and 391) in Pnrβ of *D. melanogaster*. They display homology with the GSK-3β-phosphorylation site of the mouse MAP1B (microtubule associated protein) (Fig. 3A), a peptide of which was used to raise the BUGS phospho-antibody (Trivedi et al., 2005). This antibody has been successfully used to detect phosphorylation by Sgg of *D. melanogaster* Futsch (Gogel et al., 2006). Therefore, we used this antibody to determine whether Sgg can phosphorylate Pnr.

Immunoprecipitation (IP) was used to obtain an enriched Pnr extract from wild-type larvae. Western blot analysis showed that the phospho-antibody BUGS robustly recognized a protein at about 60 kDa, which is the size expected for Pnr, as indicated by the Pnr antibody (Fig. 3B). This result suggests that *D. melanogaster* Pnr is phosphorylated at a site with the consensus sequence for phosphorylation by Sgg that is recognized by the phospho-antibody BUGS.

To determine whether Sgg can indeed phosphorylate Pnr, we employed an in vitro kinase assay. Recombinant His-tag Pnr was incubated with Sgg, obtained through IP from a *Drosophila* wing disc extract. As a control, recombinant Pnr was incubated with the immunodepleted extract (see Materials and methods). A western blot showed that the recombinant *pnr* protein alone displayed no immunoreactivity with BUGS (not shown). By contrast, after incubation with Sgg, the phospho-antibody BUGS can recognize Pnr, thus indicating that Pnr has been phosphorylated by Sgg in vitro (Fig. 3C). Immunodepleted Sgg showed reduced kinase activity towards Pnr. Furthermore, in this assay, phosphorylation of Pnr by Sgg was inhibited by lithium chloride, a specific GSK-3 inhibitor (Cohen and Frame, 2001).

In summary, these findings show that the pAb BUGS specifically recognizes a Pnr phospho-serine epitope, indicating that Pnr is phosphorylated by Sgg in vitro.

A phosphorylation-resistant form of Pannier induces ectopic bristles

To test the significance of the Sgg phosphorylation sites in Pnr we mutated the central serine residues at the two sites to alanines (Fig. 3A). Transgenic flies carrying the mutated Pnr, designated Pnr^{MUT}, in a *UAS* vector were crossed to various *Gal4* drivers. The results were compared with expression of a wild-type version of Pnr, *UAS-*Pnr^{WT}. Both Pnr^{WT} and Pnr^{MUT} induced additional macrochaetes on the lateral notum of flies of the genotype *c765-Gal4>UAS-Pnr*. Overexpression of Pnr^{WT} caused the production of an average of 9.5 extra macrochaetes per thorax (n=108) and that of Pnr^{MUT}, 19 extra macrochaetes per thorax (n=100) (Fig. 3D) (note that these numbers do not include the 11 macrochaetes normally found on the wild-type thorax). The phosphorylation-resistant form of Pnr therefore displays stronger bristle-inducing activity.

We also tested whether the phosphorylation-resistant form of Pnr rescues the DC bristles missing from pnr^{VX6}/pnr^{MD237} transheterozygotes. $pannier^{VX6}$ is a null allele and pnr^{MD237}

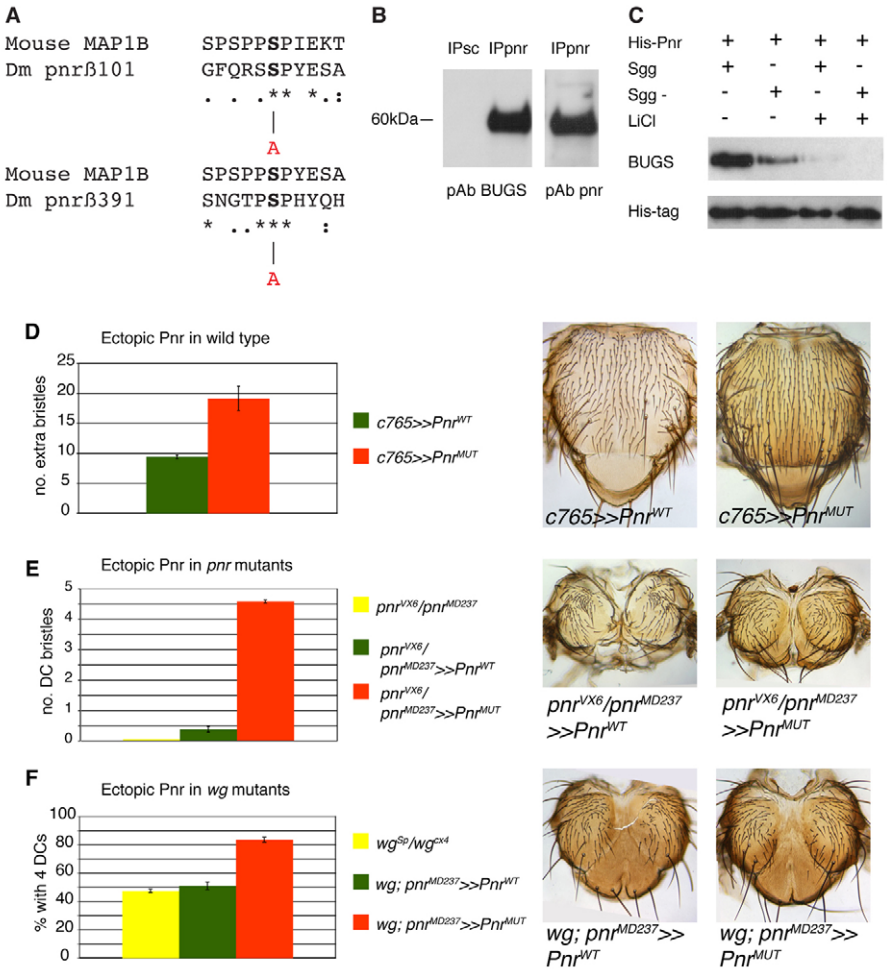

Fig. 3. Phosphorylation of Pannier by Sgg and bristle phenotypes observed after expression of Pannier bearing mutated phosphorylation sites. (**A**) The amino acid code of the mouse microtubule associated protein (MAP1B) peptide sequence used to raise the BUGS phospho-antibody (Trivedi et al., 2005) is compared with two potential phosphorylation sites in Pannier (Pnr). Asterisk indicates conserved residues. The central serine residue (S, bold) is the site for phosphorylation and is conserved in Pnrβ at amino acids 101 and 391. Red indicates bases altered in the *pnr* mutant construct. (**B**) Immunoprecipitated *pannier* (*pnr*) and *scute* (*sc*) proteins were used for western blot analysis with the Pnr and BUGS antibodies. A 60 kDa band is seen with both the phospho-antibody BUGS and the Pnr antibody in duplicate filters. No signal was seen with immunoprecipitated Sc as a control. (**C**) Western blotting showed that incubation of His-Pnr with Sgg generates the BUGS phospho-antibody epitope. The phosphorylation signal in immunodepleted Sgg supernatant (Sgg–) was largely reduced. Treatment with the GSK3β inhibitor LiCl (30 mM) during the kinase assay reaction prevents phosphorylation of Pnr. Loading controls are shown in the lower panel using an antibody against His-tag. (**D**) The number of DC bristles present after overexpression of wild-type Pnr (*c765-Gal4>UAS-Pnr^{WT}*) or a mutated form of Pnr (see A) (*c765-Gal4>UAS-Pnr^{MUT}*), is shown together with images of representative flies. (**E**) The number of DC bristles present after overexpression of wild-type or mutant Pnr in a loss-of-function *pnr* mutant devoid of DC bristles (*pnr^{VX6}/pnr^{MD237}>UAS-Pnr^{WT}* and *pnr^{VX6}/pnr^{MD237}>UAS-Pnr^{MUT}*) is shown, together with images of representative flies. Pnr^{MUT} rescues more bristles than *Pnr^{WT}*. (**F**) The number of DC bristles present after overexpression of wild-type or mutant Pnr in a hypomorphic *wg* mutant (see Fig. 1D), is shown, together with images of representative flies (*wg^{Sp}/wg^{CX4}; MD237-Gal4>UAS-Pnr^{WT}* and *wg^{Sp}/wg^{CX4}; MD237-Gal4>UAS-Pnr^{MUT}*). Pnr^{MUT} rescues more bristles than *Pnr^{WT}*. Error bars represent the mean±s.e.m. from three independent experiments.

contains a *Gal4* insert at the *pnr* locus that drives expression over the medial half of the notum (Heitzler et al., 1996). We found that rescue of DC bristles after expression of Pnr^{WT} was poor (0.4 bristles per thorax, n=113), but that rescue after expression of Pnr^{MUT} was significant (4.6 bristles per thorax, n=181; Fig. 3E). Finally we tested for rescue of DC bristles in the *wg^{Sp-1}/wg^{CX4}* mutant using the *MD237-Gal4* line. Overexpression did indeed lead to bristle rescue and again, Pnr^{MUT} rescued with a greater efficiency (Fig. 3F). Therefore Pnr^{MUT} is able to bypass the hyperactivity of Sgg caused by the loss of Wg signalling.

Taken together, these results indicate that the phosphorylation-resistant form of Pnr is hyperactive. We conclude that Pnr is phosphorylated by Sgg and that the phosphorylated form is less active. This suggests that inactivation of Sgg by the Wg signal leads to the accumulation of nonphosphorylated Pnr and consequent enhanced transcriptional activity.

Scute is a target for phosphorylation by Sgg

Clones of cells mutant for *sgg* also display ectopic macrochaetes outside the positions of proneural clusters within which the extant bristles form (Simpson and Carteret, 1989; Phillips et al., 1999).

Furthermore they bear additional microchaetes. It has been shown that formation of ectopic bristles correlates with accumulation of the *ac* protein (Phillips et al., 1999). Here we show that *sc* is also expressed at ectopic locations in *sgg* null clones (Fig. 2C). Expression of *ac-sc* at these ectopic locations is unlikely to be driven by the activity of specific cis-regulatory sequences that bind Pnr or the products of the *Iro-C*, neither of which affects microchaetes. Instead it probably results from increased transcription mediated by the basal promoters of *ac* and *sc* (Martinez and Modolell, 1991). A possible explanation for the effects of *sgg* mutants on ectopic *ac-sc* expression would be that either Ac or Sc themselves were targets for phosphorylation by Sgg.

The phosphorylation predication network NetPhosK 1.0 Server predicts two potential Sgg phosphorylation sites in *Drosophila* Sc at the positions of amino acids 85 and 268 (Fig. 4A) (Blom et al., 2004). These sites are not conserved in Ac and indeed we were unable to detect any sites in Ac that meet the criteria for a consensus sequence for Sgg phosphorylation. Multiple protein sequence alignment analysis with ClustalW2, reveals that the consensus motif at amino acid 268, but not that at amino acid 85, is highly conserved among *sc* proteins in cyclorraphous Diptera (supplementary material Fig. S3). We employed an in vitro kinase assay to examine possible phosphorylation of Sc by Sgg. The BUGS antibody did not identify any signal in IP *sc* protein, which displays a different sequence for Sgg phosphorylation from that of Pnr (Fig. 3A and Fig. 4A). Therefore, we used traditional radiolabelled [λ-P^{33}] ATP to test for protein kinase activity (see Materials and methods). Activity of Sgg should result in the incorporation of radiolabelled phosphate from [λ-P^{33}] ATP into the *sc* protein if Sc is phosphorylated by Sgg. Incubation of His-tag recombinant Sc with Sgg revealed kinase activity: a positive band of about 75 kDa indicated phosphorylated Sc (Fig. 4B). No kinase activity was detected when the recombinant *sc* protein was incubated in the absence of Sgg. Treatment with the Sgg inhibitor LiCl prevents phosphorylation of Sc (Fig. 4B). The result shows the presence of kinase activity and suggests that Sgg can phosphorylate Sc in vitro.

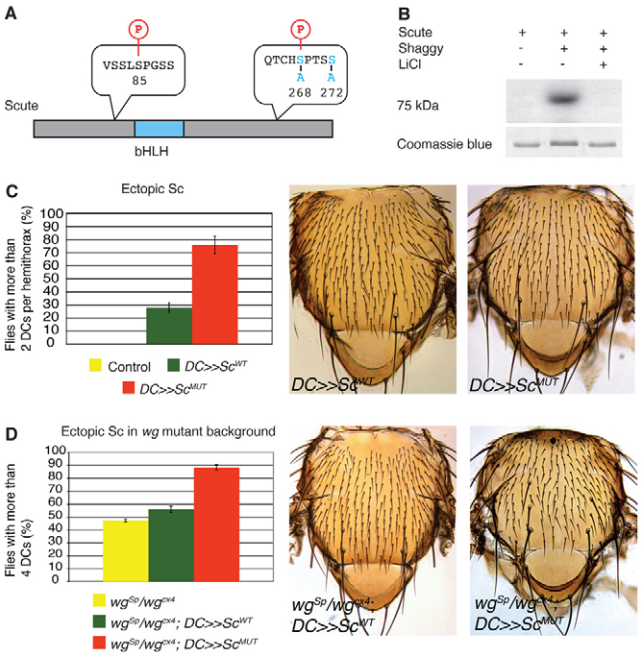

Fig. 4. Phosphorylation of Scute by Sgg and bristle phenotypes observed after expression of Scute-bearing mutated phosphorylation sites. (**A**) Sequence indicating serine residues (S) at potential sites (amino acids 85 and 268, red) for phosphorylation of Scute (Sc) by Sgg. bHLH, basic helix-loop-helix domain. (**B**) The in vitro protein kinase assay with radiolabelled [λ-P^{33}]ATP is shown. Recombinant Scute was incubated with Sgg kinase in a kinase assay buffer (see Materials and methods). A positive band at about 75 kDa indicated kinase activity on the SDS-PAGE gel. No kinase activity was detected when recombinant Sc alone is used. Treatment with the GSK3β inhibitor LiCl during the kinase assay reaction prevents phosphorylation of Sc. A duplicated gel was stained with Coomassie Blue as a loading control (lower panel). (**C**,**D**) The number of DC bristles present after overexpression of wild-type Sc (*DC-Gal4>UAS-*Sc^{WT}) or a mutant form of Sc (the central serine residue of phosphorylation site 268, as well as a possible priming phosphate site located at amino acid 272, were mutated into alanines (shown in blue in A), was examined in wild-type flies (C) (*DC-Gal4>UAS-*Sc^{WT} and *DC-Gal4>UAS-*Sc^{MUT}) and in a hypomorphic *wg* mutant (D) (Fig. 1D) (wg^{Sp}/wg^{CX4}*; DC-Gal4>UAS-*Sc^{WT} and wg^{Sp}/wg^{CX4}*; DC-Gal4>UAS-*Sc^{MUT}). Sc^{MUT} induces formation of more ectopic bristles than Sc^{WT}. Error bars represent mean±s.e.

A phosphorylation-resistant form of Scute displays hyperactivity

To test the significance of Sgg phosphorylation of Scute we performed site-directed mutagenesis at the conserved consensus site (amino acid 268). Serines in the Sgg site at amino acid 268 as well as a possible priming phosphate site located at n+4 (Cohen and Frame, 2001) at amino acid 272, were mutated into alanines (Fig. 4A) and the resulting protein designated Sc^{MUT}. Transgenic flies carrying the mutated Sc^{MUT} in a *UAS* vector were crossed to various *Gal4* drivers. The results were compared with expression of a wild-type form of Sc, Sc^{WT}. We found that the phosphorylation-resistant Sc^{MUT} displays increased proneural activity and therefore induces development of a greater number of additional bristles than Sc^{WT}. With many *Gal4* lines, expression of either Sc^{WT} or Sc^{MUT} induces so many ectopic bristles that counting bristles to obtain numbers for comparison was not possible (data not shown). We therefore used the *DC-Gal4* line that drives expression exclusively at the site of the DC proneural cluster. About 28% of *DC-Gal4>UAS-*Sc^{WT} flies display more than two DC bristles per hemithorax (Fig. 4C). This number increases to 76% in *DC-Gal4>UAS-*Sc^{MUT} flies (Fig. 4C). Expression of a phosphorylation-resistant form of Sc therefore mimics *sgg* loss of function in the notum, suggesting that phosphorylation of Sc by Sgg reduces activity.

We also tested the ability of Sc^{MUT} to rescue missing DC bristles in the wg^{Sp-1}/wg^{CX4} mutant using the *DC-Gal4* line. We find that rescue by Sc^{MUT} is more effective than rescue by Sc^{WT} (Fig. 4D). This suggests that, as for Pnr, the Wg signal, through inactivation of Sgg, leads to the accumulation of nonphosphorylated Sc that displays increased transcriptional activity.

DISCUSSION

Inactivation of Sgg by Wingless at mid third larval instar allows accumulation of Scute for macrochaete development in *Drosophila*

achaete-scute products become detectable in wing discs only at mid third larval instar (Romani et al., 1989; Cubas et al., 1991; Skeath and Carroll, 1991). The known upstream regulators, *Pnr* and the *Iro-C* genes, are selector genes that pattern the medial and lateral halves of the notum, respectively (Calleja et al., 2000; Mann and Morata, 2000). Therefore their activity is not restricted to *ac-*

sc activation and bristle patterning and they are expressed for a considerable period before *ac-sc* gene products are detected (Calleja et al., 2000; Klein, 2001; Calleja et al., 2002; Cavodeassi et al., 2002; Ghazi, 2003; Ikmi et al., 2008). Furthermore, although activation of *ac-sc* in proneural clusters by Pnr and Iro-C dramatically increases transcription at these sites, the *ac-sc* genes are also expressed at low levels over the entire disc epithelium, presumably through activity of the basal promoters (Martinez and Modolell, 1991; Ayyar et al., 2007). Indeed maintenance of proneural genes in an active state of basal transcription is a general feature of neuroepithelia (Bertrand et al., 2002). So what prevents accumulation of Ac-Sc at earlier stages in disc development?

We have shown that Sc is phosphorylated by Sgg, an enzyme that is expressed constitutively. Furthermore a mutated form of Sc that is resistant to phosphorylation has significantly greater bristle-forming activity than the wild-type protein. This suggests reduced transcriptional activity of phospho-Sc. One possibility is that the turnover of phospho-Sc is rapid, owing to phosphorylation-dependent ubiquitination and degradation (Ciechanover, 1998). It has been reported that mutations in the GSK-3β consensus motif in β-catenin abolishes ubiquitination and leads to protein stability (Yost et al., 1996; Aberle et al., 1997). GSK-3β also induces ubiquitination and degradation of *Drosophila* myc (dm – FlyBase) protein through the proteasome pathway and mutation of residues in the phosphorylation domain affects stability of this protein (Galletti et al., 2009). Indeed it has been shown that mutation of the phosphorylation site SPTS to APAA stabilizes the Sc protein (M. Kiparaki and C. Delidakis, personal communication). This suggests that before expression of *wg* at the mid third larval instar, the stability and transcriptional activity of any Sc present, whether derived from transcription mediated by the basal promoter or enhanced by Pnr and the *Iro-C* proteins, would be reduced through phosphorylation by Sgg.

Development of neural precursors requires high levels of Sc, which are needed for the process of lateral inhibition and singling out of precursors as well as for autoregulation (Culi and Modolell, 1998; Bertrand et al., 2002). During this process in *Drosophila*, Sc binds its own promoter, through a specific regulatory sequence, the sensory organ precursor enhancer (*SOPE*), to further activate transcription in presumptive precursors (Culi and Modolell, 1998). Therefore, any factors that diminish the activity of Sc itself have the potential to prevent sufficient accumulation to allow selection of precursors and maintenance of precursor cell fate. Expression of *wg* at mid third larval instar would lead to inactivation of Sgg. The consequent accumulation of a more active nonphosphorylated form of Sc might allow levels of Sc to accumulate sufficiently for precursor cell development. Achaete does not appear to be a target for Sgg. However, this protein has been shown to be dispensable for bristle development (Marcellini et al., 2005).

We find that Pnr is also a target for phosphorylation by Sgg and, that, like Sc, a mutated phosphorylation-resistant form of Pnr is hyperactive. So phosphorylation of Pnr might also result in ubiquitination and increased degradation, a situation that would be modified by Wg signalling at mid third larval instar. The effects of phosphorylation on Pnr and Sc appear to be quantitative, rather than all or nothing. Pannier has other targets before Wg signalling and activation of *ac-sc* (the *iro* genes and *wg* itself) and if the sole function of Wg were to be the inactivation of Sgg then one would expect loss of *sgg* function to have no bristle phenotype. So de-phosphorylation might just give an extra little boost to the system. Interestingly it has been shown that the *Drosophila* transcription factor Mad is also a target of Sgg and that phosphorylation-resistant Mad proteins are hyperactive (Eivers et al., 2009). Mad is activated by Dpp/TGFβ signalling, which in turn regulates expression of both *pnr* and the *Iro-C* genes in the thorax (Sato and Saigo, 2000; Tomoyasu et al., 2000; Shi and Massague, 2003; Letizia et al., 2007; Fromental-Ramain et al., 2008) (Fig. 5). Thus, it appears that inactivation of Sgg by the Wg signal can stimulate the levels of Sc via multiple routes: by increasing the levels of expression of *pnr* and the *Iro-C* genes as well as the activity of Pnr and Sc themselves. Thus, expression of *wg* at mid third larval instar might result in levels of Sc sufficient for macrochaete development. It is not known how the second phase of *ac-sc* expression for microchaetes is regulated.

Wingless is unlikely to be the only factor regulating temporal *ac-sc* expression. Indeed, although loss of *sgg* function can affect bristles over the entire notum, the effects of *wg* appear to be restricted to the medial notum (Phillips and Whittle, 1993). Other factors must be involved on the lateral notum. One possibility is NFκ-B/Rel, a factor that is required for functioning of the *SOPE* and singling out of precursors, and that also indirectly affects the

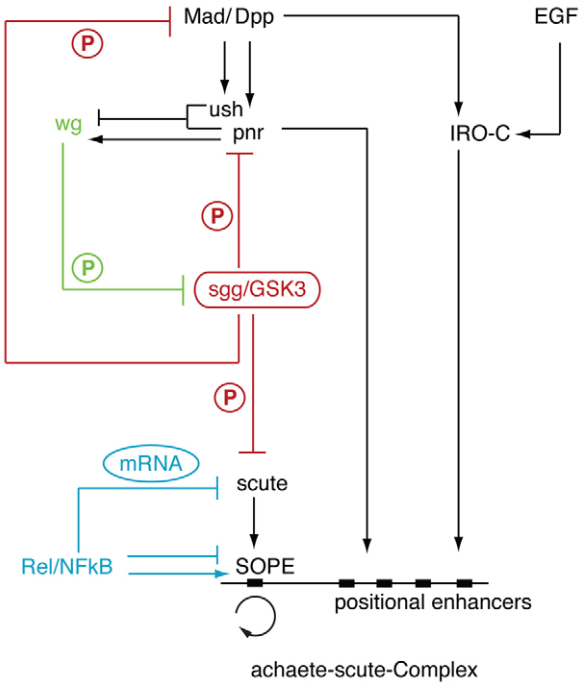

Fig. 5. The gene regulatory network controlling expression of *achaete-scute* in the notum of *D. melanogaster*. Diagram outlining the interactions between genes regulating *scute* expression. Arrows in black indicate a transcriptional response of target genes; it is not known whether activation of *wg* by Pannier is a result of direct transcriptional regulation. Pannier and the Iro-C gene products activate *sc* expression through numerous cis-regulatory sequences. Segregation of sensory organ precursors requires an autoregulatory element, the *SOPE*. Scute itself binds the *SOPE* as well as other factors including Rel/NFκ-B. Rel/NFκ-B also indirectly affects stability of *sc* transcripts. Red lines indicate three phosphorylation targets of Sgg: Mad, Pnr and Sc. These proteins are less active in the phosphorylated state and all affect *sc* expression directly or indirectly. Mad/Dpp signalling regulates expression of *pnr*, *ush* and the *Iro-C* genes, Pnr and Iro-C products activate *sc* and Sc positively autoregulates itself via the *SOPE*. Wingless signalling is known to inactivate Sgg (green). Hyperactive nonphosphorylated forms of Mad, Pnr and Sc accumulate and result in increased *sc* activity and bristle precursor development.

stability of *sc* transcripts (Culi and Modolell, 1998; Ayyar et al., 2007). Another event that coincides with the accumulation of *ac-sc* products at mid third larval instar is a small peak of 20H-ecdysteroid (not associated with a moult) (Berreur et al., 1979; Riddiford, 1993). Indeed ecdysone has been implicated in temporal regulation of expression of the proneural gene *atonal* and the development of *atonal*-dependent sense organs (Niwa et al., 2004).

An ancient function of Wingless might have been co-opted for temporal regulation of *ac-sc* expression

Wingless signalling has important functions in the thorax, likely to be ancient, that are linked to the development and patterning of flight muscles. So *wg* was probably already expressed on the notum of the ancestor of the Cyclorrapha, before the evolution of macrochaetes. The rapid development of the notum and short pupal period in many Nematocera leaves little requirement for any temporal control of expression (McAlpine, 1981). By contrast, the prolonged period of growth and patterning during the larval and pupal life of *Drosophila* allows time for two discrete phases of proneural gene expression (reviewed by Simpson and Marcellini, 2006). Wingless might then have been co-opted for the regulation of *ac-sc* and the evolution of macrochaetes in the lineage leading to the Cyclorrapha. Our results suggest that the Wg signal does not involve transcriptional regulation of target genes but instead is mediated simply through inactivation of Sgg. The phosphorylation sites are strongly conserved in the *sc* genes of *C. vicina* and *C. capitata* (supplementary material Fig. S3), two other species of Schizophora, suggesting a conserved mechanism of regulation by Wg and Sgg. By contrast, the same sites are not conserved in the other genes of the *Drosophila AS-C*, or in the *ac-sc* homologues of *A. gambiae*, although other potential Sgg phosphorylation sites can be detected in these proteins. Phosphorylation of Sc by Sgg could have been recently acquired in the Cyclorrapha. The *ac* and *sc* genes themselves have arisen from duplication events thought to have taken place during evolution of the Cyclorrapha (Skaer et al., 2002b; Negre and Simpson, 2009). Phosphorylation of Pnr by Sgg might also have been acquired in the lineage leading to the Schizophora, as one of the sites is conserved in the *pnr* protein of *C. vicina*, but not that of *Megaselia abdita* or *A. gambiae* (supplementary material Fig. S4).

Uniform proneural gene expression, together with Notch-mediated lateral inhibition (Heitzler and Simpson, 1991), is sufficient to generate a pattern of evenly spaced, but randomly positioned, bristles such as that seen in Nematocera and for the microchaetes of the Cyclorrapha (Wülbeck and Simpson, 2000; Pistillo et al., 2002; Wulbeck and Simpson, 2002). For this process, the *SOPE*, a very ancient regulatory element that predates the Diptera (Ayyar et al., 2010), is the only cis-regulatory element of *ac-sc* that would be required. Factors that act through the *SOPE* could be co-opted to modulate the temporal activity of *ac-sc*. This includes factors regulating activity of Sc, which itself binds the *SOPE* (Culi and Modolell, 1998). Control at this level could be superimposed on the ancestral state without the need to acquire new regulatory sequences for the binding of novel transcriptional repressors and activators. By contrast, the spatially restricted expression underlying the macrochaete pattern is linked to changes at the *AS-C* complex and the acquisition of novel cis-regulatory elements that possibly arose in association with gene duplication events (Skaer et al., 2002b; Negre and Simpson, 2009). This illustrates the power of evolution to make use of factors acting both in cis and in trans to effect morphological change.

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References

Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997). beta-catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.* **16**, 3797-3804.
Ayyar, S., Pistillo, D., Calleja, M., Brookfield, A., Gittins, K., Goldstone, C. and Simpson, P. (2007). NF-kappaB/Rel-mediated regulation of the neural fate in *Drosophila*. *PLoS ONE* **2**, e1178.
Ayyar, S., Negre, B., Simpson, P. and Stollewerk, A. (2010). An arthropod cis-regulatory element functioning in sensory organ precursor development dates back to the Cambrian. *BMC Biol.* **8**, 127.
Berreur, P., Porcheron, P., Berreur-Bonnenfant, J. and Simpson, P. (1979). Ecdysone levels and pupariation in a temperature sensitive mutation of *Drosophila melanogaster*. *J. Exp. Zool.* **210**, 347-352.
Bertrand, N., Castro, D. S. and Guillemot, F. (2002). Proneural genes and the specification of neural cell types. *Nat. Rev. Neurosci.* **3**, 517-530.
Biryukova, I. and Heitzler, P. (2008). *Drosophila* C-terminal binding protein, dCtBP is required for sensory organ prepattern and sharpens proneural transcriptional activity of the GATA factor Pnr. *Dev. Biol.* **323**, 64-75.
Blom, N., Sicheritz-Ponten, T., Gupta, R., Gammeltoft, S. and Brunak, S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics* **4**, 1633-1649.
Bourouis, M. (2002). Targeted increase in shaggy activity levels blocks wingless signaling. *Genesis* **34**, 99-102.
Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
Calleja, M., Herranz, H., Estella, C., Casal, J., Lawrence, P., Simpson, P. and Morata, G. (2000). Generation of medial and lateral dorsal body domains by the pannier gene of *Drosophila*. *Development* **127**, 3971-3980.
Calleja, M., Renaud, O., Usui, K., Pistillo, D., Morata, G. and Simpson, P. (2002). How to pattern an epithelium: lessons from achaete-scute regulation on the notum of *Drosophila*. *Gene* **292**, 1-12.
Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M. and Werner, T. (2005). MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* **21**, 2933-2942.
Cavodeassi, F., Rodriguez, I. and Modolell, J. (2002). Dpp signalling is a key effector of the wing-body wall subdivision of the *Drosophila* mesothorax. *Development* **129**, 3815-3823.
Ciechanover, A. (1998). The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO J.* **17**, 7151-7160.
Cohen, P. and Frame, S. (2001). The renaissance of GSK3. *Nat. Rev. Mol. Cell Biol.* **2**, 769-776.
Couso, J. P., Bate, M. and Martinez-Arias, A. (1993). A wingless-dependent polar coordinate system in *Drosophila* imaginal discs. *Science* **259**, 484-489.
Cubas, P., de Celis, J. F., Campuzano, S. and Modolell, J. (1991). Proneural clusters of achaete-scute expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Dev.* **5**, 996-1008.
Culi, J. and Modolell, J. (1998). Proneural gene self-stimulation in neural precursors: an essential mechanism for sense organ development that is regulated by Notch signaling. *Genes Dev.* **12**, 2036-2047.
Eivers, E., Fuentealba, L. C., Sander, V., Clemens, J. C., Hartnett, L. and De Robertis, E. M. (2009). Mad is required for wingless signaling in wing development and segment patterning in *Drosophila*. *PLoS ONE* **4**, e6543.

Fromental-Ramain, C., Vanolst, L., Delaporte, C. and Ramain, P. (2008). pannier encodes two structurally related isoforms that are differentially expressed during *Drosophila* development and display distinct functions during thorax patterning. *Mech. Dev.* **125**, 43-57.
Galletti, M., Riccardo, S., Parisi, F., Lora, C., Saqcena, M. K., Rivas, L., Wong, B., Serra, A., Serras, F., Grifoni, D. et al. (2009). Identification of domains responsible for ubiquitin-dependent degradation of dMyc by glycogen synthase kinase 3beta and casein kinase 1 kinases. *Mol. Cell. Biol.* **29**, 3424-3434.
Garcia-Garcia, M. J., Ramain, P., Simpson, P. and Modolell, J. (1999). Different contributions of pannier and wingless to the patterning of the dorsal mesothorax of *Drosophila*. *Development* **126**, 3523-3532.
Ghazi, A., Paul, L. and VijayRaghavan, K. (2003). Prepattern genes and signalling molecules regulate stripe expression to specify *Drosophila* flight muscle attachment sites. *Mech. Dev.* **120**, 519-528.
Gogel, S., Wakefield, S., Tear, G., Klambt, C. and Gordon-Weeks, P. R. (2006). The *Drosophila* microtubule associated protein Futsch is phosphorylated by Shaggy/Zeste-white 3 at an homologous GSK3beta phosphorylation site in MAP1B. *Mol. Cell. Neurosci.* **33**, 188-199.
Gomez-Skarmeta, J. L., Rodriguez, I., Martinez, C., Culi, J., Ferres-Marco, D., Beamonte, D. and Modolell, J. (1995). Cis-regulation of achaete and scute: shared enhancer-like elements drive their coexpression in proneural clusters of the imaginal discs. *Genes Dev.* **9**, 1869-1882.
Gomez-Skarmeta, J. L., del Corral, R. D., de la Calle-Mustienes, E., Ferre-Marco, D. and Modolell, J. (1996). *Araucan* and *caupolican*, two members of the novel *iroquois* complex, encode homeoproteins that control proneural and vein-forming genes. *Cell* **85**, 95-105.
Gonzalez, F., Romani, S., Cubas, P., Modolell, J. and Campuzano, S. (1989). Molecular analysis of the *asense* gene, a member of the *achaete-scute* complex of *Drosophila melanogaster*, and its novel role in optic lobe development. *EMBO J.* **8**, 3553-3562.
Haenlin, M., Cubadda, Y., Blondeau, F., Heitzler, P., Lutz, Y., Simpson, P. and Ramain, P. (1997). Transcriptional activity of pannier is regulated negatively by heterodimerization of the GATA DNA-binding domain with a cofactor encoded by the u-shaped gene of *Drosophila*. *Genes Dev.* **11**, 3096-3108.
Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of *Drosophila*. *Cell* **64**, 1083-1092.
Heitzler, P., Haenlin, M., Ramain, P., Calleja, M. and Simpson, P. (1996). A genetic analysis of pannier, a gene necessary for viability of dorsal tissues and bristle positioning in *Drosophila*. *Genetics* **143**, 1271-1286.
Huang, F., Dambly-Chaudiere, C. and Ghysen, A. (1991). The emergence of sense organs in the wing disc of *Drosophila*. *Development* **111**, 1087-1095.
Ikmi, A., Netter, S. and Coen, D. (2008). Prepatterning the *Drosophila* notum: the three genes of the iroquois complex play intrinsically distinct roles. *Dev. Biol.* **317**, 634-648.
Klein, T. (2001). Wing disc development in the fly: the early stages. *Curr. Opin. Genet. Dev.* **11**, 470-475.
Klein, T., Couso, J. P. and Martinez Arias, A. (1998). Wing development and specification of dorsal cell fates in the absence of apterous in *Drosophila*. *Curr. Biol.* **8**, 417-420.
Lai, E. C. (2003). *Drosophila* tufted is a gain-of-function allele of the proneural gene amos. *Genetics* **163**, 1413-1425.
Letizia, A., Barrio, R. and Campuzano, S. (2007). Antagonistic and cooperative actions of the EGFR and Dpp pathways on the iroquois genes regulate *Drosophila* mesothorax specification and patterning. *Development* **134**, 1337-1346.
Logan, C. Y. and Nusse, R. (2004). The Wnt signaling pathway in development and disease. *Annu. Rev. Cell Dev. Biol.* **20**, 781-810.
Mann, R. S. and Morata, G. (2000). The developmental and molecular biology of genes that subdivide the body of *Drosophila*. *Annu. Rev. Cell Dev. Biol.* **16**, 243-271.
Marcellini, S., Gibert, J. M. and Simpson, P. (2005). achaete, but not scute, is dispensable for the peripheral nervous system of *Drosophila*. *Dev. Biol.* **285**, 545-553.
Martinez, C. and Modolell, J. (1991). Cross-regulatory interactions between the proneural achaete and scute genes of *Drosophila*. *Science* **251**, 1485-1487.
McAlpine, J. F. (1981). *Manual of Nearctic Diptera.* Ottawa: Research Branch, Agriculture Canada.
Modolell, J. and Campuzano, S. (1998). The achaete-scute complex as an integrating device. *Int. J. Dev. Biol.* **42**, 275-282.
Negre, B. and Simpson, P. (2009). Evolution of the achaete-scute complex in insects: convergent duplication of proneural genes. *Trends Genet.* **25**, 147-152.
Neumann, C. J. and Cohen, S. M. (1996). Sternopleural is a regulatory mutation of wingless with both dominant and recessive effects on larval development of *Drosophila melanogaster*. *Genetics* **142**, 1147-1155.
Niwa, N., Hiromi, Y. and Okabe, M. (2004). A conserved developmental program for sensory organ formation in *Drosophila melanogaster*. *Nat. Genet.* **36**, 293-297.
Papadopoulou, D., Bianchi, M. W. and Bourouis, M. (2004). Functional studies of shaggy/glycogen synthase kinase 3 phosphorylation sites in *Drosophila melanogaster*. *Mol. Cell. Biol.* **24**, 4909-4919.
Phillips, R. G. and Whittle, J. R. (1993). *wingless* expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. *Development* **118**, 427-438.
Phillips, R. G., Warner, N. L. and Whittle, J. R. (1999). Wingless signaling leads to an asymmetric response to decapentaplegic- dependent signaling during sense organ patterning on the notum of *Drosophila melanogaster*. *Dev. Biol.* **207**, 150-162.
Pistillo, D., Skaer, N. and Simpson, P. (2002). scute expression in Calliphora vicina reveals an ancestral pattern of longitudinal stripes on the thorax of higher Diptera. *Development* **129**, 563-572.
Ramain, P., Heitzler, P., Haenlin, M. and Simpson, P. (1993). pannier, a negative regulator of achaete and scute in *Drosophila*, encodes a zinc finger protein with homology to the vertebrate transcription factor GATA-1. *Development* **119**, 1277-1291.
Riddiford, L. (1993). Hormones and *Drosophila* development. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), pp. 899-939. New York: Cold Spring Harbor Laboratory Press.
Rodriguez, I., Hernandez, R., Modolell, J. and Ruiz-Gomez, M. (1990). Competence to develop sensory organs is temporally and spatially regulated in *Drosophila* epidermal primordia. *EMBO J.* **9**, 3583-3592.
Romani, S., Campuzano, S., Macagno, E. R. and Modolell, J. (1989). Expression of achaete and scute genes in *Drosophila* imaginal discs and their function in sensory organ development. *Genes Dev.* **3**, 997-1007.
Ruel, L., Pantesco, V., Lutz, Y., Simpson, P. and Bourouis, M. (1993). Functional significance of a family of protein kinases encoded at the shaggy locus in *Drosophila*. *EMBO J.* **12**, 1657-1669.
Sato, M. and Saigo, K. (2000). Involvement of pannier and u-shaped in regulation of decapentaplegic-dependent wingless expression in developing *Drosophila* notum. *Mech. Dev.* **93**, 127-138.
Sato, M., Kojima, T., Michiue, T. and Saigo, K. (1999). Bar homeobox genes are latitudinal prepattern genes in the developing *Drosophila* notum whose expression is regulated by the concerted functions of decapentaplegic and wingless. *Development* **126**, 1457-1466.
Shi, Y. and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell* **113**, 685-700.
Simpson, P. and Carteret, C. (1989). A study of shaggy reveals spatial domains of expression of achaete-scute alleles on the thorax of *Drosophila*. *Development* **106**, 57-66.
Simpson, P. and Marcellini, S. (2006). The origin and evolution of stereotyped patterns of macrochaetes on the nota of cyclorraphous Diptera. *Heredity* **97**, 148-156.
Simpson, P., Woehl, R. and Usui, K. (1999). The development and evolution of bristle patterns in Diptera. *Development* **126**, 1349-1364.
Skaer, N., Pistillo, D. and Simpson, P. (2002a). Transcriptional heterochrony of scute and changes in bristle pattern between two closely related species of blowfly. *Dev. Biol.* **252**, 31-45.
Skaer, N., Pistillo, D., Gibert, J. M., Lio, P., Wulbeck, C. and Simpson, P. (2002b). Gene duplication at the achaete-scute complex and morphological complexity of the peripheral nervous system in Diptera. *Trends Genet.* **18**, 399-405.
Skeath, J. B. and Carroll, S. B. (1991). Regulation of achaete-scute gene expression and sensory organ pattern formation in the Drosophila wing. *Genes Dev.* **5**, 984-995.
Sullivan, W., Ashburner, M. and Hawley, S. (2000). *Drosophila Protocols.* New York: Cold Spring Harbor Laboratory Press.
Szuplewski, S., Sandmann, T., Hietakangas, V. and Cohen, S. M. (2009). *Drosophila* Minus is required for cell proliferation and influences Cyclin E turnover. *Genes Dev.* **23**, 1998-2003.
Tao, H., Liu, W., Simmons, B. N., Harris, H. K., Cox, T. C. and Massiah, M. A. (2010). Purifying natively folded proteins from inclusion bodies using sarkosyl, Triton X-100, and CHAPS. *Biotechniques* **48**, 61-64.
Tomoyasu, Y., Ueno, N. and Nakamura, M. (2000). The decapentaplegic morphogen gradient regulates the notal wingless expression through induction of pannier and u-shaped in *Drosophila*. *Mech. Dev.* **96**, 37-49.
Trivedi, N., Marsh, P., Goold, R. G., Wood-Kaczmar, A. and Gordon-Weeks, P. R. (2005). Glycogen synthase kinase-3beta phosphorylation of MAP1B at Ser1260 and Thr1265 is spatially restricted to growing axons. *J. Cell Sci.* **118**, 993-1005.
Usui, K., Goldstone, C., Gibert, J. M. and Simpson, P. (2008). Redundant mechanisms mediate bristle patterning on the *Drosophila* thorax. *Proc. Natl. Acad. Sci. USA* **105**, 20112-20117.
Usui-Ishihara, A. and Simpson, P. (2005). Differences in sensory projections between macro- and microchaetes in Drosophilid flies. *Dev. Biol.* **277**, 170-183.
Vaessin, H., Brand, M., Jan, L. Y. and Jan, Y. N. (1994). daughterless is essential for neuronal precursor differentiation but not for initiation of neuronal precursor formation in *Drosophila* embryo. *Development* **120**, 935-945.
van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A. et al. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. *Cell* **88**, 789-799.

Villares, R. and Cabrera, C. V. (1987). The achaete-scute gene complex of *D. melanogaster*: conserved domains in a subset of genes required for neurogenesis and their homology to myc. *Cell* **50**, 415-424.
Wülbeck, C. and Simpson, P. (2000). Expression of *achaete-scute* homologues in discrete proneural clusters on the developing notum of the medfly *Ceratitis capitata*, suggests a common origin for the stereotyped bristle patterns of higher Diptera. *Development* **127**, 1411-1420.
Wulbeck, C. and Simpson, P. (2002). The expression of pannier and achaete-scute homologues in a mosquito suggests an ancient role of pannier as a selector gene in the regulation of the dorsal body pattern. *Development* **129**, 3861-3871.
Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.
Yost, C., Torres, M., Miller, J. R., Huang, E., Kimelman, D. and Moon, R. T. (1996). The axis-inducing activity, stability, and subcellular distribution of beta-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. *Genes Dev.* **10**, 1443-1454.