Nemo is an inducible antagonist of Wingless signaling during *Drosophila* wing development

Yi A. Zeng and Esther M. Verheyen*

Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, V5A 1S6, Canada
*Author for correspondence (e-mail: everheye@sfu.ca)

Accepted 10 March 2004

Development 131, 2911-2920
Published by The Company of Biologists 2004
doi:10.1242/dev.01177

Summary

The cellular events that govern patterning during animal development must be precisely regulated. This is achieved by extrinsic factors and through the action of both positive and negative feedback loops. Wnt/Wg signals are crucial across species in many developmental patterning events. We report that *Drosophila nemo* (*nmo*) acts as an intracellular feedback inhibitor of Wingless (Wg) and that it is a novel Wg target gene. Nemo antagonizes the activity of the Wg signal, as evidenced by the finding that reduction of *nmo* rescues the phenotypic defects induced by misexpression of various Wg pathway components. In addition, the activation of Wg-dependent gene expression is suppressed in wing discs ectopically expressing *nmo* and enhanced cell autonomously in *nmo* mutant clones. We find that *nmo* itself is a target of Wg signaling in the imaginal wing disc. *nmo* expression is induced upon high levels of Wg signaling and can be inhibited by interfering with Wg signaling. Finally, we observe alterations in Arm stabilization upon modulation of Nemo. These observations suggest that the patterning mechanism governed by Wg involves a negative feedback circuit in which Wg induces expression of its own antagonist Nemo.

Key words: NLK, Wg, Wing development

Introduction

Members of the Wnt family are involved in numerous developmental events in many organisms, from the nematode *C. elegans* to mammals (Cadigan and Nusse, 1997). Most components of Wnt signal transduction pathways are highly conserved in evolution and can participate in either canonical or non-canonical pathways (http://www.stanford.edu/~rnusse/wntwindow.html). *wingless* (*wg*), which participates in the canonical signaling pathway, is the best-characterized of the seven *Drosophila* Wnt genes. Pathway activation occurs when the secreted Wg protein is received by a complex of the Frizzled2 receptor (Fz2) and LRP/Arrow. This, in turn, leads to activation of Dishevelled, which inhibits the action of a protein complex including glycogen synthase kinase 3β (GSK3β or Drosophila Zw3), Axin and APC (reviewed by Cadigan and Nusse, 1997). In the absence of Wg signaling, this protein complex targets the *Drosophila* β-catenin Armadillo (Arm) for degradation (Aberle et al., 1997; Willert et al., 1999; Yost et al., 1996). Wg signaling results in downregulation of Zw3 kinase activity which allows Arm to escape degradation and accumulate in the cytoplasm. Subsequently, Arm can proceed into the nucleus where it forms a complex with dTCF, a member of the lymphoid enhancer factor 1 (LEF1)/T-cell factor (TCF) family of transcription factors, and participates in transcriptional activation of Wg target genes (Brunner et al., 1997; van de Wetering et al., 1997).

In addition to extrinsic regulatory factors, inducible feedback loops have been found for most conserved signal transduction pathways controlling development (Freeman, 2000). In *Drosophila*, two inducible inhibitors of Wg signaling have been described that target distinct steps in the pathway. *naked cuticle* (*nkd*) encodes a cytoplasmic protein that binds to Dsh and blocks accumulation of Arm in response to Wg signaling during embryonic patterning and eye development (Rousset et al., 2001; Zeng et al., 2000). Conversely, *wingful* (*wf*) encodes a secreted extracellular feedback inhibitor that acts non-autonomously during larval imaginal disc development to inhibit Wg (Gerlitz and Basler, 2002).

Wg function is required throughout *Drosophila* development in a wide range of patterning events (Cadigan and Nusse, 1997). During wing development, Wg signaling plays at least two distinct roles. Early reductions of *wg* result in wing-to-notum transformations, indicating a requirement for Wg in defining the wing blade (Morata and Lawrence, 1977; Ng et al., 1996). Later reductions cause wing margin notching due to tissue loss, indicating the subsequent role of Wg in specifying the margin and organizing wing development (Couso et al., 1994; Diaz-Benjumea and Cohen, 1995; Rulifson and Blair, 1995). In late third larval instar wing imaginal discs, Wg is expressed in a narrow stripe of three to six cells straddling the dorsoventral (DV) boundary of the future wing blade (Baker, 1988; Couso et al., 1994; Williams et al., 1993). Directly adjacent to the stripe, Wg regulates the expression of high-threshold (or short-range) target genes, including *achaete* (*ac*) and *neuralized* (Phillips and Whittle, 1993; Couso and Arias, 1994; Zecca et al., 1996). In addition to these targets of Wg signaling, *Distal-less* (*Dll*) is expressed in a Wg-dependent manner in a wider domain radiating from the thin DV stripe (Zecca et al., 1996).

Drosophila *nemo* (*nmo*) was first identified as a gene required for epithelial planar polarity (EPP) during ommatidial

development, a process known to involve the Frizzled (Fz) receptor and which is proposed to signal through a non-canonical Wnt pathway (Choi and Benzer, 1994; Mlodzik, 2002). Subsequent analysis has shown that Nemo functions in multiple tissues and has diverse roles in development. In addition to its effect on eye polarity, we have found that disruption of *nmo* results in changes in wing shape and size, wing vein specification, fertility and viability (Verheyen et al., 2001). *nmo* is essential for embryonic development as loss of maternal and zygotic *nmo* results in embryonic lethality characterized by patterning defects in the head and ventral denticle belts as well as disruption of apoptosis (Mirkovic et al., 2002).

Nemo is the founding member of an evolutionarily conserved family of proline-directed serine/threonine protein kinases (referred to as Nemo-like kinases, NLKs) that includes the murine and human Nemo-like kinases (Nlk), *C. elegans* LIT-1, *Fugu rubripes* NLK and *Xenopus* xNLK (Choi and Benzer, 1994; Brott et al., 1998; Harada et al., 2002; Hyodo-Miura et al., 2002; Kehrer-Sawatzki et al., 2000; Meneghini et al., 1999; Rocheleau et al., 1999). NLKs can exert an inhibitory effect on the gene regulation activity of TCF/LEF transcription factors (Ishitani et al., 1999; Rocheleau et al., 1999; Shin et al., 1999). Nlk mediates phosphorylation of TCF and inhibits the DNA-binding ability of the TCF/β-catenin complex (Ishitani et al., 1999). In a *C. elegans* non-canonical pathway, activation of the LIT-1 kinase requires WRM-1, a β-catenin-like protein, and leads to phosphorylation of LIT-1 and WRM-1 and subsequent phosphorylation and inhibition of a nematode TCF, POP-1 (Rocheleau et al., 1999).

NLKs have been found to participate in both canonical and non-canonical Wnt pathways. In *C. elegans*, LIT-1 has been found to play roles in cell polarity and cell fate decisions, two processes regulated by distinct Wnt pathways (Ishitani et al., 1999; Meneghini et al., 1999; Rocheleau et al., 1999). Analysis of NLK function in *Xenopus* oocyte axis formation assays has shown that injection of murine *Nlk* and *xNLK* mRNAs can block axis formation and can rescue the axis duplication induced by β-catenin or Wnt (Hyodo-Miura et al., 2002; Ishitani et al., 1999).

Consistent with these findings, genetic and phenotypic analyses in *Drosophila* support the proposed role for Nemo in both canonical and non-canonical Wnt signaling pathways. In addition to its role in the non-canonical Fz pathway regulating epithelial planar polarity (EPP) in the eye, wing and abdomen (Choi and Benzer, 1994; Strutt et al., 1997; Verheyen et al., 2001), we have previously reported preliminary evidence that modulating levels of *nmo* results in phenotypes consistent with a role as a Wg-antagonist (Verheyen et al., 2001).

In this study, we present our thorough study of the role of Nemo in *Drosophila* canonical Wg signaling. Through detailed genetic analysis we observe that *nmo* is an antagonist of Wg during larval wing disc development and that Nemo can negatively influence Wg-dependent gene expression. In addition we present evidence that transcription of *nmo* is induced by high levels of Wg signaling in the developing wing disc. Finally, we show that cellular levels of Armadillo protein can be controlled by Nemo, such that ectopic Nemo leads to reductions in stabilized Arm. Our results indicate that Nemo is an intracellular inducible feedback antagonist of the Wingless signaling pathway that is involved with refining the Wg activity gradient during wing development.

Materials and methods

Fly strains

The following fly strains were used: nmo^{DB24} (D. Bessette and E.M.V., unpublished); nmo^{adk1} and *UAS-*$nmo^{C5–1e}$ (Verheyen et al., 2001); nmo^{P} (also referred to as *nmo-lacZ*) (Choi and Benzer, 1994); *sd-Gal4* ($sd^{SG29.1}$); *UAS-*$Daxin^{A2-4}$ (Willert et al., 1999); *UAS-fluΔarm* and *AyGal4.25-UAS-GFP.S65T* (Ito et al., 1997; Zecca et al., 1996); *vg-Gal4*, *Dll-lacZ*, *Ubi-GFP FRT* 79D, *ap-Gal4*, *dpp-lacZ* (Morimura et al., 1996); *71B-Gal4*, *UAS-lacZ*, $zw3^{m11}$ and *UAS-DFz2N* (Zhang and Carthew, 1998) and dsh^{v26}.

Clonal analysis

nmo somatic clones were induced using the FLP/FRT method (Xu and Rubin, 1993). To induce *nmo* loss-of-function clones, embryos from the appropriate crosses were collected for 24 hours and heat shocked at 38°C for 90 minutes at 48 hours of development. The genotypes examined were: for Wg and Arm staining in *nmo* clones, *y hs-Flp122*; *nmo FRT 79D/Ubi-GFP FRT79D*; for β-galactosidase staining of *Dll-lacZ* in nmo^{DB24} clones, *y hs-Flp122; Dll-lacZ/+;* nmo^{DB24} *FRT 79D/Ubi-GFP FRT79D*; and for β-gal staining in *dsh* clones, dsh^{v26}*/GFP, FRT 18A; hsFLP38/+; nmo-lacZ/+*.

To induce 'flip-out' clones ectopically expressing active Arm, *AyGal4.25-UAS-GFP.S65T; nmo-lacZ/TM6B flies* were crossed to *UAS-fluΔarm; hs-Flp* flies (Ito et al., 1997). To induce clones of ectopic Nemo expression, *AyGal4.25-UAS-GFP.S65T, UAS-*$nmo^{C5–1e}$ flies were crossed to *hs-Flp* flies. To induce clones of ectopic Daxin, *AyGal4.25-UAS-GFP.S65T; nmo-lacZ/TM6B* flies were crossed to *hs-Flp; UAS-Daxin* flies.

Immunostaining and in situ hybridization

Dissection of imaginal discs, X-Gal staining and antibody staining were carried out using the following standard protocols. The antibodies used were: mouse anti-Wg (1:100) and anti-Armadillo (1:200) concentrated supernatants from the Developmental Studies Hybridoma Bank; mouse anti-β-galactosidase (1:500) from Promega; rabbit anti β-galactosidase (1:2000) from Cappel. Secondary antibodies used were: donkey anti-mouse FITC (Jackson Immunolabs), donkey anti-mouse AlexaFluor 594 (Molecular Probes), donkey anti-rabbit CY3 and FITC (Jackson Immunolabs). All secondary antibodies were used at 1:200 dilutions. In situ hybridization was performed according to Tautz and Pfeiffle (Tautz and Pfeiffle, 1989).

Results

nmo expression in wing imaginal discs flanks the Wg expression domain

The *nmo* gene plays a role in the development of the wing. Homozygous *nmo* mutant flies display abnormal wing patterning characterized by alterations in wing size and shape and the presence of extra vein material along the longitudinal veins and emanating from the posterior crossvein (see Fig. 2D) (Verheyen et al., 2001). During pupal wing development, *nmo* is expressed in intervein regions of the wing blade, where it presumably acts to suppress vein development (Verheyen et al., 2001).

To better understand the role of *nmo* in earlier patterning events, we determined its localization pattern in larval wing imaginal discs in the nmo^{P} enhancer trap line, *nmo-lacZ* (Fig. 1A-C) (Choi and Benzer, 1994). The expression of *nmo* is quite dynamic during larval development. Staining of second instar larval discs reveals very weak expression at the anterior and posterior periphery of the wing disc (Fig. 1A). Early in the third larval stage, staining at the DV boundary becomes

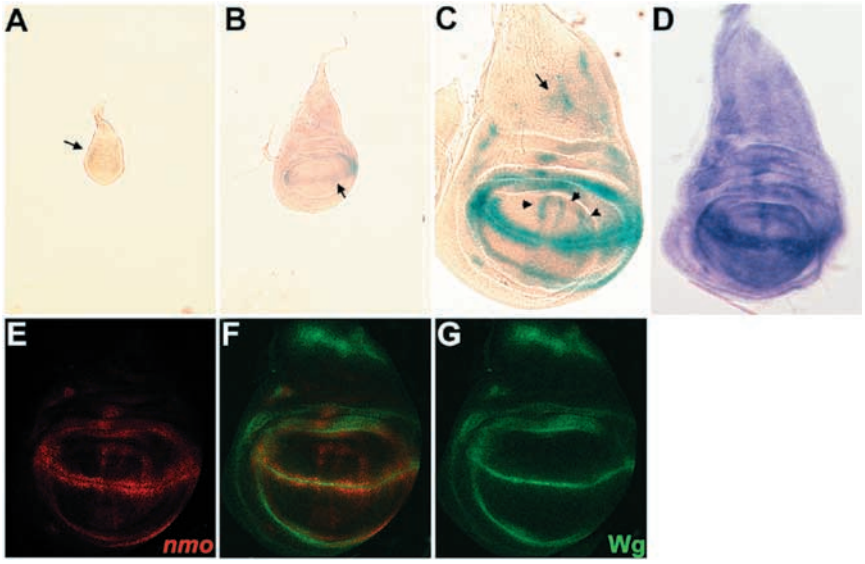

Fig. 1. *nmo* expression in the wing imaginal disc was examined in *nmo-lacZ*. (A) In second instar discs, weak *nmo* expression is seen at the periphery of the future wing pouch (arrow). (B) In early third instar discs, low level expression is at the DV boundary (arrow) and encircling the wing pouch. (C) In late third instar, high levels of *nmo* expression are seen in two stripes flanking the DV boundary and in a ring around the pouch. *nmo* is also seen in the L3, L4 and L5 vein primordia (arrowheads) and in several spots in the presumptive notum (arrow). (D) In situ hybridization using an antisense *nmo* RNA probe. (E-G) Co-localization with Wg. Discs were double stained with (E) anti-β-gal and (G) anti-Wg antibodies and the images were merged to show overlap (F). Wing imaginal discs are orientated anterior towards the left, dorsal side upwards.

evident (Fig. 1B) and the intensity of the staining increases with age. In late third instar discs, *nmo* is expressed in two thin stripes flanking the DV boundary (Fig. 1C). These two stripes of staining are weaker at the point where the anteroposterior (AP) boundary intersects the DV boundary. *nmo* expression is also seen in a ring encircling the future wing pouch in a tissue corresponding to the future proximal wing hinge, with the expression in the dorsal ring appearing darker than the ventral ring. Staining is also seen in the primordia of longitudinal wing veins 3, 4 and 5, beginning in the late third instar stage (arrowheads in Fig. 1C). Finally, *nmo* expression is also detected in spots on the wing imaginal discs that represent sites of sensory organ formation on the future notum (arrow in Fig. 1C). Consistent with such an expression pattern, we have previously shown a role for *nmo* in macrochaete bristles, as demonstrated by genetic interactions with *Hairless* (Verheyen et al., 2001).

We confirmed that this enhancer trap insertion accurately represents the expression of *nmo* by performing whole-mount RNA in situ hybridization (Fig. 1D). In addition to the localized staining seen in the enhancer trap, low level ubiquitous staining is detected throughout the disc. This ubiquitous staining is also apparent when anti-β-galactosidase antibody is used to detect the *nmo-lacZ* expression pattern (see Fig. 1E).

The *nmo-lacZ* pattern is reminiscent of the Wg expression pattern in imaginal discs (Rulifson et al., 1996). To examine the relationship between the two expression patterns, we performed double staining for β-galactosidase and Wg protein. This staining reveals that *nmo* expression at the DV boundary flanks the Wg protein domain in late third instar wing discs (Fig. 1E-G). Wg protein is detected in a narrow stripe along the presumptive wing margin (Fig. 1G) and *nmo* is seen in the cells directly adjacent to the Wg-expressing cells (Fig. 1E). In addition, *nmo* is detected in the ring domain overlapping with the Wg inner ring expression domain that encircles the wing pouch (Fig. 1F). Such a localization for *nmo* is also consistent with the observed defect in adult flies in which the wing is held away from the body at an angle and may reflect a hinge defect (Verheyen et al., 2001).

nmo antagonizes Wg signaling during wing development

Based on the expression pattern of *nmo* and data suggesting a role in Wnt signal transduction, we investigated the role of Nemo in Wg signaling using a combination of approaches, involving ectopic expression, mutant analysis, somatic loss-of-function clones and ectopic flip-out misexpression clones (Brand and Perrimon, 1993; Ito et al., 1997; Xu and Rubin, 1993). Wg is expressed along the presumptive wing margin where it is required for proneural *achaete-scute* (*AS-C*) complex gene expression and for the formation of margin bristles. Loss of Wg signaling along the wing margin leads to loss of these margin bristles and the appearance of notches along the wing margin (Couso et al., 1994; Phillips and Whittle, 1993; Rulifson et al., 1996). Ectopic expression of *UAS-nmo* in the wing using either *scalloped-Gal4* (referred to as *sd>nmo*) or *omb-Gal4* also produces such a wing notching effect (Fig. 2B, and data not shown), suggesting Nemo plays an antagonistic role in the pathway. A similar wing notching phenotype is seen when either *71B-Gal4* or *69B-Gal4* is used to drive expression of the Wg inhibitor Daxin (Hamada et al., 1999; Willert et al., 1999). The observed wing margin loss seen in *sd>nmo* flies is completely suppressed when flies are heterozygous for the $zw3^{m11}$ loss-of-function allele (Fig. 2C), consistent with the antagonistic role that Zw3 plays in Wg signaling and with the speculation that the effect of *nmo* is due to blocking the action of Wg.

To extend this study, we examined whether loss of *nmo* or ectopically expressed Nemo is able to suppress defects caused by overexpression of Wg pathway components. Ectopic expression of Dfz2N, a dominant-negative form of the Drosophila Frizzled 2 receptor (Zhang and Carthew, 1998) using the *sd-Gal4* driver induces a tiny wing phenotype characterized by loss of the wing margin and significant amounts of wing blade (Fig. 2E). Flies homozygous for nmo^{DB24}, a putative null allele of *nmo*, have a broader, shorter wing than wild type and ectopic vein material near longitudinal vein 2 and 5 and emanating from the posterior cross vein (Fig. 2D) (D. Bessette and E.M.V., unpublished). The *sd>Dfz2N* phenotype is significantly suppressed when flies are

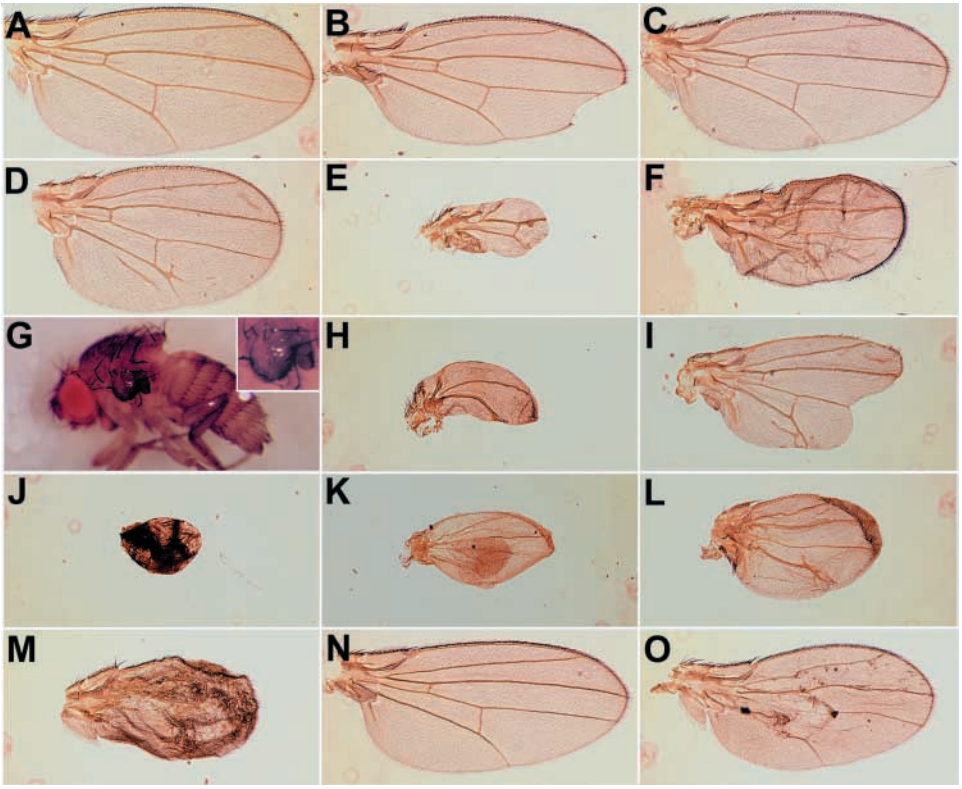

Fig. 2. *nmo* antagonizes Wg signaling during wing development. (A) A wild-type adult wing. (B) *sd-Gal4/UAS-nmo*. (C) $zw3^{m11-1}$/+; *sd-Gal4/UAS-nmo*. (D) The null allele nmo^{DB24}. (E) *sd-Gal4/UAS-DFz2N*. (F) Loss of *nmo* in *sd-Gal4/UAS-Fz2N;* nmo^{DB24}/nmo^{DB24} flies rescues the severe wing defect seen in E. (G) *sd-Gal4/+; UAS-Daxin* causes a wing-to-notum transformation (see inset). (H,I) Reductions in *nmo* rescue in a dose-dependent manner in (H) *sd-Gal4/+; UAS-Daxin,* nmo^{DB24}/+ and (I) *sd-Gal4/+; UAS-Daxin,* nmo^{DB24}/nmo^{DB24}. (J) *ap-Gal4/+; UAS-Daxin*. Reductions in *nmo* rescue this phenotype in a dose-dependent manner. (K) *ap-Gal4/+; UAS-Daxin,* nmo^{DB24}/+ and (L) *ap-Gal4/+; UAS-Daxin,* nmo^{DB24}/nmo^{DB24}. (M) *71B>fluΔarm*. (N) *71B>nmo*. (O) *UAS-fluΔarm/ UAS-nmo; 71B-Gal4/+*.

homozygous for nmo^{DB24}, resulting in restoration of most wing margin structures as well as wing blade tissue (Fig. 2F). Furthermore, we found that nmo^{DB24} also suppresses the effects of Daxin in a dose-sensitive manner (Fig. 2G-L). *sd>Daxin* causes wing-to-notum transformations (Fig. 2G) that can be rescued to a small wing by heterozygosity for nmo^{DB24} (Fig. 2H). Stronger suppression is detected in homozygous nmo^{DB24} flies, in which the ectopically produced nota are completely suppressed and the wing blade is partially restored, particularly in the anterior wing margin (Fig. 2I). The same dose-sensitive suppression is observed when the dorsally expressed *ap-Gal4* driver was used to drive Daxin. *ap>Daxin* induces a tiny blistered wing pouch (Fig. 2J). Heterozygosity for nmo^{DB24} in this background partially rescues the pouch defect (Fig. 2K), while nmo^{DB24} homozygosity strongly rescues the wing blisters and abnormal appearance (Fig. 2L). These data suggest that the block in Wg signaling caused by ectopic Daxin can be suppressed by the absence of *nmo* function.

Additional evidence supporting the involvement of Nemo as a negative player in the Wg pathway comes from examining interactions with Arm. *UAS-fluΔarm* encodes an N-terminally truncated, constitutively active form of Arm (Tolwinski and Wieschaus, 2001; Zecca et al., 1996). Using *71B-Gal4* to drive *UAS-fluΔarm* causes a very abnormal wing (Fig. 2M) characterized by excess margin bristles throughout the wing blade, loss of veins and a smaller crumpled wing blade, similar to the abnormal wing seen with ectopic expression of LEF-1 (Riese et al., 1997). While *71B>nmo* induces no visible wing defects (Fig. 2N), ectopic expression of *nmo* is able to suppress the *71B>fluΔarm* wing phenotype by restoring the size of the wing blade, reducing ectopic bristles and wing blistering (Fig. 2O).

In addition to interactions in wing patterning, *nmo* antagonizes Wg signaling in the sensory bristles of the notum. *ap>nmo* flies display a loss of notum bristles (Fig. 3B). This phenotype is opposite to that seen upon ectopic activation of Wg signaling (Phillips et al., 1999; Riese et al., 1997; Simpson and Carteret, 1989). The *ap>nmo* bristle loss phenotype is suppressed by heterozygosity for $zw3^{m11}$ (Fig. 3C) and enhanced by co-expression of *Daxin*, resulting in loss of all scutellar bristles (Fig. 3D). *ap>nmo* flies also display an abnormal wing phenotype in which the wing blades do not appose properly, forming a large blister (Fig. 3E). Heterozygosity for $zw3^{m11}$ suppresses this effect, resulting in a significant rescue of wing morphology (Fig. 3F).

All of these genetic data provide convincing evidence that Nemo can interfere with canonical Wg signaling. Both the loss and gain of *nmo* produces phenotypes consistent with the idea that Nemo acts to downregulate Wg signaling during wing development.

nmo autonomously suppresses Wg-dependent gene expression

In the wing disc, Wg signaling positively regulates *Distal-less* (*Dll*) expression (Zecca et al., 1996). Dll is expressed in a domain overlying but wider than the Wg DV expression domain and can be induced by ectopic Wg signaling (Fig. 4A) (Zecca et al., 1996). Thus the normal pattern of Dll is governed by Wg signaling and Dll expression can be used to monitor the activity of the Wg pathway. As our genetic analysis strongly indicates that Nemo antagonizes Wg signaling, we examined whether modulation of Nemo could affect Dll expression. We found that ectopic expression of *nmo* was able to suppress the expression of *Dll-lacZ* in an *ap>nmo* background (Fig. 4B).

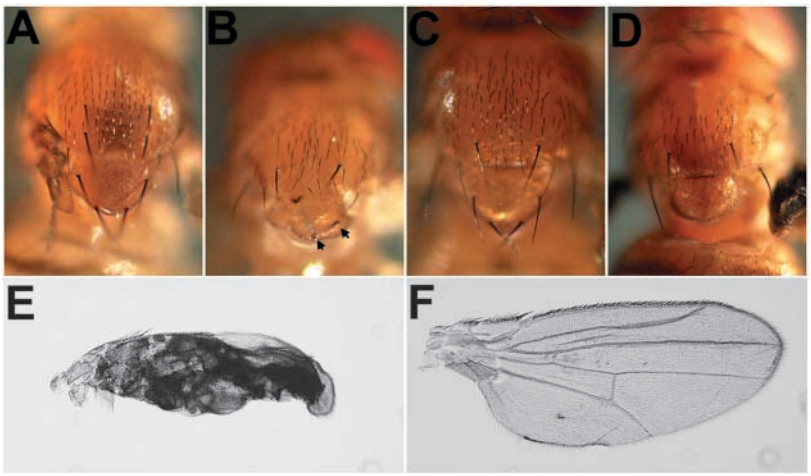

Fig. 3. Nemo plays a role in specification of macrochaete bristles on the adult notum. (A) A wild-type notum. (B) *ap>nmo* flies show loss of macrochaetes on the notum. A few scuttelar bristles remain (arrows). (C) *zw3*$^{m11-1}$*/+; ap>nmo.* (D) Ectopic expression of Daxin enhances the phenotype in *ap-Gal4, UAS-nmo/+; UAS-Daxin/+* flies. (E) *ap>nmo* wing. (F) *zw3*$^{m11-1}$*/+; ap>nmo* wing.

To understand whether Nemo is necessary for the modulation of Wg signaling, *nmo*^{DB24} somatic clones were generated. In *nmo*^{DB24} clones (Fig. 4C,D), which are located inside of, or overlapping with, the *Dll* endogenous domain, enhanced expression of *Dll* is detected (Fig. 4D,E). This effect is cell autonomous as the expression in wild-type cells neighboring the clones is not changed. Clones located outside of the *Dll* endogenous domain do not show any ectopic induction of *Dll* expression (data not shown). This result is not surprising as *nmo* most probably acts to block the activity of dTCF and Arm. Thus, we do not expect an effect outside of their zone of activity which is competent to induce Dll expression.

wg gene expression is not regulated by *nmo*

As Nemo inhibits Wg-dependent gene expression, we were interested in whether Nemo played any negative role in regulating *wg* expression itself. In embryos, *wg* gene expression is positively regulated in an autocrine fashion in response to Wg signaling (Hooper, 1994), whereas in wing discs Wg acts to repress *wg* expression in neighboring cells (Rulifson and Blair, 1995). Wg expression in *nmo* mutant somatic clones was examined and no change of Wg protein staining was detected (Fig. 5A-C). We also generated somatic flip-out clones ectopically expressing *nmo* in wing discs. Similar to what was found in mutant clones, no alterations in Wg expression were observed in flip-out clones in wing discs ectopically expressing Nemo (Fig. 5D-F).

As Wg expression is also positively regulated by Notch at the wing margin (Neumann and Cohen, 1996), and we previously described genetic interactions between *nmo* and Notch (Verheyen et al., 2001; Verheyen et al., 1996), we also investigated whether *nmo* could be influencing Wg signaling indirectly through an interaction with the Notch pathway. Notch patterns the wing margin through transcriptional regulation of *wg* and *cut* (Neumann and Cohen, 1996). We examined the expression of the Notch target gene *cut* in *ap>nmo* wing discs. We did not observe any changes in *cut*

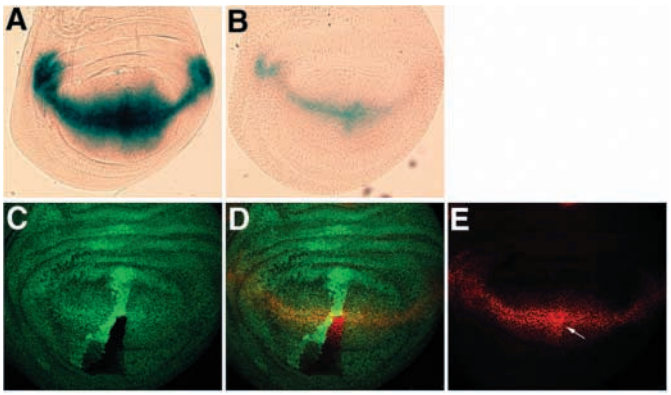

Fig. 4. Both reduction of *nmo* and ectopic *nmo* can affect Wg-dependent gene expression. (A) *Dll-lacZ* expression is seen in a broad domain centered on the DV boundary with areas of increased expression at the anterior and posterior edges of the DV boundary. (B) Ectopic Nemo in *ap>nmo* can greatly reduce the *Dll-lacZ* expression, particularly in the posterior margin region. *Dll* expression is enhanced in *nmo* mutant clones. (C,D) *nmo*^{DB24} clones (marked by the absence of GFP, green). (D,E) Expression of *Dll-lacZ* (anti β-gal, red) is also increased in *nmo*^{DB24} clones (arrow in E).

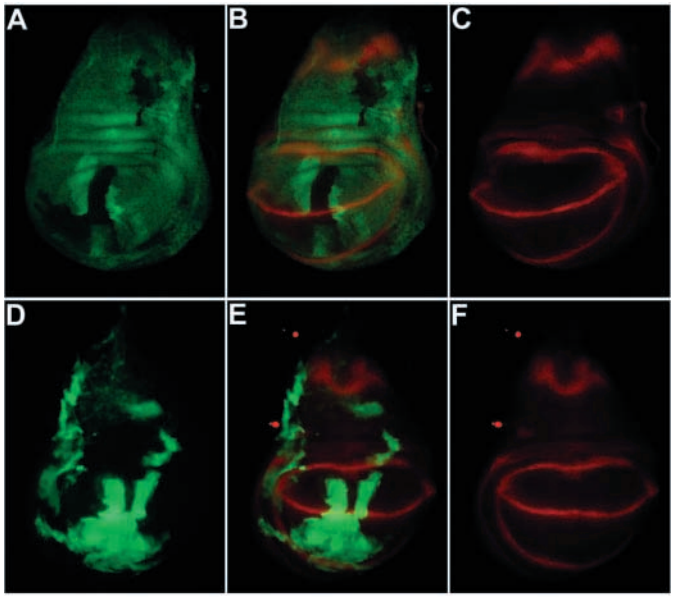

Fig. 5. Neither reduction of *nmo* nor ectopic *nmo* can affect Wg expression. Both somatic *nmo*^{DB24} clones (A,B; marked by the absence of GFP, green) and flip-out clones ectopically expressing *UAS-nmo* (D,E; marked by the areas of brighter GFP staining, green) were induced. The discs were stained for Wg protein to determine whether modulation of *nmo* could affect the Wg expression pattern (anti-Wg antibody, red in B,C,E,F). Anti-Wg stain reveals a wild-type pattern in both reduced and ectopic Nemo.

expression (data not shown), suggesting that Nemo does not affect Notch signaling, and therefore its effect on Wg is most probably not mediated indirectly through Notch. From these experiments, we conclude that the antagonistic role of Nemo in Wg signaling does not include a role in the regulation of *wg* gene expression.

nmo is a novel Wg target gene

Considering that the expression pattern of *nmo* flanks that of Wg in wing imaginal discs, we speculated that the expression of *nmo* may be regulated by Wg signaling. We first examined the effect of ectopic Wg pathway activation on *nmo-lacZ* staining. Expression of activated *UAS-fluΔarm* using *vg-Gal4* causes high levels of Wg pathway activation and leads to ectopic *nmo-lacZ* expression along the *vg-Gal4* expression domain (Fig. 6A,B) (Zecca et al., 1996). The two DV boundary stripes become less defined and appear to expand (Fig. 6B, compare to Fig. 1C). Similarly, *dpp>fluΔarm* induces *nmo-lacZ* expression along the AP boundary (Fig. 6C). These results indicate that activation of the Wg pathway can lead to *nmo* gene expression.

Next, we generated somatic flip-out clones that ectopically express *UAS-fluΔarm*. In these clones, ectopic *nmo-lacZ* expression is autonomously induced (Fig. 6D-F). The induction is observed outside of the regions of high endogenous *nmo* expression and suggests that stabilized Arm is sufficient to autonomously induce *nmo* expression.

To determine whether loss of Wg signaling activity could also affect *nmo* expression, we generated *UAS-Axin* flip-out clones and examined the effects on *nmo-lacZ* staining. In such clones, marked by GFP staining (Fig. 6G,H), *nmo* expression is suppressed (Fig. 6H,I) in both regions of high (arrow in Fig. 6I) and low (arrowhead in Fig. 6I) expression. We then examined somatic clones homozygous mutant for *dishevelled* (Fig. 6J,K) and we find a cell-autonomous inhibition of *nmo* expression (Fig. 6K,L). In all cases, we observe inhibition of not only the high levels of DV boundary *nmo* but also the low level ubiquitous staining within the wing pouch. We also examined the effect of ectopic expression of *UAS-Fz2N* and found that *vg>Fz2N* wing discs display a loss of *nmo* staining at the DV boundary which is similar to the inhibitory effect of *UAS-Fz2N* on other Wg downstream genes such as the DV boundary marker *vg-lacZ* (data not shown) (Zhang and Carthew, 1998). All of these results taken together confirm that activation of endogenous Wg signaling results in *nmo* expression, and that *nmo* is a bona fide Wg target gene.

Nemo can affect Arm stabilization

The localization of *nmo* in third instar wing discs is very reminiscent of the pattern of stabilized Arm protein observed after Wg pathway activation (Peifer et al., 1991; Mohit et al., 2003). To examine this more closely, we carried out double staining to detect *nmo* gene expression and Arm protein stabilization. First, we observed that *nmo* and stabilized Arm co-localize in the central region of the wing margin (Fig. 7A-C). In addition, we noted that in the anterior region of the wing margin where *nmo* expression is elevated, Arm protein levels are lower, relative to the rest of the margin. Third, we find that Nemo staining is reduced in the region where the DV and AP boundaries intersect and that this region shows more stabilized Arm protein.

To determine whether these observations reflected a possible mechanism for the inhibitory effect of Nemo on in the Wg signaling, we determined whether ectopic Nemo could destabilize Arm protein, thus indicating an inhibition of Wg signal transduction. In flip-out clones ectopically expressing Nemo (Fig. 7D,E), the stabilization of Arm protein appears reduced in a cell autonomous manner (Fig. 7E,F). This most probably reflects more degradation of Arm. In an attempt to address this further, we examined the stability of Arm in

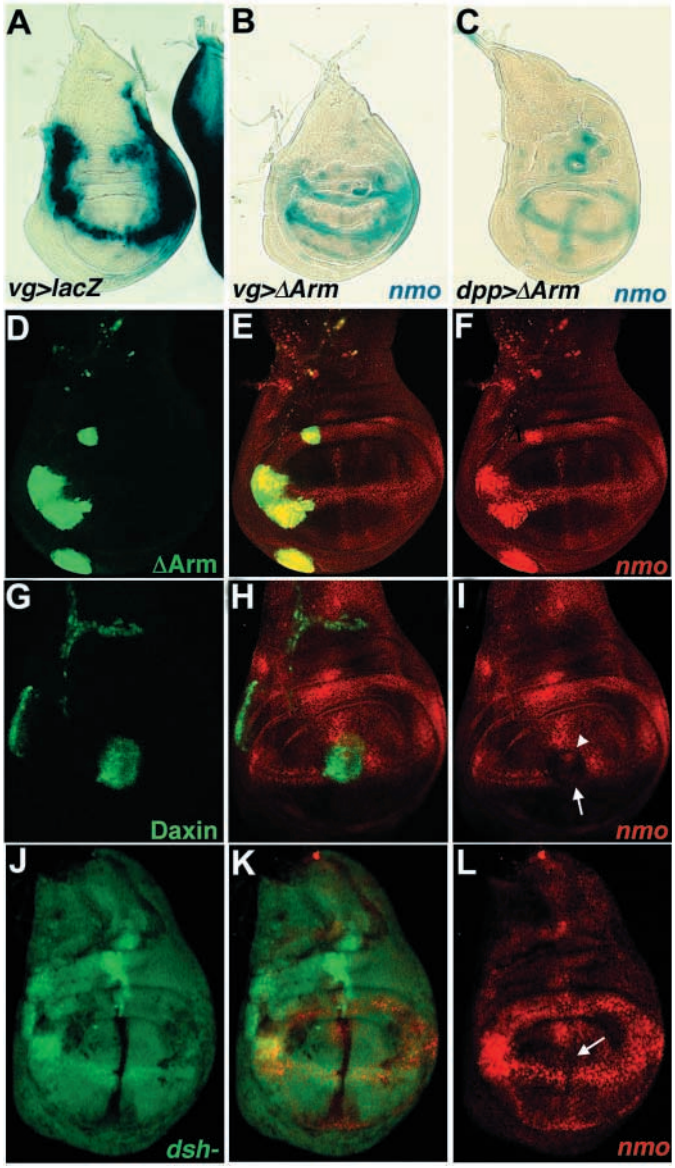

Fig. 6. Wg signaling positively regulates the expression of *nmo*. (A) *vg-Gal4* is expressed along the DV boundary in *vg>lacZ*. (B) *nmo* expression in *vg-Gal4/ UAS-fluΔarm; nmo-lacZ/+* mid third instar larval discs is greatly expanded, especially in the posterior periphery. (C) *dpp>fluΔarm* causes ectopic *nmo* expression along the AP boundary. (D-F) *nmo-lacZ* expression (E,F; anti β-gal, red) in *fluΔarm* flip-out clones (D,E; marked by the areas of brighter GFP staining). (G-I) Flip-out clones ectopically expressing *UAS-Daxin* (G,H) also result in decreased *nmo* expression (H,I). (J-L) In dsh^{v26} somatic clones (J,K; marked by the absence of GFP, green) nmo-*lacZ* expression is reduced cell autonomously (K,L; anti-β-gal, red).

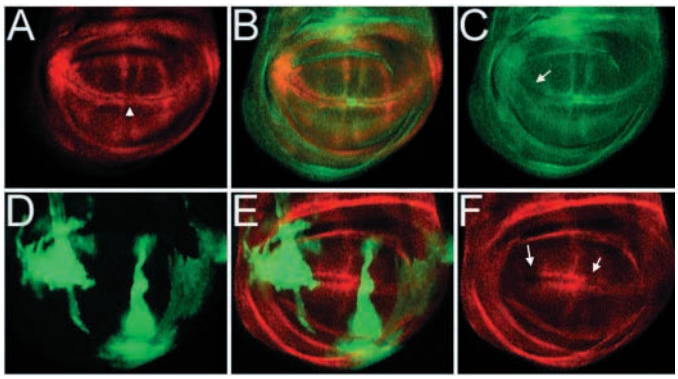

Fig. 7. Nemo can influence Arm stabilization. (A,B) *nmo* gene expression (as monitored by *nmo-lacZ*, anti β-gal, red) overlaps with stabilized Arm protein (B,C; anti-Arm, green) in third instar discs. There are distinct regions in which higher *nmo* expression in A excludes high levels of Arm (arrow in C) and in which high levels of Arm seen in C coincide with reduced *nmo* (arrowhead in A). In flip-out clones ectopically expressing Nemo (D,E; marked by the areas of brighter GFP staining), the stabilization of Arm protein appears reduced in a cell-autonomous manner (E,F; anti-Arm, red; arrows in F).

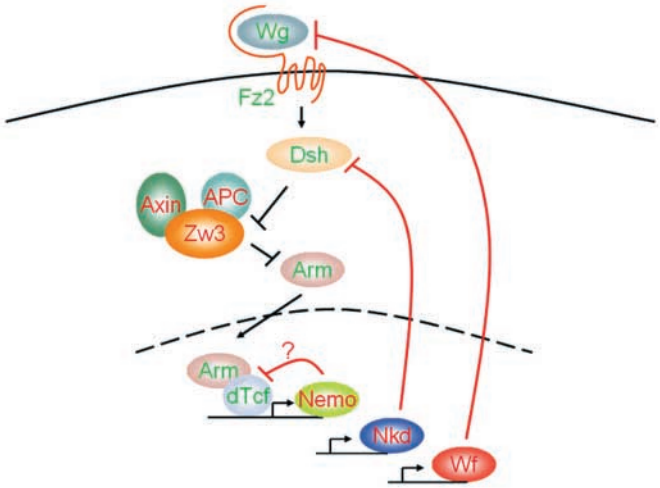

Fig. 8. The role of negative feedback inhibitors in Wg signaling. *Drosophila* Wg signaling is controlled by a number of induced inhibitors including Nkd and Wf. We show that Wg also regulates Nemo expression and that Nemo in turn can antagonize Wg during wing patterning.

nmo^{adk1} somatic clones. In this genetic background, we were unable to observe alterations in Arm stability (data not shown).

Discussion

Feedback inhibition of Wg signaling

Widespread use of feedback loops makes them important mechanisms for regulating signaling pathways during development (Anderson and Ingham, 2003; Freeman, 2000; Perrimon and McMahon, 1999). For example, a number of positive and negative feedback loops regulate the *Drosophila* EGFR, TGFβ, JNK and JAK/STAT signaling pathways to both refine and potentiate signaling. Negative feedback occurs when a signaling pathway induces expression of its own inhibitor and thereby leads to pathway downregulation. In addition, both autonomous and non-autonomous mechanisms exist to negatively regulate signaling pathways.

The canonical Wnt pathway also makes use of negative feedback mechanisms. In murine Wnt signaling, the feedback loops primarily target the activity of β-catenin. For example, Tcf1 is a target gene for β-catenin/Tcf4 in epithelial cells and is proposed to act as a repressor that counteracts β-catenin/Tcf4-mediated gene expression (Roose et al., 1999). Spiegelman et al. (Spiegelman et al., 2000) have provided evidence that the β-TrCP protein, the expression of which is induced by β-catenin/TCF signaling, targets β-catenin for ubiquitination and subsequent degradation (Spiegelman et al., 2000). It has also been shown that expression of Axin2, one of the scaffold proteins in the inhibitory APC/GSK3β complex, is also induced by Wnt signaling (Jho et al., 2002).

In *Drosophila*, several examples of Wg feedback inhibition have been identified. First, it has been shown that Wg downregulates its own transcription in the wing pouch to narrow the RNA expression domain at the DV boundary (Rulifson et al., 1996). Second, Wg signaling can repress the expression of its receptor Dfz2 in the *wg*-expressing cells of the wing disc. Wg regulation of Dfz2 creates a negative feedback loop in which newly secreted Wg is stabilized only once it moves away from the DV boundary to cells expressing higher levels of *Drosophila* Fz2 (Cadigan et al., 1998). Third, the Wg target gene *naked cuticle* (*nkd*) acts through Dsh to limit Wg activity (Rousset et al., 2001; Zeng et al., 2000). Fourth, Wingful (Wf), an extracellular inhibitor of Wg, is itself induced by Wg signaling (Gerlitz and Basler, 2002).

Nemo is an inducible inhibitor of Wg

Our research adds Nemo to this list of inducible antagonists participating in Wg signaling (Fig. 8). We show that Nemo antagonizes the Wg signal in wing development, as evidenced by phenotypic rescue, suppression of Wg-dependent gene expression in discs ectopically expressing *nmo*, and ectopic expression of a Wg-dependent gene in *nmo* mutant clones.

As both *wf* and *nmo* expression are positively regulated by Wg signaling in the wing, their expression patterns are relatively similar to that of Wg (Fig. 1B) (Gerlitz and Basler, 2002; Zeng et al., 2000). Even though *nkd* also has a similar pattern to Wg in the larval wing disc, unexpectedly, it has no detectable role in wing development. As an intracellular antagonist, Nkd regulates embryonic Wg activity in a cell-autonomous manner by acting directly with Dsh to block accumulation of Arm in response to Wg signaling (Rousset et al., 2001; Zeng et al., 2000). Wf apparently has no role during embryogenesis, although both Wf and Nkd can inhibit Wg signaling throughout development when overexpressed (Gerlitz and Basler, 2002; Zeng et al., 2000). Wf is an extracellular protein that functions non-autonomously to regulate Wg signaling (Gerlitz and Basler, 2002). This mechanism of inhibition parallels that of Argos, a secreted feedback antagonist in the EGFR pathway.

The effect of Nemo on the Wg-dependent reporter gene *Dll* is confined to regions of endogenous gene expression. In the absence of *nmo* expression, ectopic *Dll* expression is only seen at elevated levels within the endogenous expression domain, thus being dependent on Wg activity. This is in contrast to inhibition of the Dpp pathway by Brinker (Campbell and

Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999). Brinker acts independently of Dpp in its repression of Dpp target genes, such that in the absence of both *brk* and Dpp the target genes are expressed ectopically (Campbell and Tomlinson, 1999). We speculate that the role of Nemo in the Wg pathway is analogous to the role of Daughters against Dpp (Dad) in Dpp signaling (Tsuneizumi et al., 1997). Dpp induces the expression of *dad*, which in turn antagonizes the pathway through an as yet undefined mechanism. These might include either interactions with the intracellular transducer Mothers against Dpp (Mad) or with TGFβ receptors.

Nemo does not participate in the self-refinement of Wg expression

It is intriguing that Nemo does not play a role in regulating *wg* expression; however, this is most probably because of the point of action of Nemo within the Wg pathway. The self-refinement of *wg* expression in the wing is dependent on Dsh but independent of Arm (Rulifson et al., 1996). Recent work has raised some questions about the factors involved in Wg self-refinement, specifically postulating a role for dTCF in this process (Schweizer et al., 2003). dTCF (*pan*) somatic clones were shown to have elevated Wg protein, suggesting that TCF plays an active role in repressing Wg gene expression. The authors, however, indicate that they fail to distinguish between increased *wg* gene expression and stabilized Wg protein. Another recent paper examined regulation of Wg signaling by Twins (*tws*), a protein phosphatase subunit, and found that it is required for Arm stabilization (Bajpai et al., 2004). Modulation of *tws* resulted in aberrant Wg signaling, as monitored by Dll expression, that are not accompanied by alterations in *wg* gene expression. Our data are consistent with the findings of Bajpai et al. and suggest that the mechanism of *wg* refinement most probably does not involve Arm or dTCF. Our genetic analyses support the placement of Nemo at or below the level of Arm within the pathway. The apparent absence of a role for Nemo in regulating *wg* expression contrasts with the other inducible feedback inhibitors. Modulation of either the extracellular inhibitor Wf or the Dsh-antagonist Nkd can influence *wg* gene expression in wing discs and embryos, respectively (Gerlitz and Basler, 2002; Zeng et al., 2000). As stated above, neither loss of nor ectopic expression of *nmo* during imaginal disc development has an effect on the pattern of Wg expression.

nmo expression is induced by high levels of Wg signaling

The developing wing is bisected by a narrow stripe of Wg-expressing cells. Wg protein has a short half-life near the DV boundary, which causes a rapid decrease in Wg concentration and forms a steep symmetric gradient of the Wg protein (Cadigan et al., 1998). Radiating out from the source of Wg, there are three concentric domains of Wg-dependent gene expression (reviewed by Martinez Arias, 2003). First, a very narrow domain of cells adjacent to the highest concentration of Wg expresses *achaete* (*ac*). Second, Dll is expressed in a median range domain of Wg and third, a long-range domain expresses *vg*. Our results suggest that *nmo* is a short-range target, like *ac*, the activation of which is limited by the high threshold of Wg signal. This may be the explanation for the very narrow pattern of enriched *nmo* expression at the DV boundary and the ring domain and the cell-autonomous induction of *nmo* in the ectopic ΔArm clones.

If higher levels of Wg protein induce *nmo* expression, it raises the question of why *nmo* is not expressed in DV boundary cells. One possibility is that there are genes that are expressed between the two stripes of *nmo* that prevent its expression. In Fig. 6B, *vg-Gal4*, which is mainly expressed at the DV boundary, drives *UAS-fluΔarm* to induce ectopic *nmo* expression. In this case, the ectopic expression of *nmo* fills the gap between the two endogenous bands. This observation supports a model in which there is a suppressor(s) located along the DV boundary to silence *nmo* expression. The balance between the Wg signal and the suppressor(s) would refine *nmo* expression into two thin stripes flanking the DV boundary. In the case of ectopic *UAS-fluΔarm*, the Wg signal may overpower the suppressor, thereby allowing *nmo* to be expressed at the boundary. In a similar mechanism, it has been shown that Wg can direct the expression of *ac* at the margin but that this expression is prevented, at least partially, by the activity of Cut (Couso et al., 1994).

Although the wing margin, ring expression and low level ubiquitous staining of *nmo* in imaginal wing discs reflects regulation by Wg signaling, the other developmental expression patterns, such as staining in primordia of wing veins, may reflect regulation by other signaling pathways. For example, the staining in the wing vein primordia that emerges in late third instar and the gene expression pattern observed in pupal wings reflects the later role of *nmo* in wing vein patterning (Verheyen et al., 2001), which may involve interactions with EGFR and TGFβ signaling.

In further support that Wg signaling regulates the transcription of *nmo*, we find several dTCF consensus binding sites in the 5′ region of the *nmo* gene which may represent enhancer elements (B. Andrews and E.M.V., unpublished). Indeed, two sites match 9 out of 11 bp (GCCTTTGAT) of the T1 site (GCCTTTGATCT) in the *dpp* BE enhancer that has been shown both in vitro and in vivo to bind and respond to dTCF (Yang et al., 2000). The presence of these sites suggests that the observed transcriptional regulation of *nmo* by Wg may involve direct binding to the *nmo* DNA sequence by dTCF.

Nemo may target Arm for degradation

As a result of comparing the endogenous expression pattern of *nmo* with stabilized Arm, we noticed that the highest levels of Nemo excluded Arm stabilization, while high levels of Arm were present in cells in which *nmo* levels were lower. As Arm protein stabilization is a direct consequence of Wg pathway activation, we sought to examine whether Nemo may function to inhibit Wg by promoting Arm destabilization and subsequent breakdown. Indeed, ectopic expression of Nemo can lead to cell-autonomous reduction in Arm protein levels. This preliminary result suggests a mechanism in which Nemo may contribute to the destabilization of Arm that involves the Axin/APC/GSK3 complex. One explanation to account for such a finding would concern the interaction with TCF in the nucleus and the role of dTCF as an anchor for Arm (Behrens et al., 1996; Tolwinski and Wieschaus, 2001). Given what is known about NLKs, it is likely that Nemo may act on the ability of the dTCF/Arm complex to bind DNA and activate transcription (Ishitani et al., 1999). Tolwinksi and Wieschaus (Tolwinksi and Wieschaus, 2001) propose that dTCF acts as an anchor for Arm in the nucleus. It remains to be determined how efficient this anchor is and whether there are conditions in which the interaction may become

compromised, such as we see with elevated Nemo. NLKs have been shown to affect the DNA-binding ability of TCF/β-catenin (Ishitani et al., 1999). Perhaps in the absence of DNA binding, this complex is less stable and Arm could be free to shuttle to the cytoplasm where it could associate with Axin or APC and become degraded (Henderson and Fagotto, 2002). We propose that the ectopic *nmo* in our assay is leading to destabilization of the dTCF/Arm/DNA complex and thus causing Arm to exit the nucleus and be degraded through interaction with Axin, APC and GSK3. The observation that ectopic expression of full-length Arm cannot induce any activated Wg phenotypes (Orsulic and Peifer, 1996) have been explained by the hypothesis that even these high levels of protein are not sufficient to overcome the degradation machinery (Tolwinski and Wieschaus, 2001). Thus, our finding that there is no elevated Arm in *nmo* clones is consistent with an inability to overcome the endogenous degradation machinery; even though less Nemo could lead to more stabilized DNA interactions, this would not lead to higher levels of stabilized Arm than is normally found.

Model for Nemo and NLK function in Wnt/Wg signaling

Studies of homologs of Nemo in other species have provided clues to its function, although it is still not clear if the same mechanism in used in *Drosophila*. Our studies in this paper establish that Drosophila Nemo does in fact play a negative regulatory role in canonical Wg signaling. Although *nmo* was originally identified as playing a role in the non-canonical Fz pathway that regulates tissue planar polarity, its precise role in that pathway has not been further defined (Brown and Freeman, 2003; Choi and Benzer, 1994; Strutt et al., 1997).

In addition to the findings that NLKs can bind to and phosphorylate TCF and LEF-1 proteins (Ishitani et al., 2003) and thereby decrease the DNA-binding affinity of the TCF/β-catenin complex, a model is emerging that NLKs regulate multiple HMG-box containing proteins. Recently, it was shown that *Xenopus* NLK (xNLK) binds to a novel HMG-domain containing protein HMG2L1, which can inhibit Wnt signaling in several assays (Yamada et al., 2003). In addition, xNLK binds to xSox11, another HMG-box containing transcription factor, and they cooperatively induce neural development in *Xenopus* (Hyodo-Miura et al., 2002).

Although our results do not directly address the molecular mechanism, we speculate that activated Nemo can inhibit the interaction of the Arm-dTCF complex with DNA. The genetic data presented in this paper support the molecular mechanism that Nemo acts downstream of or at the same level as Arm. Indeed, the finding that increased levels of *nmo* can block accumulation of Arm is intriguing as it suggests that Nemo may regulate Wg at the level of Arm stabilization and dTCF function. At this point, further biochemical experiments are in progress to address these issues. They should shed light on the exact mechanism of function that allows Nemo to be an inducible antagonist of canonical Wg signaling in *Drosophila*.

We are grateful to the many people who supplied fly strains, antibodies and other reagents: Richard Carthew, Hideki Nakagoshi, Roel Nusse, Shelagh Campbell, Konrad Basler, Masahiro Go, Kwang-wook Choi, Mariann Bienz, Seth Blair, Kathy Matthews and the Bloomington Drosophila Stock Center. We thank Mark Peifer for his insight, Hideki Nakagoshi for helpful suggestions, Desiree Essen for technical help, Bryan Andrews for finding the dTCF site and Eric Accili for use of his confocal microscope. We thank Nick Harden, Minna Roh and members of the Verheyen laboratory for comments on the manuscript. This work is supported by grants from the National Cancer Institute of Canada (NCIC) and the Canadian Institutes for Health Research (CIHR).

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.

Anderson, K. V. and Ingham, P. W. (2003). The transformation of the model organism: a decade of developmental genetics. *Nat. Genet.* **33**, 285-293.

Bajpai, R., Makhijani, K., Rao, P. R. and Shashidhara, L. S. (2004). Drosophila Twins regulates Armadillo levels in response to Wg/Wnt signal. *Development* **131**, 1007-1016.

Baker, N. E. (1988). Transcription of the segment-polarity gene wingless in the imaginal discs of Drosophila, and the phenotype of a pupal-lethal wg mutation. *Development* **102**, 489-497.

Behrens, J., von Kries, J. P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R. and Birchmeier, W. (1996). Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature* **382**, 638-642.

Brand, A. and Perrimon, N. (1993). Targetted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brott, B., Pinsky, B. and Reikson, R. (1998). Nlk is a murine protein kinase related to Erk/MAP kinases and localized in the nucleus. *Proc. Natl. Acad. Sci. USA* **95**, 963-968.

Brown, K. E. and Freeman, M. (2003). Egfr signalling defines a protective function for ommatidial orientation in the Drosophila eye. *Development* **130**, 5401-5412.

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.

Campbell, G. and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by brinker. *Cell* **96**, 553-562.

Choi, K.-W. and Benzer, S. (1994). Rotation of photoreceptor clusters in the developing *Drosophila* eye requires the *nemo* gene. *Cell* **78**, 125-136.

Couso, J. P. and Arias, A. M. (1994). Notch is required for *wingless* signaling in the epidemis of Drosophila. *Cell* **79**, 259-272.

Couso, J. P., Bishop, S. A. and Martinez-Arias, A. (1994). The wingless signalling pathway and the patterning of the wing margin in *Drosophila*. *Development* **120**, 621-636.

Diaz-Benjumea, F. and Cohen, S. (1995). Serrate signals through Notch to establish a Wingless-dependent organizer at the dorsal/ventral compartment boundary of the Drosophila wing. *Development* **121**, 4215-4225.

Freeman, M. (2000). Feedback control of intercellular signalling in development. *Nature* **408**, 313-319.

Gerlitz, O. and Basler, K. (2002). Wingful, an extracellular feedback inhibitor of Wingless. *Genes Dev.* **16**, 1055-1059.

Hamada, F., Tomoyasu, Y., Takatsu, Y., Nakamura, M., Nagai, S., Suzuki, A., Fujita, F., Shibuya, H., Toyoshima, K., Ueno, N. et al. (1999). Negative regulation of Wingless signaling by D-axin, a Drosophila homolog of axin. *Science* **283**, 1739-1742.

Harada, H., Yoshida, S., Nobe, Y., Ezura, Y., Atake, T., Koguchi, T. and Emi, M. (2002). Genomic structure of the human NLK (nemo-like kinase) gene and analysis of its promoter region. *Gene* **285**, 175-182.

Henderson, B. R. and Fagotto, F. (2002). The ins and outs of APC and beta-catenin nuclear transport. *EMBO Rep.* **3**, 834-839.

Hooper, J. E. (1994). Distinct pathways for autocrine and paracrine Wingless signalling in Drosophila embryos. *Nature* **372**, 461-464.

Hyodo-Miura, J., Urushiyama, S., Nagai, S., Nishita, M., Ueno, N. and Shibuya, H. (2002). Involvement of NLK and Sox11 in neural induction in Xenopus development. *Genes Cells* **7**, 487-496.

Ishitani, T., Ninomiya-Tsuji, J., Nagai, S., Nishita, M., Meneghini, M., Barker, N., Waterman, M., Bowerman, B., Clevers, H., Shibuya, H. et

al. (1999). The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta- catenin and transcription factor TCF. *Nature* **399**, 798-802.
Ishitani, T., Ninomiya-Tsuji, J. and Matsumoto, K. (2003). Regulation of lymphoid enhancer factor 1/T-cell factor by mitogen-activated protein kinase-related Nemo-like kinase-dependent phosphorylation in Wnt/beta-catenin signaling. *Mol. Cell. Biol.* **23**, 1379-1389.
Ito, K., Awano, W., Suzuki, K., Hiromi, Y. and Yamamoto, D. (1997). The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* **124**, 761-771.
Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S. and Rushlow, C. (1999). The Drosophila gene brinker reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563-573.
Jho, E. H., Zhang, T., Domon, C., Joo, C. K., Freund, J. N. and Costantini, F. (2002). Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Mol. Cell. Biol.* **22**, 1172-1183.
Kehrer-Sawatzki, H., Moschgath, E., Maier, C., Legius, E., Elgar, G. and Krone, W. (2000). Characterization of the Fugu rubripes NLK and FN5 genes flanking the NF1 (Neurofibromatosis type 1) gene in the 5′ direction and mapping of the human counterparts. *Gene* **251**, 63-71.
Martinez Arias, A. (2003). Wnts as morphogens? The view from the wing of Drosophila. *Nat. Rev. Mol. Cell Biol.* **4**, 321-325.
Meneghini, M. D., Ishitani, T., Carter, J. C., Hisamoto, N., Ninomiya-Tsuji, J., Thorpe, C. J., Hamill, D. R., Matsumoto, K. and Bowerman, B. (1999). MAP kinase and Wnt pathways converge to downregulate an HMG-domain repressor in Caenorhabditis elegans. *Nature* **399**, 793-797.
Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H. and Tabata, T. (1999). brinker is a target of Dpp in Drosophila that negatively regulates Dpp-dependent genes. *Nature* **398**, 242-246.
Mirkovic, I., Charish, K., Gorski, S. M., McKnight, K. and Verheyen, E. M. (2002). Drosophila nemo is an essential gene involved in the regulation of programmed cell death. *Mech. Dev.* **119**, 9-20.
Mlodzik, M. (2002). Planar cell polarization: do the same mechanisms regulate Drosophila tissue polarity and vertebrate gastrulation? *Trends Genet.* **18**, 564-571.
Mohit, P., Bajpai, R. and Shashidhara, L. S. (2003). Regulation of Wingless and Vestigial expression in wing and haltere discs of Drosophila. *Development* **130**, 1537-1547.
Morata, G. and Lawrence, P. A. (1977). The development of wingless, a homeotic mutation of Drosophila. *Dev. Biol.* **56**, 227-240.
Morimura, S., Maves, L., Chen, Y. and Hoffmann, F. M. (1996). decapentaplegic overexpression affects Drosophila wing and leg imaginal disc development and wingless expression. *Dev. Biol.* **177**, 136-151.
Neumann, C. J. and Cohen, S. M. (1996). A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the Drosophila wing. *Development* **122**, 3477-3485.
Ng, M., Diaz-Benjumea, F. J., Vincent, J. P., Wu, J. and Cohen, S. M. (1996). Specification of the wing by localized expression of wingless protein. *Nature* **381**, 316-318.
Orsulic, S. and Peifer, M. (1996). An in vivo structure-function study of armadillo, the beta-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.
Peifer, M., Rauskolb, C., Williams, M., Riggleman, B. and Wieschaus, E. (1991). The segment polarity gene armadillo interacts with the wingless signaling pathway in both embryonic and adult pattern formation. *Development* **111**, 1029-1043.
Perrimon, N. and McMahon, A. P. (1999). Negative feedback mechanisms and their roles during pattern formation. *Cell* **97**, 13-16.
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.
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.
Riese, J., Yu, X., Munnerlyn, A., Eresh, S., Hsu, S. C., Grosschedl, R. and Bienz, M. (1997). LEF-1, a nuclear factor coordinating signaling inputs from wingless and decapentaplegic. *Cell* **88**, 777-787.
Rocheleau, C. E., Yasuda, J., Shin, T. H., Lin, R., Sawa, H., Okano, H., Priess, J. R., Davis, R. J. and Mello, C. C. (1999). WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in C. elegans. *Cell* **97**, 717-726.
Roose, J., Huls, G., van Beest, M., Moerer, P., van der Horn, K., Goldschmeding, R., Logtenberg, T. and Clevers, H. (1999). Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science* **285**, 1923-1926.
Rousset, R., Mack, J. A., Wharton, K. A., Jr, Axelrod, J. D., Cadigan, K. M., Fish, M. P., Nusse, R. and Scott, M. P. (2001). Naked cuticle targets dishevelled to antagonize Wnt signal transduction. *Genes Dev.* **15**, 658-671.
Rulifson, E. J. and Blair, S. S. (1995). *Notch* regulates *wingless* expression and is not required for reception of the paracrine *wingless* signal during wing margin neurogenesis in *Drosophila*. *Development* **121**, 2813-2824.
Rulifson, E. J., Micchelli, C. A., Axelrod, J. D., Perrimon, N. and Blair, S. S. (1996). wingless refines its own expression domain on the Drosophila wing margin. *Nature* **384**, 72-74.
Schweizer, L., Nellen, D. and Basler, K. (2003). Requirement for Pangolin/dTCF in Drosophila Wingless signaling. *Proc. Natl. Acad. Sci. USA* **100**, 5846-5851.
Shin, T., Yasuda, J., Rocheleau, C., Lin, R., Soto, M., Bei, X., Davis, R. and Mello, C. (1999). MOM-4, a MAP kinase kinase kinase-related protein, activates WRM-1/LIT-1 kinase to transduce anterior/posterior polarity signals in *C. elegans*. *Mol. Cell* **4**, 275-280.
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.
Spiegelman, V. S., Slaga, T. J., Pagano, M., Minamoto, T., Ronai, Z. and Fuchs, S. Y. (2000). Wnt/beta-catenin signaling induces the expression and activity of betaTrCP ubiquitin ligase receptor. *Mol. Cell* **5**, 877-882.
Strutt, D., Weber, U. and Mlodzik, M. (1997). The role of RhoA in tissue polarity and Frizzled signaling. *Nature* **387**, 292-295.
Tautz, D. and Pfeiffle, C. A. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. *Chromosoma* **98**, 81-85.
Tolwinski, N. S. and Wieschaus, E. (2001). Armadillo nuclear import is regulated by cytoplasmic anchor Axin and nuclear anchor dTCF/Pan. *Development* **128**, 2107-2117.
Tsuneizumi, K., Nakayama, T., Kamoshida, Y., Kornberg, T. B., Christian, J. L. and Tabata, T. (1997). Daughters against dpp modulates dpp organizing activity in Drosophila wing development. *Nature* **389**, 627-631.
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.
Verheyen, E. M., Purcell, K. J., Fortini, M. E. and Artavanis-Tsakonas, S. (1996). Analysis of dominant enhancers and suppressors of activated *Notch* in *Drosophila*. *Genetics* **144**, 1127-1141.
Verheyen, E. M., Mirkovic, I., MacLean, S. J., Langmann, C., Andrews, B. C. and MacKinnon, C. (2001). The tissue polarity gene *nemo* carries out multiple roles in patterning during Drosophila development. *Mech. Dev.* **101**, 119-132.
Willert, K., Logan, C. Y., Arora, A., Fish, M. and Nusse, R. (1999). A Drosophila Axin homolog, Daxin, inhibits Wnt signaling. *Development* **126**, 4165-4173.
Williams, J. A., Paddock, S. W. and Carroll, S. B. (1993). Pattern formation in a secondary field: a hierarchy of regulatory genes subdivides the developing Drosophila wing disc into discrete subregions. *Development* **117**, 571-584.
Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult Drosophila tissues. *Development* **117**, 1223-1237.
Yamada, M., Ohkawara, B., Ichimura, N., Hyodo-Miura, J., Urushiyama, S., Shirakabe, K. and Shibuya, H. (2003). Negative regulation of Wnt signalling by HMG2L1, a novel NLK-binding protein. *Genes Cells* **8**, 677-684.
Yang, X., van Beest, M., Clevers, H., Jones, T., Hursh, D. A. and Mortin, M. A. (2000). decapentaplegic is a direct target of dTcf repression in the Drosophila visceral mesoderm. *Development* **127**, 3695-3702.
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.
Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a wingless morphogen gradient. *Cell* **87**, 833-844.
Zeng, W., Wharton, K. A., Jr, Mack, J. A., Wang, K., Gadbaw, M., Suyama, K., Klein, P. S. and Scott, M. P. (2000). naked cuticle encodes an inducible antagonist of Wnt signalling. *Nature* **403**, 789-795.
Zhang, J. and Carthew, R. W. (1998). Interactions between Wingless and DFz2 during Drosophila wing development. *Development* **125**, 3075-3085.