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Original Article

dTcf antagonises Wingless signalling during the development and patterning of the wing in *Drosophila*

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ABSTRACT Members of the Tcf family of HMG box-containing transcriptional regulators mediate Wnt signalling in the nucleus. Current models suggest that in the absence of Wnt signalling, Tcf interacts with the repressor protein Groucho and suppresses the expression of Wnt targets. Wnt signalling leads to increases in the level of cytoplasmic βcatenin, which enters the nucleus, displaces Tcf from Groucho and leads to transcriptional activation. In order to test this model we have studied the effects of *Drosophila* Tcf (dTcf) on signalling by Wingless, a *Drosophila* member of the Wnt family. We show that overexpression of wild-type dTcf during the development and patterning of the wing antagonises Wingless signalling. Furthermore, increases in the concentration of Armadillo, the *Drosophila* homologue of βcatenin, do not appear to be sufficient to trigger the change from antagonism to activation. This leads us to suggest that the inactivation of the repressive activity of dTcf requires the activity of Wingless in a manner that is independent of Armadillo. We observe that a Groucho molecule devoid of the WD40 repeats can interact with dTcf and acts as a dominant repressor of Wingless signalling *in vivo* and *in vitro*. Coexpression of this molecule with dTcf however, does not lead to enhancement of the repressive effects of dTcf alone. This observation suggests that repression by dTcf might not simply be mediated by an interaction with Groucho but that dTcf may have an intrinsic repressive activity that has to be antagonised by Wingless signalling.

KEY WORDS: *dTcf, Wingless, Drosophila, development.*

Introduction

The Wnt signalling pathway is a conserved system of signals, receptors and transducers that play an important role in the patterning of developing embryos. Experiments using both genetic analysis and biochemical assays in insect and vertebrate systems, as well as in cell culture, have led to a model of how a Wnt signal is relayed to its targets. The current model (reviewed in Miller *et al.*, 1999) contends that Wnt ligands act through receptors encoded by members of the *frizzled* gene family to activate a down-stream effector, Dishevelled (Dsh). Dsh seems to act by inhibiting a complex containing GSK3/Shaggy, Axin and APC whose function it is to target cytosolic βcatenin for degradation (reviewed in Bienz, 1999). The activation of Dsh leads to an intracellular increase and post-translational modification of βcatenin, which under these conditions enters the nucleus and forms a complex with members of the Tcf/LEF family of nuclear proteins. This complex acts to alter gene expression directly and, consistent with this model, Tcf binding sites have been reported in promoters of Wnt responsive genes (Brannon *et al.*, 1997; Riese *et al.*, 1997).

Although the prevalent view about Tcf is that it is an activator, there is evidence to suggest that it may also act as a repressor. A screen in *Drosophila* for modifiers of the segment polarity phenotypes caused by loss of *wingless* (a *Drosophila* Wnt gene) or *armadillo* (the gene encoding the *Drosophila* homologue of βcatenin) identified mutations in *Drosophila* Tcf (dTcf) as suppressers of these phenotypes (Cavallo *et al.*, 1998). Given current views of Wingless (Wg) signalling, mutations in dTcf should behave as enhancers rather than suppressers of defects in this pathway. In addition, ectopic expression of dTcf was found to enhance rather than suppress the phenotype of a weak *wingless* (*wg*) mutant allele (Cavallo *et al.*, 1998). This raises the possibility that in *Drosophila*, Tcf acts as a repressor of Wingless signalling. The finding that dTcf binds the transcriptional co-repressor Groucho (Gro) lends support to this possibility and suggests that the repressive effects of dTcf are mediated through its association with Gro (Roose *et al.*, 1998).

Abbreviations used in this paper: Tcf, T-cell factor; dTcf, *Drosophila* T-cell factor; Wg, Wingless; Dsh, Dishevelled; Arm, Armadillo; Gro, Groucho; *sd*G4, *scalloped*Gal4; *ms1096*G4, *ms1096*Gal4

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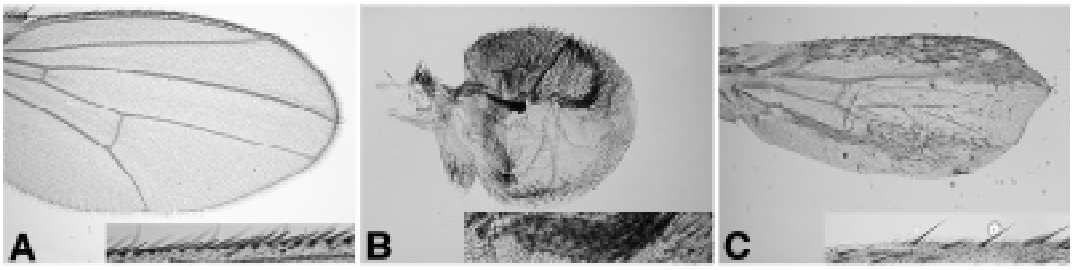

Fig. 1. Effects of overexpression of dTcf under the control of *scalloped*Gal4 (*sd*G4). (A) *A wild-type wing for comparison.* **(B)** *Overexpression of Wg leads to a dramatic expansion of the wing margin. (It is likely that the size of the wing is reduced as neural cell types are increased at the expense of epidermis).* **(C)** *In contrast, the overexpression of dTcf leads to a loss of wing margin bristles. All wings are from female flies and are at the same magnification. The insets show a 4x magnification of the corresponding anterior wing margin.*

To analyse further the function of dTcf *in vivo* we have carried out overexpression experiments in the developing *Drosophila* wing. These experiments show that dTcf on its own antagonises rather than implements Wingless signalling. In addition, we find that coexpression of dTcf with Armadillo (Arm) can suppress aspects of the phenotype generated by overexpression of dTcf alone but surprisingly this never leads to ectopic Wingless signalling as the current model would predict. Overexpression of dTcf together with modified Gro proteins in the *Drosophila* wing led us to conclude that dTcf has a repressive function which may not rely simply on its interaction with Gro.

Results

The adult wing of *Drosophila* is made up of two sheets of cells, one dorsal and one ventral, separated by a neurogenic region, the wing margin. The wing develops from the wing imaginal disc in which the expression of Wg outlines the hinge and the margin during the third larval instar. To investigate the role of dTcf *in vivo* we have directed the expression of dTcf using the Gal4/UAS system in the developing wing (Brand and Perrimon, 1993). As Wg is involved in various steps during wing development we would expect that interference with Wingless signalling would have effects on the development of this structure.

dTcf behaves as a repressor of Wingless signalling in vivo

Overexpression of dTcf in a wild-type background leads to phenotypes typical of loss rather than gain of the Wingless signal. When Wg is over-expressed with *scalloped*Gal4 (*sd*G4) the neurogenic region of the wing margin is greatly expanded (Fig. 1B). However, overexpression of dTcf throughout the developing wing with *sd*G4 produces reduced wings with severe patterning defects including extensive loss of wing margin (Fig. 1C); a structure whose development requires inputs from both the Notch and Wingless signalling pathways (Kim *et al.*, 1996; Klein and Martinez Arias, 1998). At a low frequency (about 4%, n=37), *sd*G4>UAS*dTcf* flies display wing to notum transformations. This phenotype is characteristic of a loss of Wingless signalling during larval life (Couso *et al.,* 1994). In a *wg* heterozygous background the instances of wing to notum transformations in *sd*G4>UAS*dTcf* flies increase from 4% to 25% (n=39) suggesting that the effects of UAS*dTcf* result from an antagonism of the Wg pathway. This result supports the idea that, in addition to being an activator of Wingless signalling, dTcf is involved in repressing targets downstream of Wingless signalling in the absence of Wg itself, see also (Cavallo *et al.*, 1998; Roose *et al.*, 1998).

Due to the high lethality induced by expression of dTcf with *sd*G4, *ms1096*Gal4 was used for further experiments with dTcf, (see Materials and Methods) with this giving a higher adult survival rate. Overexpression of dTcf with *ms1096*Gal4 produces severe patterning defects in the wing. In these experiments we observed that the expression of dTcf reduces the size of the wing, eliminates bristles in the margin and produces some extra bristles and veins over the wing blade (Fig. 2B). The effects on the size of the wing and on the ectopic bristles are partially suppressed by coexpression of dTcf with wild-type Arm (Fig. 2D). This observation supports the idea that dTcf is antagonising Wingless signalling. However, Arm fails to rescue the loss of wing margin bristles caused by overexpression of dTcf. This is surprising since expression of Arm alone leads to the production of ectopic bristles close to the wing margin (Fig. 2C). These results indicate that, at least at the wing margin, an interaction between Arm and dTcf is not sufficient to promote Wingless signalling. Perhaps the complex between these proteins requires a further modification that is limiting at this position.

Coexpression of dTcf with a truncated Armadillo molecule (ArmΔC), which has greatly reduced signalling ability and which has been shown to have dominant negative properties (White *et al.*, 1998) fails to rescue the ectopic bristle phenotype and reduces further the size of the wing (Fig. 2F). The loss of bristles at the wing margin is not enhanced suggesting again that at the margin, the effects of the interactions between Arm and dTcf are not solely dependent on the relative amount of these products.

The extra bristles that appear over the wing blade after expression of dTcf could be interpreted as the result of ectopic Wg activity (see for example Fig. 1B). However, it is worth stressing that these bristles are suppressed, rather than enhanced as would be expected if this were the case, when dTcf is coexpressed with wild-type Arm (Fig. 2D). These ectopic bristles produced by dTcf overexpression seem to be associated with ectopic vein tissue. There is evidence to show that the expression of the pro-neural genes *achaete* and *scute* is repressed in the pro-vein regions of the wing disc during pupal development (Skeath and Carroll, 1991). Therefore the ectopic bristles elicited by dTcf might reflect an interference with this repression rather than be a direct result of Wingless signalling.

Gro^{Nterm} antagonises Wingless signalling

The possibility that dTcf mediates repression was first suggested by the finding that dTcf and the co-repressor Groucho (Gro) interact physically and functionally with each other (Roose *et al.*, 1998). The Groucho protein can be separated into five separate domains (Stifani *et al.*, 1992) (see Fig. 3). The N-terminal domains contain the sites of interaction with other Gro proteins and dTcf, (Pinto and Lobe, 1996; Chen *et al.*, 1998; Roose *et al.*, 1998) as well as the ability to provide a repression domain when bound directly to DNA (Fisher *et al.*,

1996). On the other hand, the C-terminal domain contains the WD40 repeats which are thought to be important in binding H/E(spl) proteins, (Paroush *et al.*, 1994; Jimenez *et al.*, 1997) and has some repressive activity when bound to DNA, (Fisher *et al.*, 1996).

In order to further our studies of the repressive functions of dTcf we looked at the effects of overexpression of Gro and of an N-terminal Gro construct (Gro^{Nterm}) (see Materials and Methods). Whilst overexpression of Gro had little effect on the development and patterning of the wing (data not shown) we found that expression over the developing wing of Gro^{Nterm} with *sd*G4 led to phenotypes in the wing similar to those seen when dTcf is over-expressed. *sd*G4>UASGro^{Nterm} flies display loss of wing margin and wing to notum transformations. Furthermore, these phenotypes can be enhanced by expression of the constructs in a Wg heterozygous background (Fig. 3). This reveals that the effects of this construct are dependent on the dosage of *wg.* The phenotypes seen when Gro^{Nterm} is over-expressed with *sd*G4 are accompanied by loss of *wg* expression along the wing margin and more significantly, by loss of Vestigial boundary enhancer expression (vgBE) (Fig. 4). The activity of the vgBE is required for the expression of Wg at the wing margin (Klein and Martinez Arias, 1998) but it also depends on early Wingless signalling (Klein and Martinez Arias, 1999).

The possibility that Gro^{Nterm} is antagonising Wg is supported by the observation that overexpression of Gro^{Nterm} during embryogenesis generates weak but reproducible segment polarity defects characteristic of *wg* mutants (data not shown). Altogether these results indicate that Gro^{Nterm} can act to antagonise Wingless signalling. The fact that full length Gro does not have these effects suggests that deletions of the C-terminal region of Groucho are revealing the potential of this antagonism (see Discussion).

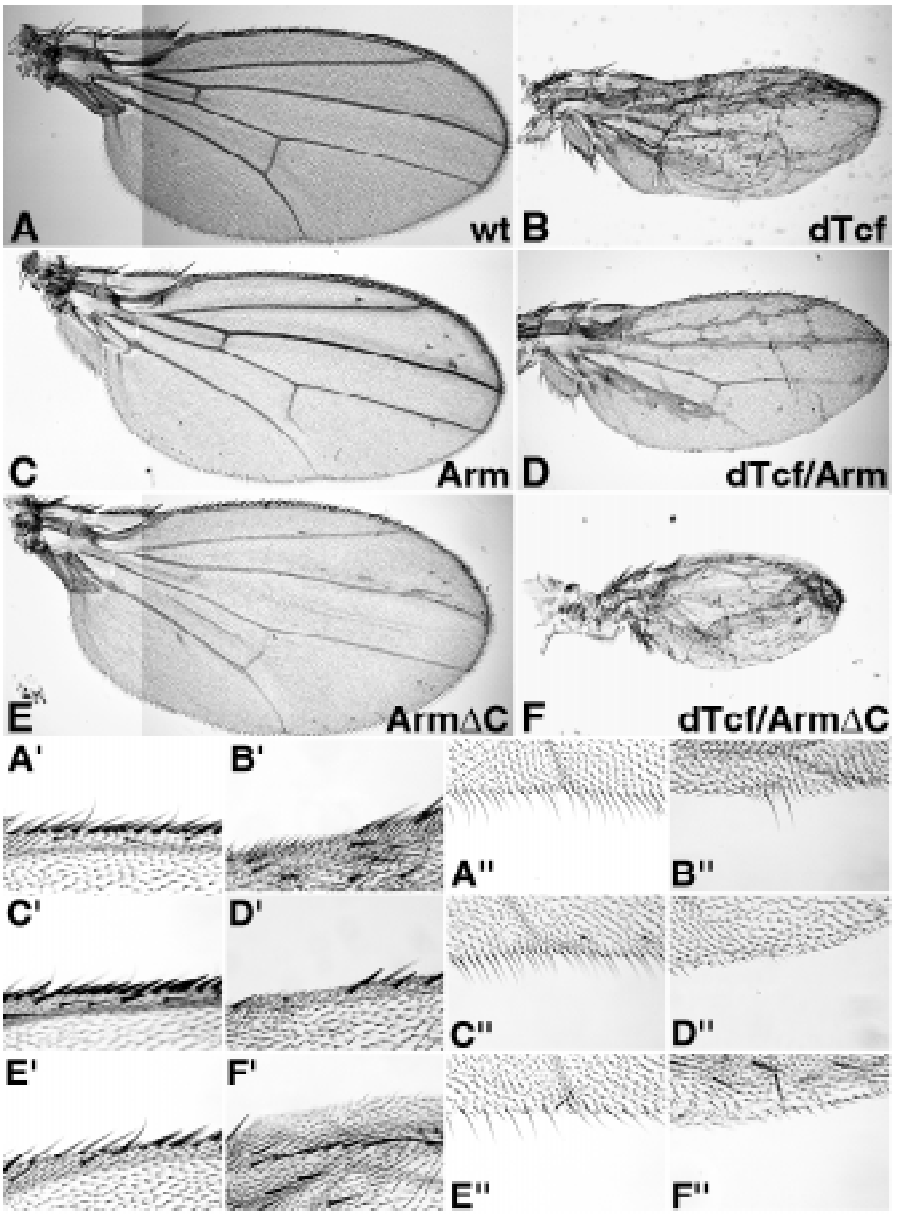

Fig. 2. Effects of overexpression of dTcf under the control of *ms1096*G4 alone or in combination with Arm. (A) *A wild-type wing.* **(B)** *Overexpression of dTcf leads to a reduction in the size of the wing. This is accompanied by ectopic bristles and vein tissue throughout the wing blade. The wing margin is consistently nicked (see* **B′** *and* **B″***).* **(C)** *Overexpression of Arm leads to ectopic bristles close to the wing margin (see* **C′** *and* **C″***) and some ectopic vein tissue.* **(D)** *Coexpression of Arm and dTcf leads to a consistent rescue of the size of the wing. The number of ectopic bristles is also reduced relative to the effect of overexpression of dTcf alone and the venation pattern is somewhat restored. The nicking of the wing margin is not rescued (see* **D′** *and* **D″***).* **(E)** *Overexpression of an Arm construct deleted for the C-terminus (ArmΔC) has been shown to have dominant negative effects in the embryo (White et al., 1998). In the wing, overexpression of this construct leads to some disruption of the wing margin (see* **E′** *and* **E″***) and the cross-veins and a few ectopic bristles.* **(F)** *Coexpression of ArmΔC and dTcf leads to a small wing with a highly disrupted venation pattern and many ectopic bristles. (*__A__ *to* __F__*) All the wings are shown at the same magnification. (*__A′__ *to* __F′__*) 4x magnification of a section of anterior wing margin from the main panels* __A__ *to* __F__ *respectively. (*__A″__ *to* __F″__*) 4x magnification of a section of posterior wing margin from the main panels* __A__ *to* __F__ *respectively.*

Gro^{Nterm} affects the action of a Wingless Response Element (WRE)

In order to test how direct the effects of Gro^{Nterm} are, we have also assayed the effects of Gro^{Nterm} on the activity of an enhancer of the Ubx gene (UbxB) which is responsive to Wingless signalling (Thuringer *et al.*, 1993; Riese *et al.*, 1997). In wild-type embryos, Wingless signalling promotes the activity of this enhancer in a spatially restricted domain of the visceral mesoderm. This activity requires the presence of a Wg response element (WRE) in the enhancer that contains two dTcf binding sites (Riese *et al.*, 1997). Overexpression of Gro^{Nterm} in the developing mesoderm significantly reduces expression of UbxB*lacZ* in the visceral mesoderm, particularly in the regions of low Wingless signalling (Fig. 5).

To further test the possibility that Gro^{Nterm} antagonises Wingless signalling directly, we have probed the ability of this molecule to interfere with the Arm/dTcf mediated activation of specific targets

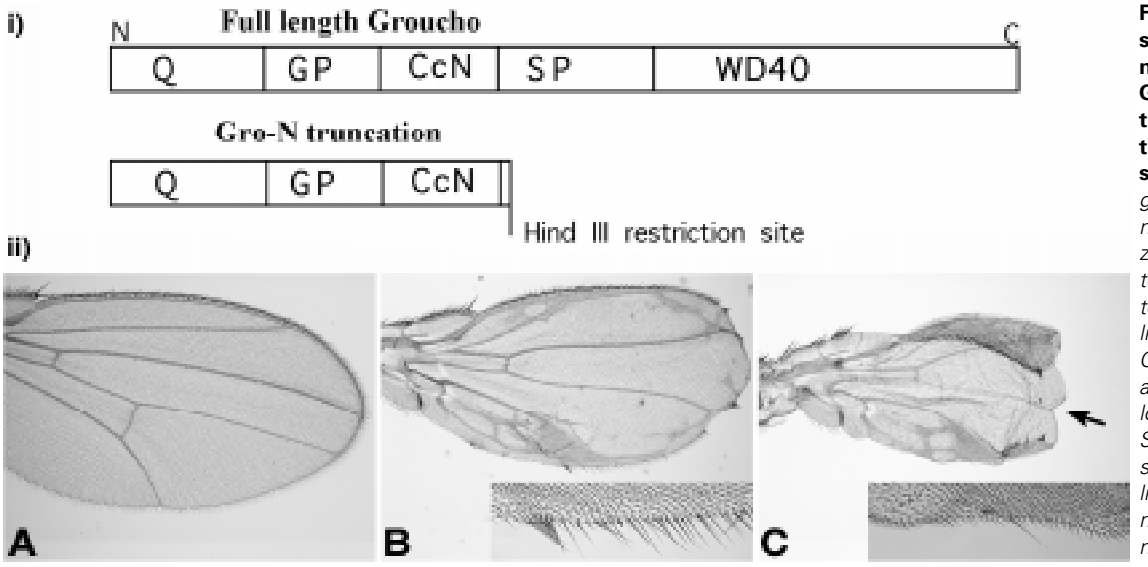

Fig. 3. (i) Schematic diagram showing the different domains of the full length Groucho protein (Gro) and those present in the truncation (Gro^{Nterm}) used in this study. *The Q domain is a glutamine-rich conserved domain which contains a leucine zipper-like motif involved in Gro tetramerization. The site of interaction between Gro and Tcf lies within this domain. The CcN region is highly conserved and contains a nuclear localisation signal. The GP and SP domains are poorly conserved regions, glycine and proline rich or serine and proline rich respectively. The C terminus is highly conserved and contains many WD40 repeats.*

(ii) The effects of over-expressing Gro^{Nterm} in the developing wing under the control of *sd*G4. (A) *A wild-type wing for comparison.* **(B)** *Overexpression of Gro^{Nterm} leads to large nicks in the wing margin (see inset) and disruption in the venation pattern of the wing. The overall size of the wing is reduced.* **(C)** *The effects of overexpression of Gro^{Nterm} are enhanced when in a heterozygous wingless (wg) background (see inset and arrow). All wings are at the same magnification. Each inset shows a 4x magnification of the posterior wing margin of the main panel.*

in IIAI.6 B cells (see Roose *et al.*, 1998). Coexpression of Arm and dTcf in these cells results in a transcriptional response of a transiently transfected Tcf reporter. (Fig. 6, see also Roose *et al.*, 1998). Gro^{Nterm} represses the activity of the Tcf reporter in the same manner as the wild-type Gro molecule (Fig. 6). This observation does suggest that the Gro^{Nterm} mediated antagonism of Wingless signalling that we observe *in vivo* is direct. The fact that full length Gro appears not to show an antagonism of Wingless signalling *in vivo* suggests that the C-terminus may be involved in regulating this activity of Gro *in vivo*.

Gro^{Nterm} titrates dTcf in vivo

In principle Gro^{Nterm} can bind both to dTcf and to other Gro molecules and it could be argued that, as suggested above, Gro^{Nterm} antagonises Wingless signalling by enhancing the repressive action of dTcf. To test this we coexpressed Gro^{Nterm} and Tcf in the developing wing. We reasoned that, if the effects of Gro^{Nterm} are mediated by dTcf, coexpression of Gro^{Nterm} and dTcf would result in a synergistic dominant negative activity on Wingless signalling.

In these experiments we have driven expression of the different proteins in the developing wing with the *ms1096*GAL4 line. When UASGro^{Nterm} is expressed using this Gal4 driver we only observe mild defects in wing pattern (Fig. 7C), but as mentioned above, dTcf overexpressed with *ms1096*Gal4 generates reductions in the size of the wing and significant deletions of the wing margin and associated bristles (Fig. 7B). When both Gro^{Nterm} and dTcf are coexpressed we do not observe an enhancement of the wing

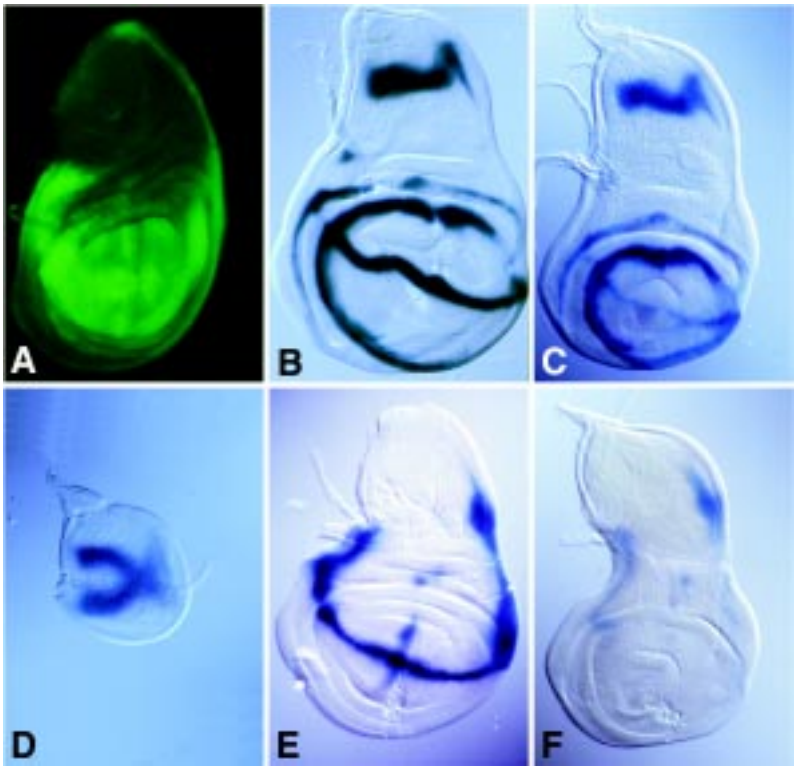

Fig. 4. Overexpression of Gro^{Nterm} in the wing imaginal disc under the control of *sd*G4 leads to loss of wing margin markers. (A) *The expression pattern of sdG4 as visualised by UASGFP in a third larval instar wing imaginal disc.* **(B)** *The expression pattern of wg in a wild-type wing imaginal disc.* **(C)** *The overexpression of Gro^{Nterm} driven by sdG4 leads to a disruption of wg expression along the presumptive wing margin.* **(D)** *In 5% of cases wing to notum transformations are seen. In such cases there is a duplication of the notal Wg stripe.* **(E, F)** *The expression pattern of the vestigial boundary enhancer (vgBE) in a third larval instar wing imaginal disc.* **(E)** *The wild-type expression from this enhancer.* **(F)** *Overexpression of Gro^{Nterm} leads to a loss of expression from the vgBE throughout the wing pouch.*

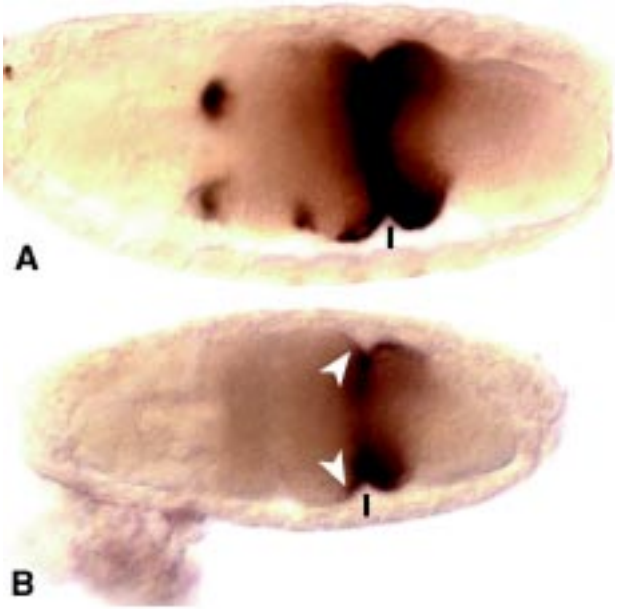

Fig. 5. Overexpression of Gro^{Nterm} in the mesoderm leads to repression of *lacZ* expression from a Ubx enhancer element. (A) *Expression from a 250 base pair region of the Ubx enhancer upstream of a lacZ reporter gene (the Bhz element, Thüringer et al., 1993) in a wild-type background. Dorsolateral view of a stage 16 embryo. Anterior is to the left. Expression of the reporter extends from parasegments (ps) 6 to 9 in the visceral mesoderm with the strongest expression in ps 7 and 8 around the second midgut constriction (marked with a vertical bar). There is also a domain of expression in ps 3 in the gastric caecae. Expression is dependent on Wg and Decapentaplegic (Dpp) signalling (Thüringer et al., 1993).* **(B)** *When Gro^{Nterm} is overexpressed under the control of 24B Gal4, the enhancer is repressed anteriorly. Expression is missing from the gastric caecae and is also absent from ps 6 and is weak in ps 7 (arrowheads mark extent of anterior expression).*

margin defects induced by dTcf alone. In fact we observe a consistent recovery of the wing margin close to the hinge although neither the size of the wing nor the ectopic bristle phenotypes are consistently altered (Fig. 7D and inset).

This result suggests that, in this assay, Gro^{Nterm} may titrate dTcf rather than promote transcriptional repression. In the light of the repressive effects of dTcf, the partial rescue of the dTcf phenotype by Gro^{Nterm} might reflect the fact that additional factors may be required by dTcf to mediate repression. It would also appear that the wing margin is more sensitive to the concentration of Gro^{Nterm} than the blade.

Discussion

Members of the Tcf family of HMG box containing transcription factors are thought to play an important role in Wnt signalling (Eastman and Grosschedl, 1999). Although on their own they are unable to promote transcription, they can interact with βcatenin/Arm and this complex can elicit transcription from reporter constructs containing Tcf consensus binding sites (van de Wetering *et al.*, 1997; Roose *et al.*, 1998). While the interactions with βcatenin/Arm reveal an activity of Tcf in transcriptional activation, interactions with other proteins, particularly with members of the Groucho family of co-repressors, reveal a potential for Tcf to participate in transcriptional repression.

Here we have provided further evidence for an interaction between Gro and dTcf. A Gro protein that lacks the WD40 repeats, Gro^{Nterm}, is very effective in antagonising Wingless signalling *in vivo* and in tissue culture. Because the WD40 repeats are thought to be involved in the interaction between Groucho and bHLH proteins (Paroush *et al.*, 1994), this result suggests that the effects of Gro on Wingless signalling do not require, nor are likely to be mediated by, the interaction of Gro with bHLH proteins. Since full length Gro has little effect in our overexpression assay *in vivo* it could be argued that molecules that interact with the C-terminal WD40 repeats play a role in negatively regulating the interaction between Gro and dTcf. Such interactions could limit the amount of Gro available for association with dTcf. Thus, the action of full length Gro in the cell culture assay could be explained by the absence of such molecules.

The interaction between Gro and Tcf is an important element in the current model of Wnt signalling (Miller *et al.*, 1999; Bejsovec, 1999). In this model, in the absence of Wnts, Tcf is associated with Gro and does not activate transcription. Wnt signalling increases the cytoplasmic pool of βcatenin/Arm and promotes its entry into the nucleus where it displaces Tcf from Gro and forms a complex that can activate transcription. In this model, the repressive activity of Tcf is deemed to be a basal state of Wnt signalling rather than an activity of Tcf.

In our experiments with *Drosophila* we find that overexpression of dTcf during the development of the wing disc antagonises Wingless signalling. The possibility that dTcf indeed represses Wingless signalling is most compelling when considering the effects that overexpression of dTcf has on the wing margin. The development,

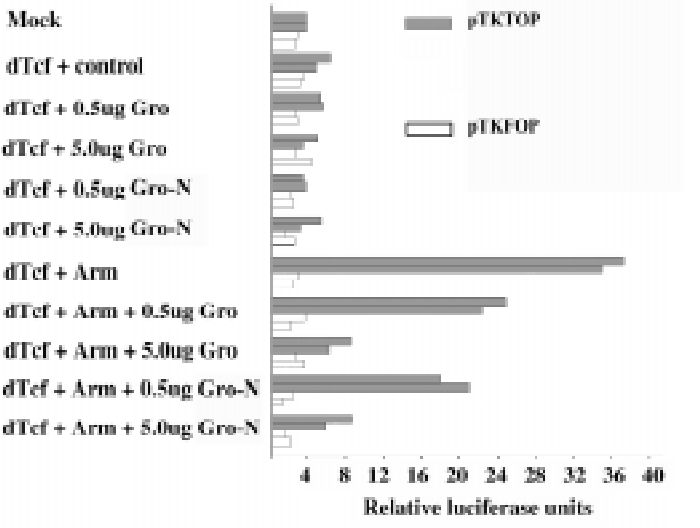

Fig. 6. Gro^{Nterm} antagonises Arm-Tcf driven transactivation of a luciferase reporter. *The reporter construct contains three optimal Tcf binding sites upstream of a minimal HSV-TK promoter (TKTOP) (Roose et al., 1998). TKFOP contains mutated Tcf sites as a control. 11A1.6 B cells were transiently transfected with optimal amounts of the expression vectors indicated (as described in Roose et al., 1998). Co-transfection of Arm and dTcf in these cells results in a transcriptional response from the Tcf reporter TKTOP. This activity was antagonised to the same degree by addition of either Gro^{Nterm} or full length Gro in a dose dependant manner.*

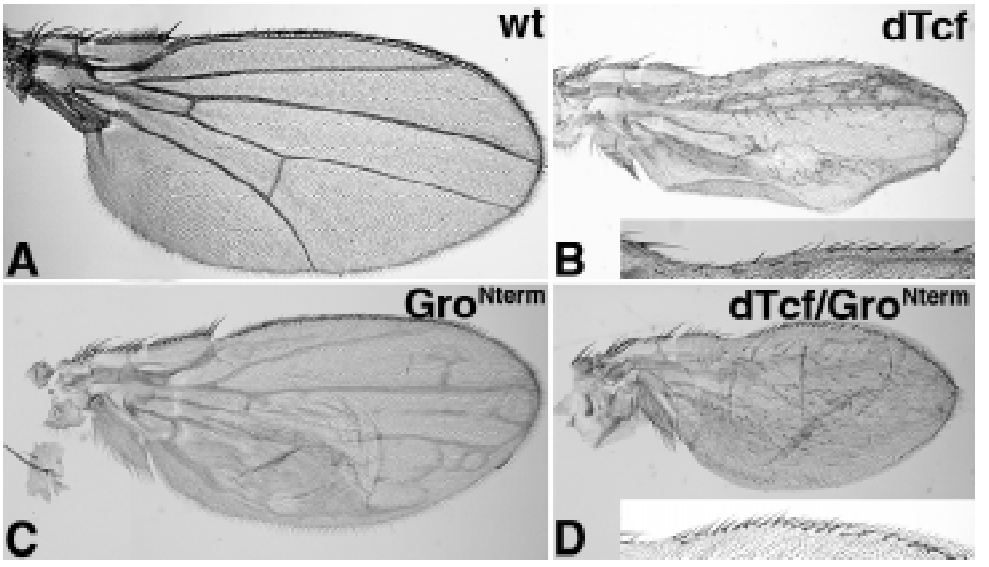

Fig. 7. Coexpression of Gro^{Nterm} with dTcf can rescue some aspects of the phenotype caused by overexpression of dTcf alone. (A) *A wild-type wing.* **(B)** *As shown in Fig. 2, overexpression of dTcf in the wing with ms1096G4 leads to a small wing with disrupted pattern elements. The inset highlights the presence of a nick in the anterior margin.* **(C)** *Overexpression of Gro^{Nterm} with this driver leads to disruptions in the venation pattern of the wing as well as a slight reduction in the size of the wing.* **(D)** *Coexpression of Gro^{Nterm} with dTcf leads to a rescue of the nick in the wing margin (inset) while other aspects of the pattern are not rescued. All wings are at the same magnification.*

and more clearly the patterning, of this structure are dependent on Wingless signalling (Couso *et al.*, 1994) and we find that overexpression of dTcf results in a loss of bristles at the margin similar to that which we observe when *wg* function is lowered. The dominant negative effect of dTcf on Wingless signalling could be mediated by the association of dTcf with an excess of Gro, which might exist in the wing primordium. If this were the case we would expect a synergistic effect of expressing both dTcf and Gro^{Nterm} at the same time. However, we observe that Gro^{Nterm} suppresses rather than enhances the antagonistic effects of dTcf on Wingless signalling at the wing margin. While these results support the evidence of an interaction between Gro and dTcf, they suggest that the antagonistic effects of dTcf that we have observed are not simply mediated by its interactions with Gro. In *Xenopus*, Tcf has been shown to repress transcription in a Gro independent manner through an interaction with CtBP (Brannon *et al.*, 1999). There is a *Drosophila* homologue of CtBP (Zhang and Levine, 1999) which could mediate the repressive effects that we observed but it is also possible that dTcf does this through an interaction with other molecules.

It is interesting to note that the phenotypes produced by overexpression of dTcf, such as ectopic veins and nicking of the wing margin, resemble phenotypes observed when Notch signalling is disrupted. It has been suggested that there is a close relationship between Notch and Wingless signalling (reviewed in Martinez Arias, 1998) and these phenotypes might in part be a reflection of that relationship.

An important observation from our experiments is that coexpression of dTcf with Arm during wing development does not result in increased nor ectopic Wingless signalling as would be expected from the synergy observed between these two molecules in tissue culture. Overexpression of Arm suppresses some of the effects of ectopic expression of dTcf supporting the well documented interaction between Arm and dTcf but this does not support the simple model that dTcf promotes Wingless signalling in the presence of high levels of Arm. It may be that the turnover mechanisms that control the cytoplasmic levels of Arm are very effective in reducing the input levels of our experiment and therefore Arm never reaches the critical functional concentration. However, it is also possible that the nuclear translocation of βcatenin depends on post-translational modifications that follow Wingless signalling. Recent studies of Wnt/βcatenin signalling in a cell free system indicate that this might indeed be the case since Tcf mediated transcription of Wnt targets appear not to correlate with the steady state levels of βcatenin (Nelson and Gumbiner, 1999). An additional possibility is that Wnt signalling antagonises the repressive effects of Tcf through a molecular pathway that is different from the one it uses to modulate the activity of Arm/βcatenin. Thus Wnt signalling would be composed of two simultaneous and convergent events: one that targets the concentration and activity of Arm/βcatenin and another one that modulates the activity of Tcf. Wnt signalling is only efficient when both signalling events converge in the nucleus.

Evidence for this more complex view of Wingless signalling can be found in other experimental systems (reviewed in Sharpe and Martinez Arias, 2000). For example, during the early development of *C. elegans*, Wnt signalling acts to block the activity of the *C. elegans* Tcf family member POP-1 (Lin *et al.*, 1998). It may be that a Wnt signalling event results from the balance between activator and repressor activities of Tcf and that this balance is tuned to specific situations. So, some targets might display an absolute requirement for activation whilst neutralising the repressive activity of Tcf can activate others. Hedgehog signalling provides a precedent for this possibility. The mediator of Hedgehog signalling, Cubitus interuptus (Ci), also has repressive and activator activities and different targets display different requirements for the activator and repressor forms (Methot and Basler, 1999).

Materials and Methods

Drosophila *strains used*

Ectopic expression of different constructs was achieved through the Gal4/UAS system of Brand and Perrimon (1993). The Gal4 driver stocks used in this study were *scalloped*Gal4; IF/CyO^{wglacZ}, *scalloped*Gal4; vgBElacZ/CyO^{wglacZ}, *ms1096*Gal4; IF/CyO^{wglacZ}, and *24B*Gal4,UbxB*lacZ*.

The first two stocks allow the expression of the different UAS constructs throughout the wing from the early second larval instar under the control of the *scalloped* promoter (Klein *et al.*, 1997). *ms1096*Gal4 drives expression in a dynamic pattern in the wing pouch during the third larval instar (Capdevila and Guerrero, 1994; Klein *et al.*, 1997). The expression of the endogenous *wg* gene and the *vestigial* boundary enhancer can be monitored by the expression of a β-gal reporter gene. *24B*Gal4 drives expression throughout the mesoderm (Brand and Perrimon, 1993). UbxB is an enhancer fragment from the Ubx regulatory region that drives expression of a β-gal reporter gene in the visceral mesoderm in response to Wg and Dpp signalling (Thuringer *et al.*, 1993). The following UAS constructs were used: UAS*wgE1*, UAS*Arm*, UAS*ArmΔC,* UAS*ArmS10*, UAS*dTcf*, and UAS*Gro*^{Nterm} (see below).

Making the Groucho N terminal construct

The Gro^{Nterm} construct was assembled from a clone of the open reading frame of *groucho* kindly provided by Dr. J. Terol-Alcayde by creating a stop codon after the CcN domain of *groucho* at amino acid 263. To produce this stop codon a full-length clone was digested with *Bam*H1 and *Hin*dIII (see Fig. 2i) and then ligated to an adapter fragment made by annealing two phosphorylated oligonucleotides according to the methods of White and Butler, 1995. Oligonucleotides used were AGCTTGTAACCCT and CTAGAGGGTTACA, which, when annealed, produce an adapter containing a *Hin*dIII site, an in-frame stop codon and an *Eco*R1 site. The *groucho* fragment and the adapter were cloned into *Bam*H1, *Eco*R1 cut BlueScript (Stratagene) and the resulting clones fully sequenced. This construct was then sub-cloned into pUAST and transformed, using established techniques, into w^{1118} *Drosophila*.

X-Gal Stains

The expression of the endogenous *wg* gene and of the *vestigial Boundary Enhancer* was detected by the expression of a β-gal reporter gene inserted in the two loci. The expression of β-gal was determined by X-gal staining. Selected larvae were dissected in cold PBS and fixed for 4 min in 2.5% glutaraldehyde in PBS. After washing in 0.3% Triton X-100 in PBS the presence of the lacZ protein was revealed using X-gal following standard procedures (Ashburner, 1989). The specimens were then mounted in 75% glycerol.

Wing preparations

The flies used for wing preparations were collected and stored in SH solution (25% glycerol and 75% ethanol). Wings were prepared by removing them from the notum with watchmaker's forceps in a dissecting dish containing tap water and were mounted in Hoyer's medium (Ashburner, 1989). All the wings shown are taken from female flies.

Cell transfection assays

2 x 10^6 IIAI.6 B cells were transfected by electroporation with 1.0 μg of a luciferase reporter plasmid containing three optimal dTcf sites upstream of the minimal HSV-TK promoter (pTKTOP) or its negative control containing mutated dTcf sites (pTKFOP). The internal transfection control was 0.5 μg of SV40CAT. These were co-transfected with 2 μg of dTcf expression vector; 0.5 or 5.0 μg Gro expression plasmids and 0.5 μg of Arm expression plasmid. cDNAs encoding tagged versions of dTcf and Gro were inserted into pCDNA3. Total amount of plasmid was balanced with pCDNA3. PTKTOP and pTKFOP are described in (Roose *et al.*, 1998). Luciferase and CAT activity were determined as in (van de Wetering *et al.*, 1997); luciferase activity was corrected by CAT activity.

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