The C-terminal domain of Armadillo binds to hypophosphorylated Teashirt to modulate Wingless signalling in *Drosophila*

Armél Gallet, Corinne Angelats, Alfrun Erkner, Bernard Charroux¹, Laurent Fasano and Stephen Kerridge²

Laboratoire de Génétique et Physiologie du Développement, UMR 9943, CNRS-Université, IBDM CNRS-INSERM-Université de la Méditerranée, Campus de Luminy Case 907, F-13288 Marseille, Cedex 09, France and ¹Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, 326 Clinical Research Building, 415 Curie Boulevard, Philadelphia, PA 19104-6148, USA

²Corresponding author
e-mail: kerridge@ibdm.univ-mrs.fr

Wnt signalling is a key pathway for tissue patterning during animal development. In *Drosophila*, the Wnt protein Wingless acts to stabilize Armadillo inside cells where it binds to at least two DNA-binding factors which regulate specific target genes. One Armadillo-binding protein in *Drosophila* is the zinc finger protein Teashirt. Here we show that Wingless signalling promotes the phosphorylation and the nuclear accumulation of Teashirt. This process requires the binding of Teashirt to the C-terminal end of Armadillo. Finally, we present evidence that the serine/threonine kinase Shaggy is associated with Teashirt in a complex. We discuss these results with respect to current models of Armadillo/ β -catenin action for the transmission of the Wingless/Wnt pathway.

Keywords: Armadillo/*Drosophila*/Teashirt phosphorylation/Wingless/Wnt pathway

Introduction

The *Drosophila* Armadillo (Arm) protein, which is the homologue of vertebrate β -catenin, has a dual function during development. First, it plays a role in cell adhesion, participating in the formation of adherens junctions by binding directly to DE-cadherin and α -catenin (Cox *et al.*, 1996; Orsulic and Peifer, 1996; Pai *et al.*, 1996). The second function is to transduce signalling upon induction by the Wingless/Wnt family of secreted proteins (reviewed by Cadigan and Nusse, 1997).

Wingless (Wg)/Wnt signalling is essential for embryonic patterning in insects and vertebrates. The ventral side of the *Drosophila* larva consists of alternate patterns of naked and denticled cuticle in thoracic and abdominal segments (the trunk). This patterning depends in part on the secreted protein Wg, which is produced in homologous stripes of cells in each segment primordium during embryogenesis. Absence of Wg signalling leads all cells to choose a denticle cell fate whereas excess Wg induces only naked cuticle (Nusslein-Volhard and Wieschaus, 1980; Martinez-Arias *et al.*, 1988; Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992; Noordermeer *et al.*, 1992).

Genetic and biochemical experiments have allowed the identification of several components that act to transduce Wg signal. Wg binding to its receptor(s) (probably D-*Frizzled* 2; Bhanot *et al.*, 1996; Hays *et al.*, 1997; Bhat, 1998; Cadigan *et al.*, 1998; Zhang and Carthew, 1998; Muller *et al.*, 1999) activates the cytoplasmic protein Dishevelled which in turn inhibits the serine/threonine kinase Shaggy (Sgg; homologous to the vertebrate glycogen synthase kinase 3 β) (Siegfried *et al.*, 1992; Peifer *et al.*, 1994c; Pai *et al.*, 1997). This allows the stabilization of the non-junctional, intracellular Arm (reviewed in Cadigan and Nusse, 1997; Cox and Peifer, 1998). Upon its cytoplasmic stabilization, Arm interacts with the ubiquitous DNA-binding transcription factor dTCF (T-cell factor)/Pangolin (Brunner *et al.*, 1997; van de Wetering *et al.*, 1997) and then regulates the Wg target genes.

These versatile functions of Arm can be explained by its capacity to bind distinct proteins (Miller and Moon, 1996; Orsulic and Peifer, 1996; van de Wetering *et al.*, 1997). Arm can be considered to possess three different domains: an N-terminal domain containing potential sites for phosphorylation by Sgg (probably inducing the degradation of Arm; Peifer *et al.*, 1994b; Pai *et al.*, 1997) and the binding site for α -catenin (Orsulic and Peifer, 1996). The central domain is characterized by 13 Armadillo repeats (Peifer *et al.*, 1994a) repeats 3 to 8 are sufficient to bind DE-cadherin (Pai *et al.*, 1996), dTCF (van de Wetering *et al.*, 1997) and, for vertebrate β -catenin, adenomatous polyposis coli (APC) (Hulsken *et al.*, 1994). The function of the last domain at the C-terminal end is still unclear although analysis of different *arm* mutants has shown that it plays a key role for Wg signalling (Peifer and Wieschaus, 1990), possibly with a transactivation role for transcriptional regulation (van de Wetering *et al.*, 1997).

Arm also binds to the zinc finger protein Teashirt (Tsh) (Gallet *et al.*, 1998), which was first described as a region-specific homeotic gene (Fasano *et al.*, 1991). Tsh is expressed in the embryonic trunk segments (but not the head and tail) and is necessary for trunk identity in cooperation with certain Hox proteins (Roder *et al.*, 1992; de Zulueta *et al.*, 1994). Tsh is also necessary for the late signalling functions of the Wg pathway in the ventral ectoderm of the embryo (e.g. for the maintenance of *wg* transcription and not the earlier *engrailed* regulation). Wg-dependant stabilization of intracellular Arm results in the upregulation of nuclear Tsh in each trunk segment in cells destined to make naked cuticle (Gallet *et al.*, 1998).

In this study, we dissect in more detail the intimate relation between Arm, Tsh and Wg signalling. We show that Tsh is a nuclear phosphoprotein and that its nuclear accumulation depends in part on Wg signal. We determine that Tsh binds to a specific C-terminal domain of Arm. Finally we show that Tsh is in a complex with Sgg, allowing regulation of the Wg target genes specific to the trunk.

Fig. 1. Tsh is a phosphoprotein. (A) Treatment of overnight embryonic extracts with alkaline phosphatase. (B) Developmental Western blot of Tsh. Ten micrograms of total protein from staged embryos were loaded per lane on SDS–PAGE then transferred and immunoblotted with anti-Tsh. Note that the hyperphosphorylated isoforms of Tsh increase during embryonic development indicated in hours.

Results

Teashirt phosphorylation and subcellular localization during development

Teashirt was previously characterized as a protein with different isoforms on Western blots (Alexandre *et al.*, 1996). At least three isoforms are detectable: two predominant slow-migrating bands (at ~120–130 kDa) and one minor fast migrating band (116 kDa) (Figure 1A). By using immunoprecipitation experiments, we have previously shown that a faster migrating form of Tsh co-immunoprecipitates with Arm (Gallet *et al.*, 1998). In order to test whether these isoforms of Tsh result from differential phosphorylation we treated embryonic lysates with alkaline phosphatase for different times (Figure 1A). After 1 h treatment, Tsh was detected exclusively as a 116 kDa band, suggesting that the more slowly migrating isoforms are due to phosphorylation. Consistent with this idea, after 1 h treatment in the presence of phosphatase inhibitors, no effect on the Tsh phosphorylation pattern was detectable. The 116 kDa form could still be partially phosphorylated since not all phosphorylations alter protein migration on SDS–PAGE, however in order to clarify the text, we consider the 116 kDa isoform as the hypophosphorylated one.

We then examined at different stages of embryogenesis Tsh phosphorylation on developmental Western blots (Figure 1B) and Tsh localization by confocal analyses (Figure 2A). A 116 kDa form of Tsh is detected between 1 and 3 h (stages 5–8) of development resulting from zygotic product (Figure 1B); at this stage Tsh is detected in the region of the future three thoracic and the first

abdominal segments as a diffuse labelling within cells (Alexandre *et al.*, 1996). This staining shows that Tsh is present in both the cytoplasmic and nuclear compartments (Figure 2A, upper panel). Between 4 and 6 h (stages 9–11), the lower band is detected more intensively and the more slowly migrating forms are becoming stronger (Figure 1B). At this stage, Tsh is highly concentrated in the nuclei in the posterior part of each trunk segment corresponding to the Wg-responding cells (Gallet *et al.*, 1998; Figure 2A, middle panel). Anteriorly in the segments, Tsh continues to be diffuse and localizes both in the nuclei and the cytoplasm (see also Figure 3A, upper left panel). During later stages, the upper bands are the predominant forms of Tsh, although the 116 kDa is still present (Figure 1B); Tsh staining at this stage is high in all nuclei irrespective of segment position (Figure 2A, lower right panel). Note that in the developmental Western blot, the more slowly migrating forms are the more highly phosphorylated ones, since dephosphorylation studies on lysates from this stage give rise to a single 116 kDa band (not shown) as described in Figure 1A.

By subcellular fractionation, we sought to determine the cellular location of phosphorylated and hypophosphorylated Tsh forms. Cytoplasmic and nuclear extracts were isolated from wild-type embryos. In cytoplasmic extracts (Figure 2B, lane 2), Tsh is detected as a single band at 116 kDa corresponding to the hypophosphorylated isoform, whereas in the nuclear extract a diffuse band is present (Figure 2B, lane 3) corresponding mainly to the more slowly migrating phosphorylated isoforms (compare lane 1 with lane 3). A faint band is also detected at ~116 kDa in the nuclear preparations (lane 3). To assess the quality of purification, we probed the same blot with an antibody against histone H2, known to be associated with the DNA, or with an antibody against β -tubulin, a component of the cytoplasmic cytoskeleton.

In conclusion (Figures 1 and 2), Tsh phosphorylation is regulated during embryogenesis: the first appearance of phosphorylated isoforms occurs after 3 h of development; these isoforms become more predominant by 5 h and gradually increase until the end of embryogenesis. Phosphorylation state is correlated with the nuclear accumulation of Tsh in stage 9 embryos (5 h). These results suggest that hypophosphorylated Tsh is essentially cytoplasmic whereas hyperphosphorylated Tsh is nuclear.

Mutants in Wg signalling affect Tsh phosphorylation

We have previously shown that intrasegmental nuclear modulation of Tsh between stages 8–10 (Figure 3A) is Wg dependent (Gallet *et al.*, 1998). Excess Wg signalling (ubiquitous Wg in Figure 3A, upper right panel) promotes a high level of Tsh in all trunk nuclei, while absence of Wg signalling results in uniform lower levels of nuclear Tsh (Figure 3A, lower panels). High levels of Tsh (Gallet *et al.*, 1998), stabilized Arm (Pai *et al.*, 1997) or Wg (Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992; Noordermeer *et al.*, 1992; Perrimon, 1994; Hays *et al.*, 1997) promote production of naked cuticle in the larva. Strikingly, Tsh phosphorylation is first detected during this phase (Figure 1B), correlating phosphorylation of Tsh with a high level of nuclear Tsh and the production

Fig. 2. Tsh subcellular localization. **(A)** Confocal analyses of wild-type embryos from stage 8, 10 and 12 (anterior is to the left). Embryos were stained with anti-Tsh in green and 7AAD in red that specifically labels the nuclei. Note the evolution of Tsh localization with time: Tsh is first in both the nuclear and cytoplasmic compartments at stage 8 and then becomes modulated at stage 10, where Tsh is highly nuclear in the posterior part of each segment. Finally at stage 12, Tsh is highly nuclear in all cells of the trunk segments. **(B)** Subcellular fractionation from stage 8–11 embryos. Upper panel was immunoblotted with anti-Tsh. Twenty micrograms of total protein (lane 1), 40 μ g of cytoplasmic protein (lane 2) and 10 μ g of nuclear protein (lane 3) were loaded on SDS–PAGE. Middle panel was immunoblotted with anti- β -tubulin, which is a marker for the cytoplasmic compartment. Lower panel was immunoblotted with anti-histone H2, which is specific to the nucleus. The panels are from the same gel.

of naked cuticle (Noordermeer *et al.*, 1992; Gallet *et al.*, 1998).

To investigate the effects of Wg signalling on Tsh phosphorylation, we performed Western blots on proteins extracted from stage 9–11 embryos mutant for different components of the Wg pathway (Figure 3B). We selected mutant embryos that constitutively transduce Wg (*sgg*[−] germ-line clones, UASWg or UASArmS10c) and those lacking signal transmission (*arm*^{XM19} germ-line clones and

wg^{CX4} homozygotes). In wild-type embryos, different hyperphosphorylated forms are present (square bracket, Figure 3B). In constitutive Wg signalling mutant embryos the most hyperphosphorylated forms are predominant (Figure 3B, upper arrow). Conversely, in Wg signalling loss-of-function mutants, the upper band is fainter and the 116 kDa form is more apparent (Figure 3B, lower arrow). By probing the same blot with an anti-tubulin antibody (Figure 3B, lower panel) and by densitometric analysis

Fig. 3. Wg signalling promotes Tsh nuclear accumulation and phosphorylation. (A) Tsh confocal analyses of wild-type (upper left panel), 69BGal4×UASWg (upper right panel), *arm*^{XM19} (lower left panel) and *wg*^{CX4} (lower right panel) stage 10 embryos. Mutant embryos for *arm* or *wg* were identified by the loss of *engrailed* expression. The two lower panels were overexposed to show the low level of non-modulated Tsh. In all panels anterior is to the left. (B) Tsh immunoblotting in different Wg signalling mutants. Three embryos (between stages 9–11) of the indicated genotypes were loaded per lane. Upper panel was immunoblotted with an anti-Tsh and the lower panel (from the same gel) was blotted with anti-β-tubulin for loading control. The square bracket highlights the different hyperphosphorylated forms of Tsh. In the loss of Wg signalling (two right-most lanes) arrows show more hypophosphorylated (lower arrow) and less hyperphosphorylated (upper arrow) Tsh.

(data not shown), we can correlate the relative amount of Tsh in the different mutants: there are equal amounts of Tsh in wild-type and in embryos with gain of Wg signalling function, but there is less Tsh in mutants lacking Wg signalling function. This is consistent with a decreased level of nuclear Tsh observed in the absence of Wg function (Gallet *et al.*, 1998; Figure 3A).

Taken together, the results indicate that the Tsh phosphorylation and the increasing nuclear level of Tsh is in part dependent on the Wg pathway. Nevertheless, even in mutants lacking signal transmission, Tsh is still phosphorylated and localized in the nucleus (Figure 3A, lower panels), indicating that other factors are acting on Tsh independently of Wg.

Tsh binds to the C-terminal end of Arm

Arm has a dual function, one is to contribute to the formation of adherens junctions and the other is to transduce Wg signalling. Several isoforms of Arm are

Fig. 4. Hypophosphorylated Tsh interacts with hypophosphorylated Arm. (A) Tsh (left panel) and Arm (right panel) immunoblotting. Lanes 2 and 4 are whole embryonic extracts from wild-type embryos. Lane 1 is whole embryonic extract immunoprecipitated with anti-Arm antibody. Only hypophosphorylated Tsh is detected (arrowhead). Lane 3 is a whole embryonic extract immunoprecipitated with anti-Tsh antibody. Only the hypophosphorylated intracellular Arm is seen (arrow). Note that the neural form of Arm (Loureiro and Peifer, 1998; see Figure 5B) is not detected since the embryos were at stages 9–10 when this form is not detectable. (B) Immunoprecipitation of Tsh by anti-Arm antibody on staged embryos. Upper panel shows that Tsh co-immunoprecipitates with Arm at all stages of embryogenesis except between 0 and 1 h when Tsh is not expressed. Lower panel shows immunoblot of Arm as an internal control for immunoprecipitation experiments. The arrowhead indicates the neural isoform of Arm that is only detectable after 14 h of development.

detectable on Western blots (Riggelman *et al.*, 1990; Peifer *et al.*, 1994a,b,c; Loureiro and Peifer, 1998): a hyperphosphorylated form that migrates at 103 kDa, a hypophosphorylated form at 99 kDa (Figure 4A, lane 4) and an alternative spliced form at 82 kDa (Figure 4B, arrowhead in lower panel). The 103 kDa isoform is localized at the membrane in adherens junctions. The 99 kDa form accumulates in the cytoplasm upon Wg signalling and finally the 82 kDa isoform is a neural splicing product that is only detectable after stage 14.

In order to identify which are the Arm isoforms interacting with Tsh, we performed immunoprecipitation experiments. Using the monoclonal N2 7A1 anti-Arm antibody (Peifer *et al.*, 1994c) we show that the hypophosphorylated Tsh isoform co-immunoprecipitates with Arm (Figure 4A, compare lanes 1 and 2). Secondly, we performed immunoprecipitation experiments with an affinity-purified anti-Tsh antibody and detected Arm on the blot. Only the hypophosphorylated intracellular form of Arm is detected, which is necessary to transduce the Wg pathway (Peifer *et al.*, 1994c). To determine whether or not the Arm–Tsh interaction occurs at all development stages, we have performed immunoprecipitation with anti-Arm N2 7A1 on staged embryos (Figure 4B). These experiments show that Arm and Tsh are in a complex in all stages of embryogenesis when Tsh is produced. Together, these results suggest that hypophosphorylated Tsh interacts with non-junctional Arm throughout embryogenesis.

In order to dissect precisely the domain(s) of Arm interacting with Tsh we used *in vivo* and *in vitro* approaches. We performed epistasis experiments, overexpressing Tsh in Arm mutant backgrounds. The first allele, *arm^{XM19}*, behaves as a null allele for Wg signalling and correspond to the presence of a stop codon within the 13th Arm repeat at amino acid (aa) 680. The second allele is *arm^{H8.6}*, a thermosensitive allele deficient for wg signalling at 25°C. *arm^{H8.6}* has an insertion of a transposable element after aa 722 (Figure 5D; Klingensmith *et al.*, 1989; Peifer and Wieschaus, 1990; Peifer *et al.*, 1993b). Tsh overexpression in wild-type embryos gives rise to naked larva with few denticles on their ventral side (Gallet *et al.*, 1998). In combination with *arm^{XM19}*, Tsh overexpression gives little or no naked cuticle (Gallet *et al.*, 1998; Figure 5A, middle panel). With the *arm^{H8.6}* allele however, the naked cuticle was more extensive (Figure 5A, lower panel). If we compare the molecular structure of these two mutants, Arm^{H8.6} possesses 42 aa more than Arm^{XM19} at the C-terminal end. Tsh could interact inside this 42 aa domain and promote naked cuticle following entry into the nucleus. To test this hypothesis we performed immunoprecipitation with the anti-Tsh antibody in wild-type, *arm^{XM19}* and *arm^{H8.6}* heterozygous embryos. In wild-type embryos two forms of Arm co-immunoprecipitate with Tsh: the cytoplasmic full-length form (as shown before) and a smaller form at 82 kDa (Figure 5B, arrowhead). This neural-specific isoform is obtained by alternative splicing of the 6th exon generating a stop codon after aa 721 (Loureiro and Peifer, 1998). We used heterozygous Arm mutant embryos because mutant Arm protein coexists with wild-type Arm, providing an internal positive control. In *arm^{H8.6}* heterozygous embryos, we co-immunoprecipitated the three forms of Arm (Figure 5B, lane 6): the wild-type (99 kDa), Arm^{H8.6} (86 kDa, arrow in lane 6) and the neural (82 kDa; arrowhead) forms indicating that the amino acids after 721 are dispensable for the interaction with Tsh. Conversely, in *arm^{XM19}* heterozygotes, only wild-type and not Arm^{XM19} protein is co-immunoprecipitated with Tsh (lane 5).

We then analysed the Tsh-binding domain of Arm using glutathione *S*-transferase (GST) pull-down experiments with different parts of Arm translated *in vitro* and then tested for binding to GST-Tsh. Three Arm N-terminal truncations were produced using internal methionines at positions 279, 549 and 579. We also tested an Arm repeat construct (Arm R1–8) which failed to bind to Tsh (Figure 5C). In summary, Tsh binds only to the C-terminal part of Arm (Figure 5C and D).

Taken together, these *in vivo* and *in vitro* experiments allow us to pinpoint the domain of Arm interacting and required for Tsh function (outlined by a black box in Figure 6A; see also Figure 6B). The C-terminal part of Arm is the least conserved compared with vertebrate β -catenins. However, the binding domain with Tsh shows blocks of homology between Arm and vertebrate β -catenins (Figure 6A, black boxes; Peifer *et al.*, 1993a). Tsh is the first example of a protein binding to the 'transactivating' domain of Arm (van de Wetering *et al.*, 1997).

Tsh is in a complex with Sgg

Wg signal acts by inhibiting the activity of Sgg, which would otherwise promote the degradation of Arm inside

the cell. Thus Arm accumulates inside the cell and can interact with its partners. Loss of Sgg activity causes the stabilization of intracellular Arm everywhere in the segment promoting the production of naked cuticle in the trunk (Siegfried *et al.*, 1992; Peifer *et al.*, 1994c; Figure 7A). When Wg does not signal, Sgg is thought to promote phosphorylation of Arm on an N-terminal motif, leading to Arm degradation via the ubiquitination pathway (Peifer *et al.*, 1994c; Rubinfeld *et al.*, 1996; Aberle *et al.*, 1997; Korinek *et al.*, 1997; Pai *et al.*, 1997).

In order to test the interaction between Tsh and Sgg, we induced germ-line clones of *sgg*. We first examined the distribution of Tsh in such embryos. As expected nuclear Tsh level is high as in embryos constitutively expressing the Wg pathway (Figure 7A). In order to analyse the epistasis between *tsh* and *sgg*, we examined *sgg* cuticles with or without *tsh* activity. Whereas *sgg* germ-line clones give naked cuticle as described before (Siegfried *et al.*, 1992), absence of *tsh* gives larvae with reduced naked cuticle and a lawn of denticles (Figure 7A). Therefore Tsh acts downstream of Sgg. Finally we performed immunoprecipitations to test whether Sgg and Tsh are in a complex. Using affinity-purified anti-Tsh, Sgg co-immunoprecipitates with Tsh (Figure 7B).

Together, these results show that Tsh is epistatic to Sgg, that the nuclear Tsh level is also Sgg-dependent and that *in vivo* Tsh is in a protein complex with Sgg.

Discussion

A number of ubiquitous factors have been identified which are responsible for transmission of the Wg pathway. The secreted protein Wg acts as a morphogen with different Wg concentrations specifying which of the target genes are induced in particular cells around the source of morphogen (reviewed by Neumann and Cohen, 1997). Tsh acts as a regionalized factor in the trunk of the embryo and allows the specification of the late Wg-signalling events (e.g. for the maintenance of *wg* expression and the production of naked cuticle but not for the expression of the early Wg target gene *en*; Gallet *et al.*, 1998). In this study, we have shown that the Wg pathway is able to regulate Tsh post-translationally and that the C-terminal domain of Arm binds to Tsh.

Recruitment of Tsh by the Wg pathway

Tsh is necessary for trunk segment identity. Loss of *tsh* function leads both to an homeotic transformation of the prothoracic segment to a labial one and to a segment polarity phenotype by reduction of naked cuticle and disorganization of the denticle belts (Fasano *et al.*, 1991; Roder *et al.*, 1992; Gallet *et al.*, 1998). Gain-of-function of *tsh* leads to an homeotic transformation of the labial to prothoracic segment identity and to the production of naked cuticle in all trunk segments (de Zulueta *et al.*, 1994; Gallet *et al.*, 1998). Here we show that Wg signal is able to modify Tsh post-translationally, resulting in the recruitment of Tsh to this pathway. By 5 h of development (stage 9), Tsh becomes intrasegmentally modulated and the first signs of phosphorylation can be seen (Figures 1 and 2). We have shown that Wg signalling is in part required for phosphorylation and nuclear accumulation of Tsh (Figure 3). Nevertheless, Tsh is still phosphorylated

Fig. 5. *In vivo* and *in vitro* interaction between Tsh and Arm. (A) Epistasis experiments of germ-line clones homozygous for *arm*^{XM19} (top panel) or *arm*^{XM19} and *arm*^{H8.6} overexpressing Tsh (lower panels). Note that denticles are more efficiently suppressed in *arm*^{H8.6} embryos (bottom panel) than in *arm*^{XM19} ones (middle panel). (B) Left panel shows an immunoblot with anti-Arm on whole embryonic lysates from wild-type (lane 1), *arm*^{XM19/+} (lane 2) and *arm*^{H8.6/+} (lane 3) embryos. Right panel shows immunoprecipitation by anti-Tsh from the same genotypes respectively in lanes 4, 5 and 6. Arrows indicate the mutant proteins at 75 kDa for Arm^{XM19} and 86 kDa for Arm^{H8.6}. Note that the 75 kDa Arm^{XM19} does not co-immunoprecipitate (lane 5) whereas the Arm^{H8.6} one does (arrow in lane 6). Arrowhead indicates the neural isoform (82 kDa). In lane 5, a novel band appears between the neural and hypophosphorylated forms of Arm; this band is probably a breakdown product of slow migrating forms of Arm. (C) GST pull-down experiments showing that the C-terminal part of Arm binds GST-Tsh full-length (right panel) but not the Arm repeat region (R1–8) or the control GST-Modulo (middle panel). Left panel shows the [³⁵S]Arm constructs. (D) Schematic drawing of mutant Arm proteins and their interaction with Tsh *in vivo* and *in vitro*. (ND: not determined; numbers indicate the amino acid end points of each construct.)

in the absence of Wg signalling, suggesting that other Wg-independent kinase(s) phosphorylate Tsh. Although multiple isoforms of Tsh are detectable, only the hypophosphorylated form seems to interact with Arm throughout embryogenesis (Figure 4) and this form of Tsh appears to be localized both in the cytoplasmic and nuclear compartments (Figure 2). Gain of Wg function induces

the hyperphosphorylation of Tsh and a high Tsh level in all nuclei in the trunk correlating high nuclear Tsh with the production of naked cuticle in larvae. Conversely, loss of Wg function gives rise to less hyperphosphorylated Tsh and lower levels of Tsh in the nuclei (Gallet *et al.*, 1998; Figure 3). One possibility is that during the stabilization phase (stages 8–10) when initial cell-fate decisions are

Fig. 6. (A) Sequence of the C-terminal end of Armadillo and its Human (Hum) and *Xenopus* (Xen) orthologues. Identities between the three sequences are shaded in black. The deduced amino acid domain of Arm which binds to Tsh is outlined in black. (B) Summary of the proteins which interact with different domains of Arm/β-catenin.

taken (Bejsovec and Martinez-Arias, 1991; Dougan and DiNardo, 1992; Noordermeer *et al.*, 1992; Hooper, 1994; Manoukian *et al.*, 1995; Hays *et al.*, 1997; reviewed by Perrimon, 1994) Wg signal could recruit hypophosphorylated Tsh via its interaction with intracellular Arm, stimulating its entrance into the nucleus. This phenomenon may allow Tsh to enhance its own transcription (since Tsh autoregulates its own transcription; de Zulueta *et al.*, 1994) leading to increased levels of Tsh in the nuclei. Thus Wg signalling with high Tsh levels induces cells to secrete naked cuticle in the posterior part of each segment. On the other hand in cells that do not receive Wg, Arm is not stabilized due to Sgg activity, allowing free low levels of Tsh to participate in the formation of the denticle belt. Another non-exclusive possibility, raised by the existence of a Sgg/Tsh complex *in vivo* (Figure 7), is that Sgg could directly or indirectly regulate Tsh phosphorylation and/or stability.

We do not know whether Sgg is directly responsible for the hyperphosphorylation of Tsh observed in this study. In wild-type two major forms of hyperphosphorylated Tsh are detected on Western blots (Figure 3B), which are present in the nuclear compartments (Figure 2B). Loss of Sgg activity seems to remove one of these forms leaving the other intact (Figure 3B). Furthermore in *sgg*⁻ embryos Tsh is detected strongly in all nuclei of the trunk segments (Figure 3A). Sgg is known to act negatively to phosphorylate Arm causing its degradation. The phosphorylation of Arm due to Sgg activity is therefore transitory and difficult to detect (Peifer *et al.*, 1994c; Pai *et al.*, 1997). Sgg may have the same effect on Tsh. If so the observed hyperphosphorylation of Tsh may be due to the activities of other unknown kinases, as is the case with Arm (Peifer *et al.*, 1994b). Finally we stress that the present results cannot distinguish between the hypotheses of whether Tsh is phosphorylated in the nuclear and/or cytoplasmic compartments.

Tsh binds to the C-terminal domain of Arm

Many proteins are known to bind Arm/β-catenin. Many of these partners bind to repeats 3–8; one exception is α-catenin that binds to the N-terminal domain (Figure 6B)

Fig. 7. Tsh is epistatic to and is in a complex with Sgg. (A) Epistasis experiments between *sgg*^{MXII} hemizygotes derived from *sgg* germ-line clones (glc) and lacking *tsh*⁺ activity. The double mutant embryos (lower right panel) display denticles on their ventral surface whereas *sgg* glc in the presence of *tsh*⁺ activity have naked cuticles (middle left panel). This indicates that Tsh is genetically downstream of Sgg. Confocal analyses of stage 10 *sgg*^{MXII} glc embryos indicates that Tsh is highly nuclear everywhere in the trunk (lower left panel). All views are ventro-lateral and anterior is to the left. (B) Immunoprecipitation by anti-Tsh from whole embryonic lysates show that Sgg is in a complex with Tsh (lane 3). In wild-type embryos, with this Sgg antibody, we detected two isoforms of Sgg at ~56 and 58 kDa (M.Bourouis, personal communication). Note that we detect only the more slowly migrating isoform of Sgg in this experiment (arrow). Lanes 1 and 4 correspond to negative controls for the immunoprecipitation experiments.

(Hulsken *et al.*, 1994; Miller and Moon, 1996; Orsulic and Peifer, 1996; van de Wetering *et al.*, 1997). By *in vivo* and *in vitro* approaches we defined the Arm C-terminal domain as essential for the interaction with Tsh (Figure 5). Taken together, our results allow us to determine a 23 aa domain (between aa 692 and 715) in Arm as necessary for the interaction with Tsh. Interestingly, this domain lies in the most conserved part of the C-terminal domain of Arm (Figure 6A).

Genetic analysis has shown that the Arm C-terminal domain is essential for Wg signalling: mutants lacking the C-terminal domain (following the repeat domain) abolish the transduction of Wg signal without affecting Arm function in cell adhesion. The larger the C-terminal deletion the more severe is the mutant phenotype (Peifer and Wieschaus, 1990; reviewed by Peifer *et al.*, 1993a).

The prevailing model proposes that TCF (a DNA-binding protein) binds directly the target gene in a complex with Arm/ β -catenin activating the transcription of the Wg/Wnt target genes. Using a chloramphenicol acetyl transferase (CAT) reporter gene in cell culture, Molenaar *et al.* (1996) and van de Wetering *et al.* (1997) have shown that X-TCF3 did not activate transcription unless cotransfected with β -catenin. Moreover, a fusion between the Gal4 DNA-binding domain and the C-terminal domain of Arm is able to transactivate a CAT reporter gene (van de Wetering *et al.*, 1997). A second model is that Arm/ β -catenin could act as a shuttle protein between cytoplasmic and nuclear compartments. Arm/ β -catenin shares homology with the importins/karyopherins which are cytoplasmic receptors for proteins containing nuclear localization sequences (NLS) allowing their docking to the nuclear pore (Peifer *et al.*, 1994a; Yano *et al.*, 1994; Fagotto *et al.*, 1998). In agreement with this, it has been shown that Arm is able to interact with the nuclear pore machinery (Fagotto *et al.*, 1998).

Considering these two models, how could the C-terminal domain of Arm act as transactivator? In the first model, Arm/ β -catenin could be considered as a bridge between a general DNA-binding factor (e.g. TCF) and a specific transcription factor like Tsh which modulates a specific function for signalling. In this model the transactivating domain of Arm could bind several different and localized factors allowing tissue specific output.

In the shuttle model, upon Wg signalling, Arm could interact with general (dTCF) as well as specific factors (Tsh) and translocate them into the nucleus to activate Wg target genes. In this model Arm/ β -catenin are not in a transcription complex with dTCF/LEF, but act rather to catalyse the entry of ubiquitous and localized transcription factors into the nucleus. This model can explain the results described above in cell culture: if Arm/ β -catenin can bind many different nuclear factors present in cells, cotransfection of Arm/ β -catenin with dTCF/xTCF could promote the nuclear entry of several factors which collaborate with TCF to transactivate the reporter gene. Moreover, the Gal4-Arm C-terminus fusion protein which binds directly the DNA could also trap such transactivating factors affecting reporter gene activity.

In accord with either hypothesis Arm/ β -catenin are detected in the nucleus in cells where Wg is signalling. Furthermore, Tsh is able to produce naked cuticle in the absence of zygotic dTCF (Gallet *et al.*, 1998), suggesting that Tsh and dTCF act independently for Wg signalling. However we note that the maternal source of dTCF in this experiment may be sufficient to produce the naked cell-fate choice in combination with high levels of Tsh. Further experiments are required to establish whether Tsh acts directly with dTCF and especially to determine whether or not Arm/ β -catenin are part of a transcription complex or act simply as nuclear shuttles.

Finally, we can consider these two models together where, upon an extracellular signal (e.g. Wg/Wnt), a cytoplasmic protein (e.g. Arm/ β -catenin) recruits specific factors (e.g. Tsh) and stimulates their entry into the nucleus, where this bipartite complex could collaborate with general DNA-binding factors (e.g. TCF) to regulate specific target genes of the pathway (Fagotto *et al.*, 1998). There are several results in favour of this model: first, TCF

is localized in the nucleus independently of Arm (reviewed by Clevers and van de Wetering, 1997). Secondly, TCF is able to bind other specific factors including Groucho or dCBP to repress Wg/Wnt target genes (Cavallo *et al.*, 1998; Roose *et al.*, 1998; Waltzer and Bienz, 1998). Thirdly, Tsh does not contain canonical NLS signal and requires an unknown mechanism for entry into the nucleus. Nevertheless, Tsh is the first example of a protein binding Arm in its C-terminal domain and potentially is able to participate in the transactivating process.

Materials and methods

Fly strains

arm^{XM19} is a null allele for Wg signalling but normal for cell adhesion; *arm^{H8.6}* is a thermosensitive Wg signalling but cell adhesion plus allele (Klingensmith *et al.*, 1989; Peifer and Wieschaus, 1990). *sgg^{MX11}* is described by Siegfried *et al.* (1992) and Ruel *et al.* (1993). We made a *wg^{CX4}/CyO[Act5cGFP, w⁺]* strain for embryo selection (see below). The other strains are described in Gallet *et al.* (1998) or obtained from the Bloomington stock center. All experiments with the UAS-Gal4 system (Brand and Perrimon, 1993) were performed at 29°C.

Germ-line clones and genetic epistasis

arm^{XM19} and *sgg^{MX11}* (both *in cis* with *FRT101*) were crossed with *ovo^D FRT101/FM6; HS-FLP38/HS-FLP38* males. Progeny were heat shocked at the third instar larvae stage and *ovo^D FRT101/arm* (or *sgg*) *FRT101* females were crossed with *y w* males. For epistasis experiments between *sgg^{MX11}* and *tsh⁸*, *sgg^{MX11}FRT101/FM7; tsh⁸/CyO* females were crossed with *ovo^D FRT101/FM6; HS-FLP38/HS-FLP38* males. Progeny were heat shocked as described above and *ovo^D FRT101/sgg^{MX11}FRT101; tsh⁸/HS-FLP38* females were crossed with *tsh⁸/CyO* males.

Immunostaining of embryos

Immunodetection was performed as described in Gallet *et al.* (1998) except for stage 5 and 8 embryos where anti-Tsh was used at 1/200. 7-aminoactinomycin D (7AAD) (5 μ g/ml) in PBT was used to label nuclei following incubation for 5 min incubation at room temperature. Secondary anti-rat fluorescent fluorescein isothiocyanate (FITC) antibodies were from Jackson laboratories. Embryos were analysed by confocal microscopy (Zeiss). Embryos mutant for *wg^{CX4}* and *arm^{XM19}* germ-line clones were identified by the loss of Invected expression.

Immunoblotting

Anti-Tsh (rat) was described in Gallet *et al.* (1998) and was used at 1/1000. The monoclonal anti-Arm (mouse) N2 7A1 antibody (Peifer *et al.*, 1994c) was used at 1/500. Mouse monoclonal 4G1E11 anti-Sgg was a gift from Marc Bourouis and was used at 1/500. Anti-Modulo (a gift from J.Pradel) was a mouse monoclonal antibody and was used at 1/4000. Anti-histone H2 (mouse) was used at 1/500. Secondary antibodies were all peroxidase-coupled and revelation performed using the ECL technology (Pierce).

Teashirt dephosphorylation assay

Overnight embryos were dechorionated in 50% bleach and extensively washed with water. Embryos were then dounced in lysis buffer (Tris pH 9.5, 1.5% Triton X-100, 150 mM NaCl, 10% glycerol), spun at 12 000 g for 20 min at 4°C. Supernatant was collected and proteins quantified by the Bradford method (Bio-Rad). One hundred micrograms of total proteins were incubated at 37°C with 200 U of alkaline phosphatase (Boehringer Mannheim) in the presence of alkaline phosphatase buffer. Dephosphorylation was stopped by adding 2 $\times$ Laemmli buffer and stored at -20°C. In parallel, one assay was performed under the same conditions but with addition of phosphatase inhibitors: NaF, β -glyceraldehyde and orthovanadate. Tsh phosphorylation was analysed by SDS-PAGE (7.5%) and immunoblotting.

Developmental Western blot and genotypes analyses

Proteins extracts were prepared as described above and proteins were separated by SDS-PAGE by loading 10 μ g/lane of total protein on 7.5% acrylamide gel. Blots were probed with anti-Tsh antibody. For Western blots of mutant embryos, stage 10–12 embryos were selected. *wg^{CX4}* homozygotes were selected for their non-CyOGFP phenotype. Hemi-

zygotes for *arm^{XM19}* and *sgg^{MX11}* were selected on the basis of their phenotypes. Three embryos per lane were loaded and these experiments were repeated several times to test for their reproducibility. Blots were reprobed with anti- β -tubulin to verify that similar amounts of protein were loaded (Peifer *et al.*, 1994c).

Nuclear and cytoplasmic extract preparation

For cytoplasmic preparations, stage 8–11 embryos were dechorionated in 50% bleach, washed extensively with distilled water and dounced gently in HEPES 20 mM (pH 7.9), 10 mM KCl in presence of proteases and phosphatase inhibitors. Homogenates were spun at 20 000 g for 20 min at 4°C. Protein amounts were quantified by the Bradford method. For nuclear preparation, embryos were also dechorionated, then washed in water and in phosphate-buffered saline. Embryos were resuspended and dounced in NEB 0.3 buffer (300 mM sucrose, 10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol plus phosphatase and protease inhibitors). Embryonic homogenates were loaded on a sucrose cushion made with NEB 1.7 (same composition as NEB 0.3 but with 1.7 mM sucrose) and spun at 4°C in a microfuge for 20 min. The supernatant was discarded and nuclei resuspended in NEB 0.3 to purify again on a sucrose cushion. This operation was performed twice to ensure high quality of purification.

Co-immunoprecipitation and binding studies

Immunoprecipitation and binding studies were performed as described in Gallet *et al.* (1998). Arm M279, M549 and M579 (with translation starting at methionine 279, 549 and 579) were generated by PCR and cloned in Blue Script vector for *in vitro* translation. Arm R1-8 was taken off from pCK2 (Pai *et al.*, 1996) and cloned in BS.

Acknowledgements

We thank Mark Peifer who suggested the immunoprecipitation experiments with *armadillo* mutants and for his advice on the manuscript. We thank Marc Bourouis for providing Sgg antibodies and Françoise Pages for advice on biochemistry. The Arm antibodies (N2 7A1; developed by Eric Wieschaus) was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences at Johns Hopkins University of Medicine, and the department of Biological Sciences at University of Iowa, under contract NO1-HD-2-3144 from NICHD. A.G. and A.E. were supported by grants from Association de la Recherche contre le Cancer.

References

- Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997) β -catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.*, **16**, 3797–3804.
- Alexandre, E., Graba, Y., Fasano, L., Gallet, A., Perrin, L., De Zulueta, P., Pradel, J., Kerridge, S. and Jacq, B. (1996) The *Drosophila* Teashirt homeotic protein is a DNA-binding protein and *modulo*, a HOM-C regulated modifier of variegation, is a likely candidate for being a direct target gene. *Mech. Dev.*, **59**, 191–204.
- Bejsovec, A. and Martinez, A. (1991) Roles of wingless in patterning the larval epidermis of *Drosophila*. *Development*, **113**, 471–485.
- Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J. and Nusse, R. (1996) A new member of the *frizzled* family from *Drosophila* functions as a Wingless receptor. *Nature*, **382**, 225–230.
- Bhat, K. (1998) *frizzled* and *frizzled-2* play a partially redundant role in Wingless signaling and have similar requirements to *wingless* in neurogenesis. *Cell*, **95**, 1027–1036.
- Brand, A. and Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotype. *Development*, **118**, 401–415.
- Brunner, E., Peter, O., Schweizer, L. and Basler, K. (1997) *pangolin* encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in *Drosophila*. *Nature*, **385**, 829–833.
- Cadigan, K.M. and Nusse, R. (1997) Wnt signaling: a common theme in animal development. *Genes Dev.*, **11**, 3286–3305.
- Cadigan, K.M., Fish, M.P., Rulifson, E.J. and Nusse, R. (1998) Wingless repression of *Drosophila* *frizzled 2* expression shapes the Wingless morphogen gradient in the wing. *Cell*, **93**, 767–777.
- Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M. and Bejsovec, A. (1998) *Drosophila* Tcf and Groucho interact to repress Wingless signalling activity. *Nature*, **395**, 604–608.
- Clevers, H. and van de Wetering, M. (1997) TCF/LEF factor earn their wings. *Trends Genet.*, **13**, 485–489.
- Cox, R.T. and Peifer, M. (1998) Wingless signaling: the inconvenient complexities of life. *Curr. Biol.*, **8**, R140–R144.
- Cox, R.T., Kirkpatrick, C. and Peifer, M. (1996) Armadillo is required for adherens junction assembly, cell polarity and morphogenesis during *Drosophila* embryogenesis. *J. Cell Biol.*, **134**, 133–148.
- de Zulueta, P., Alexandre, E., Jacq, B. and Kerridge, S. (1994) Homeotic complex and *teashirt* genes co-operate to establish trunk segmental identities in *Drosophila*. *Development*, **120**, 2287–2296.
- Dougan, S.T. and DiNardo, S. (1992) *wingless* generates cell type diversity among *engrailed* expressing cells. *Nature*, **360**, 347–350.
- Fagotto, F., Gluck, U. and Gumbiner, B.M. (1998) Nuclear localization signal-independent and Importin/Karyopherin-independent nuclear import of β -catenin. *Curr. Biol.*, **8**, 181–190.
- Fasano, L., Roder, L., Core, N., Alexandre, E., Vola, C., Jacq, B. and Kerridge, S. (1991) The gene *teashirt* is required for the development of *Drosophila* embryonic trunk segments and encodes a protein with widely spaced zinc finger motifs. *Cell*, **64**, 63–79.
- Gallet, A., Erker, A., Charroux, B., Fasano, L. and Kerridge, S. (1998) Trunk-specific modulation of Wingless signalling in *Drosophila* by Teashirt binding to Armadillo. *Curr. Biol.*, **8**, 893–902.
- Hays, R., Gibori, G.B. and Bejsovec, A. (1997) Wingless signaling generates pattern through two distinct mechanisms. *Development*, **124**, 3727–3736.
- Hooper, J.E. (1994) Distinct pathways for autocrine and paracrine Wingless signalling in *Drosophila* embryos. *Nature*, **372**, 461–464.
- Hulsken, J., Birchmeier, W. and Behrens, J. (1994) E-cadherin and APC compete for the interaction with β -catenin and the cytoskeleton. *J. Cell Biol.*, **127**, 2061–2069.
- Klingensmith, J., Noll, E. and Perrimon, N. (1989) The segment polarity phenotype of *Drosophila* involves differential tendencies toward transformation and cell death. *Dev. Biol.*, **134**, 130–145.
- Korinek, V., Barker, N., Morin, P., van Wichen, D., de Weger, R., Kinzler, K., Vogelstein, B. and Clevers, H. (1997) Constitutive transcriptional activation by a β -catenin-Tcf complex in APC-/- colon carcinoma. *Science*, **275**, 1784–1787.
- Loureiro, J. and Peifer, M. (1998) Roles of Armadillo, a *Drosophila* catenin, during central nervous system development. *Curr. Biol.*, **8**, 622–632.
- Manoukian, A.S., Yoffe, K.B., Wilder, E.L. and Perrimon, N. (1995) The *porcupine* gene is required for *wingless* autoregulation in *Drosophila*. *Development*, **121**, 4037–4044.
- Martinez-Arias, A., Baker, N.E. and Ingham, P.W. (1988) Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development*, **103**, 157–170.
- Miller, J.R. and Moon, R.T. (1996) Signal transduction through β -catenin and specification of cell fate during embryogenesis. *Genes Dev.*, **10**, 2527–2539.
- Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O. and Clevers, H. (1996) XTcf-3 transcription factor mediates β -catenin-induced axis formation in *Xenopus* embryos. *Cell*, **86**, 391–399.
- Muller, H.-Arno, J., Arno, J., Samanta, R. and Wieschaus, E. (1999) Wingless signaling in the *Drosophila* embryo: zygotic requirements and the role of *frizzled* genes. *Development*, **126**, 577–587.
- Neumann, C. and Cohen, S. (1997) Morphogens and pattern formation. *Bioessays*, **19**, 721–729.
- Noordermeer, J., Johnston, P., Rijsewijk, F., Nusse, R. and Lawrence, P.A. (1992) The consequences of ubiquitous expression of the *wingless* gene in the *Drosophila* embryo. *Development*, **116**, 711–719.
- Nusslein-Volhard, C. and Wieschaus, E. (1980) Mutations affecting segment number and polarity in *Drosophila*. *Nature*, **287**, 795–801.
- Orsulic, S. and Peifer, M. (1996) An *in vivo* structure-function study of Armadillo, the β -catenin homologue, reveals both separate and overlapping regions of the protein required for cell adhesion and for wingless signaling. *J. Cell Biol.*, **134**, 1283–1300.
- Pai, L.M., Kirkpatrick, C., Blanton, J., Oda, H., Takeichi, M. and Peifer, M. (1996) *Drosophila* α -catenin and E-cadherin bind to distinct regions of *Drosophila* Armadillo. *J. Biol. Chem.*, **271**, 32411–32420.
- Pai, L.M., Orsulic, S., Bejsovec, A. and Peifer, M. (1997) Negative regulation of Armadillo, a Wingless effector in *Drosophila*. *Development*, **124**, 2255–2266.

- Peifer,M. and Wieschaus,E. (1990) The segment polarity gene *armadillo* encodes a functionally modular protein that is the *Drosophila* homolog of human plakoglobin. *Cell*, **63**, 1167–1176.
- Peifer,M., Orsulic,S., Pai,L.M. and Loureiro,J. (1993a) A model system for cell adhesion and signal transduction in *Drosophila*. *Dev. Suppl.*, 163–176.
- Peifer,M., Orsulic,S., Sweeton,D. and Wieschaus,E. (1993b) A role for the *Drosophila* segment polarity gene *armadillo* in cell adhesion and cytoskeletal integrity during oogenesis. *Development*, **118**, 1191–1207.
- Peifer,M., Berg,S. and Reynolds,A.B. (1994a) A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell*, **76**, 789–791.
- Peifer,M., Pai,L.M. and Casey,M. (1994b) Phosphorylation of the *Drosophila* adherens junction protein Armadillo: roles for Wingless signal and Zeste-white 3 kinase. *Dev. Biol.*, **166**, 543–556.
- Peifer,M., Sweeton,D., Casey,M. and Wieschaus,E. (1994c) Wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. *Development*, **120**, 369–380.
- Perrimon,N. (1994) The genetic basis of patterned baldness in *Drosophila*. *Cell*, **76**, 781–784.
- Riggelman,B., Schedl,P. and Wieschaus,E. (1990) Spatial expression of the *Drosophila* segment polarity gene *armadillo* in post-transcriptionally regulated by wingless. *Cell*, **63**, 549–560.
- Roder,L., Vola,C. and Kerridge,S. (1992) The role of the *teashirt* gene in trunk segmental identity in *Drosophila*. *Development*, **115**, 1017–1033.
- Roose,J., Molenaar,M., Peterson,J., Hurenkamp,J., Brantjes,H., Moerer,P., van de Wetering,M., Destree,O. and Clevers,H. (1998) The *Xenopus* Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature*, **395**, 608–612.
- Rubinfeld,B., Albert,I., Porfiri,E., Fiol,C., Munemitsu,S. and Polakis,P. (1996) Binding of GSK3 β to the APC- β -catenin complex and regulation of complex assembly. *Science*, **272**, 1023–1026.
- Ruel,L., Bourouis,M., Heitzler,P., Pantescio,V. and Simpson,P. (1993) *Drosophila* shaggy kinase and rat glycogen synthase kinase-3 have conserved activities and act downstream of *Notch*. *Nature*, **362**, 557–560.
- Siegfried,E., Chou,T.B. and Perrimon,N. (1992) Wingless signaling acts through Zeste-white, 3, the *Drosophila* homolog of Glycogen synthase kinase-3, to regulate *engrailed* and establish cell fate. *Cell*, **71**, 1167–1179.
- van de Wetering,M. *et al.* (1997) Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene *dTcf*. *Cell*, **88**, 789–799.
- Waltzer,L. and Bienz,M. (1998) *Drosophila* CBP represses the transcription factor TCF to antagonize Wingless signalling. *Nature*, **395**, 521–525.
- Yano,R., Oakes,M.L., Tabb,M.M. and Nomura,M. (1994) Yeast Srp1p has homology to armadillo/plakoglobin/ β -catenin and participates in apparently multiple nuclear functions including the maintenance of the nucleolar structure. *Proc. Natl Acad. Sci. USA*, **91**, 6880–6884.
- Zhang,J. and Carthew,R. (1998) Interactions between Wingless and Dfz2 during *Drosophila* wing development. *Development*, **125**, 3075–3085.

Received January 22, 1999; accepted February 26, 1999