Notch modulates Wnt signalling by associating with Armadillo/β-catenin and regulating its transcriptional activity

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Summary

The establishment and stability of cell fates during development depend on the integration of multiple signals, which ultimately modulate specific patterns of gene expression. While there is ample evidence for this integration at the level of gene regulatory sequences, little is known about its operation at other levels of cellular activity. Wnt and Notch signalling are important elements of the circuitry that regulates gene expression in development and disease. Genetic analysis has suggested that in addition to convergence on the transcription of specific genes, there are modulatory cross-regulatory interactions between these signalling pathways. We report that the nodal point of these interactions is an activity of Notch that regulates the activity and the amount of the active/oncogenic form of Armadillo/β-catenin. This activity of Notch is independent of that induced upon cleavage of its intracellular domain and which mediates transcription through Su(H)/CBF1. The modulatory function of Notch described here, contributes to the establishment of a robust threshold for Wnt signalling which is likely to play important roles in both normal and pathological situations.

Key words: Notch, Armadillo, Signalling, Wnt

Introduction

The *Drosophila* Notch gene encodes a member of a family of single transmembrane receptors that play a central role in the assignation of cell fates during development (Artavanis-Tsakonas et al., 1999; Kopan, 2002). The extracellular domain of Notch is composed of an array of EGF-like repeats that are involved in ligand binding and three cysteine-rich domains (LNR) required for signal transduction (Brennan et al., 1999b; Lawrence et al., 2000; Lieber et al., 1993; Rebay et al., 1991). The intracellular domain is the signalling moiety of the receptor and its most prominent structural feature is a group of six cdc10/Ankryn (ANK) repeats that are involved in a variety of molecular interactions (Kopan et al., 1994; Lieber et al., 1993; Rebay et al., 1993; Struhl et al., 1993). Upon binding its ligand Delta, Notch undergoes a sequence of proteolytic cleavage events that release the intracellular domain (NICD) from the membrane (Schroeter et al., 1998; Schweisguth and Lecourtois, 1998; Struhl and Adachi, 1998). NICD then enters the nucleus where it interacts with Suppressor of Hairless (Su(H)/CBF1) (Artavanis-Tsakonas et al., 1999; Barolo et al., 2002; Kidd et al., 1998; Kopan, 2002) and regulates the transcription of specific targets. This signalling event is used in some inductive events but more importantly in multiple binary cell fate decisions in which Notch signalling favours one of two alternative fates by suppressing the onset of the genetic programme that would lead to the other fate (Artavanis-Tsakonas et al., 1999; Kopan, 2002).

There is evidence that Notch can also signal in a Su(H)-independent manner (Endo et al., 2002; Endo et al., 2003; Martinez Arias et al., 2002). A number of experiments in *Drosophila* indicate that this alternative pathway modulates signalling by Wingless, a member of the Wnt family of signalling molecules (Martinez Arias et al., 2002). Loss of function of *Notch*, but not of *Delta* or of *Su(H)*, can bypass loss of function of *wingless*, or of *dishevelled*, a gene that encodes a core element in the transduction of the Wnt signal (Brennan et al., 1999a; Lawrence et al., 2001). This suggests that Notch can downregulate Wnt signalling in a Su(H)-independent manner, a notion reinforced by the existence of gain-of-function mutations in *Notch,* which antagonise Wingless signalling (Brennan et al., 1999b; Martinez Arias et al., 2002; Ramain et al., 2001). Consistent with these observations, removal of *Notch1* in the skin leads to tumours associated with Wnt signalling and with high levels of the nuclear form of β-catenin (Nicolas et al., 2003). However, even though the interaction between Notch and Wingless signalling is well established at the genetic level its molecular mechanism remains unclear.

It is generally accepted that the key parameter of Wnt signalling is the stability and precise intracellular location of a soluble pool of Armadillo/β-catenin (Arm/β-cat) (Gottardi and Gumbiner, 2001). In the absence of Wnt this pool interacts with a destruction complex where it is phosphorylated by Shaggy/GSK3β and degraded via the proteasome. Wnt acting through the Frizzled and Arrow/LRP receptors activates the cytoplasmic adaptor protein Dishevelled which, in a poorly

understood manner leads to the inactivation of the destruction complex and allows the accumulation of a hypophosphorylated form of Armadillo/β-catenin. This form then enters the nucleus where it interacts with members of the TCF family of transcription factors to influence the transcriptional state of the cell (Tolwinski and Wieschaus, 2004a). While the central role of Armadillo/β-catenin is well established, the mechanism by which it is activated remains open to discussion (Giles et al., 2003; Tolwinski and Wieschaus, 2004b). It has recently been observed that Axin has effects on Wnt signalling that are independent of Shaggy/GSK3β (Tolwinski et al., 2003), suggesting that the central event in the activation of Armadillo/β-catenin is the activity of Axin.

Here we analyse the molecular nature of the interactions between Notch and Wingless signalling in *Drosophila* and between mouse Notch1 and β-catenin.

Materials and methods

Immunohistochemistry and genetic analysis

The activity of various genes was eliminated by generating clones of mutant cells in an otherwise heterozygous background through the FLP recombinase system as described before (Klein et al., 2000). The following *Drosophila melanogaster* stocks were used: *Df(1)N*^{81k} *y* [FRT101 *w*$^{+}$]/FM6: *sgg*^{D127}; *N*^{55e11} [FRT101 *w*$^{+}$]/FM6*f* and *w*^{a} *sgg*^{m11} *sn*^{3} [FRT101 *w*$^{+}$]/FM6*f*, all are null alleles of the different genes. Each stocks was outcrossed to *yw ubiq*GFP^{x1} [FRT101 *w*$^{+}$]; *ptc*GAL4; UASFLP A101lacZ/SM6a^TM6B. As a result, clones were induced continuously throughout development over the domain of *ptc* expression and were identified by loss of GFP. Where indicated, ectopic expression of particular forms of Notch or Armadillo over the domain of *ptc* was induced using the stocks: *w*^{a} *sgg*^{m11} [FRT101 *w*$^{+}$]/FM7c; UASTNotch and *Df(1)N*^{81k} *y* [FRT101 *w*+]/FM6:; UASArmadillo. TNotch (Seugnet et al., 1997) and UASArmadillo (Pai et al., 1997) have been described before. The TNotch construct is unable to activate a Su(H) reporter in vivo but nevertheless promotes dominant gain-of-function phenotypes during neurogenesis. On the possibility that there are different functions of Notch (Brennan et al., 1997; Ramain et al., 2001), the activity of TNotch would involve some but not all functions. Consistent with this, TNotch cannot rescue a complete loss of function of Notch (A.M.A., unpublished). This behaviour mimics that of the Abruptex and Mcd alleles of Notch, which have been classified as antimorphic mutations, i.e. the activity of the proteins they encode reflects aspects of that of the wild-type protein and competes with it (for details, see Brennan et al., 1997; Brennan et al., 1999b; Ramain et al., 2001). While it is true that TNotch is an experimental creation, its activity responds to the dosage of endogenous Notch and therefore is likely to reflect one of its wild-type functions.

Imaginal wing disc were dissected from third instar larvae in fix solution [4% paraformaldehyde in balanced salt solution (BBS) with 1 mM $CaCl_2$]. Discs were fixed for 30 minutes and then immunostained with the indicated antibodies in BBS [50 mM BES, 280 mM NaCl, 1.5 mM $Na_2HPO_4.2H_2O$]+ 0.1% Triton X-100, 0.5% BSA 1 mM $CaCl_2$] using standard antibody staining protocols. Discs were mounted in Vectashield and viewed using a confocal microscope (note, the same gain was used in each figure set).

Analysis of Armadillo protein levels

Wing discs from third instar larvae expressing Armadillo^{s10}, TNotch, FLNotch or NICD under the control of *dpp*GAL4 were lysed in 2× Laemmli buffer (Harlow and Lane, 1988). Proteins were separated by 8% SDS-PAGE, the equivalent of five wing discs were loaded per lane. Western blot analysis for Armadillo (N2 7A1), Armadillo^{s10} (anti-MYC, 9E10) and tubulin (E7) was performed.

Cell based reporter assays

Assays in insect cells

Transfections were performed in triplicate in 96-well plates using 8×10^4 cells per well and Effectene transfection reagent (Qiagen). The amount of Firefly and Renilla luciferase was measured 4 days after transfection using Dual-Glo reagent (Promega). Data is normalised with respect to Renilla luciferase and presented as relative light units (RLU), all data represents at least three independent experiments.

RNAi experiment

Clone 8 cells (Peel et al., 1990) were used. dsRNAs were synthesised as described previously (Boutros et al., 2004); 80 ng of dsRNA was added to each transfection reaction along with luciferase reporter (Top12X-HS-luciferase; R.G. and N.P., unpublished data), and normalisation vector (pPOLIII-Renilla) in a 1:1 ratio, 50 ng of total DNA added per well.

Gain of function assays

SL2 (Nagao et al., 1996) and S2R+ (Yanagawa et al., 1998) cells were used. For each transfection the ratio of luciferase reporter (Top12X-HS-luciferase), normalisation vector (pPOLIII-Renilla) and inducer [pPac-S37Aβcat (Schweizer and Varmus, 2003)] DNA was 1:1:2, the remaining DNA was composed of variable amounts of pPACTN and pPAC with a total amount 200 ng DNA added per well.

Assays in mammalian cells

Two Notch1 (Nye et al., 1994) constructs bearing extracellular deletions were generated in pSecTag2 (Invitrogen): LNR-N1, which lacks amino acids 19-1654, so the encoded protein should be identical to that produced following furin cleavage at the S1 site, and ΔN-N1, which lacks amino acids 19-1710, so the encoded protein should be identical to that produced upon cleavage at the S2 site during ligand-induced signalling. Plasmids encoding mouse Wnt1 (Shimizu et al., 1997), *Xenopus* dishevelled (Sokol, 1996) and *Xenopus* β-catenin (Kypta et al., 1996) have been described previously. The Lef1-VP16 fusion protein and the CBF reporter were obtained from Dr R. Kemler, Max-Planck Institut für Immunbiologie and Dr G. McKenzie, Lorantis Ltd. Triplicate transfections were performed in 24-well plates with HEK-293T cells (1×10^5 cells/well), using the calcium phosphate co-precipitation method with a plasmid cocktail containing 0.22 μg of DNA (including 50 ng pTOPFLASH or CBF1 luciferase reporter, and 20 ng pRL-CMV). Lysates were prepared 48 hours after transfection, and Firefly and Renilla luciferase activities in 5 μl of lysate were measured with the Dual Luciferase Reagent (Promega).

Immunoprecipitation experiments

Wild-type *Drosophila* embryos were dechorionated and lysed in RIPA or NP-40 buffer (Harlow and Lane, 1988). Each immunoprecipitation reaction contained the equivalent of 5 μl packed volume of embryos homogenised in 250 μl lysis buffer. Notch proteins were immunoprecipitated with 20 μl anti-NICD sheep antiserum or 50 μl anti-Notch (C17.9C6) and 20 μl protein G Sepharose. Armadillo proteins were immunoprecipitated using 10 μl anti-Armadillo rabbit antiserum and 20 μl protein A Sepharose. Control reactions with protein G, protein A or anti-GFP rabbit antiserum with Protein A were undertaken. Immune complexes were released by boiling in 60 μl Laemmli buffer and separated by 8% SDS-PAGE, 20 μl immunoprecipitate per lane. Proteins were detected by western blot.

Results

Notch modulates Wingless signalling by regulating the activity of Armadillo

A soluble form of the intracellular domain of Notch, NICD, acts as an activated Notch receptor and provides constitutive Su(H)-dependent Notch signalling (Schweisguth, 2004).

Whereas a chimera between the extracellular and transmembrane domains of the receptor tyrosine kinase (RTK) Torso and the intracellular domain of Notch (TNotch) prevents the cleavage of Notch and the translocation of its intracellular domain to the nucleus (Struhl and Adachi, 2000). However, this chimeric molecule is still capable of signalling, as reflected by the loss of neural precursors during neurogenesis (Seugnet et al., 1997; Zecchini et al., 1999) (A.M.A., unpublished data). This signalling event is likely to be independent of Su(H) because while NICD and full length Notch are able to activate transcription of either a Su(H) reporter in vivo (Furriols and Bray, 2001) or the Notch target gene *wingless* (Diaz-Benjumea and Cohen, 1995; Klein and Martinez Arias, 1998), TNotch is unable to do so (A.M.A., unpublished data). Thus TNotch behaves as a gain-of-function allele but one specific for a particular function of Notch which might not involve Suppressor of Hairless. In agreement with this, TNotch is unable to rescue a complete loss of function of Notch (A.M.A., unpublished data). For details of the genetic properties of this construct see Materials and methods.

The inputs of Notch and Wingless signalling on the development of the wing are well characterised (Klein and Martinez Arias, 1998; Klein and Martinez Arias, 1999; Martinez Arias, 2003). Notch and Wingless signalling cooperate in the development of the wing and in the case of Notch the effects are mediated by NICD. To test if the cleavage-independent function of Notch modulates Wingless signalling, we have expressed NICD and TNotch at the same time that we activate Wingless signalling either with ectopic expression of Wingless or of a constitutively active form of Armadillo, Armadillo^{s10}. This form of Armadillo lacks the Shaggy/GSK3β phosphorylation sites and provides Wingless-independent signalling by escaping degradation by the Axin-based destruction complex (Pai et al., 1997). Expression of either Wingless or Armadillo^{s10} along the AP boundary results in an expansion of the hinge region and the occasional appearance of extra wing tissue off the notum (Klein and Martinez Arias, 1998) (Fig. 1C). However, the effects of the intracellular domain of Notch depend on its molecular disposition. Expression of NICD along the AP boundary induces the appearance of an ectopic wing margin and promotes the growth of the wing (Diaz-Benjumea and Cohen, 1995; Klein and Martinez Arias, 1998), while expression of TNotch leads to a slight reduction in the overall size of the wing pouch region of the disc (Fig. 1B). In the developing wing, co-expression of NICD with either Wingless or Armadillo^{s10} leads to a synergistic effect of extra growth of the wing tissue (Klein and Martinez Arias, 1998). In contrast to NICD, TNotch is very effective in suppressing the effects of ectopic expression of Wingless and, surprisingly, also of Armadillo^{s10} (Fig. 1D, also see Fig. S1 in supplementary material).

Since Armadillo^{s10} provides Wingless signalling constitutively (Pai et al., 1997) and expression of TNotch does not affect the expression of Wingless in the third instar discs (see Fig. S1 in supplementary material), these results argue that a Su(H)-independent Notch activity modulates Wingless signalling by targeting the activity of Armadillo. To test this further, we analysed the effects of TNotch on the ability of Armadillo^{s10} to induce expression of Wingless target genes, *Distalless* (*Dll*) a low threshold target of Wingless, and the proneural gene *senseless* (*sens*), which like other proneural genes, provides a high threshold target (Zecca et al., 1996). Both are elevated and ectopic in the presence of Armadillo^{s10}, and in both cases TNotch markedly suppresses this effect (Fig. 2).

To test whether the effects observed are restricted to the developing wing, we have monitored the effects of TNotch on the cuticle pattern of the first instar larva. In the wild-type each segment contains an anterior region decorated with

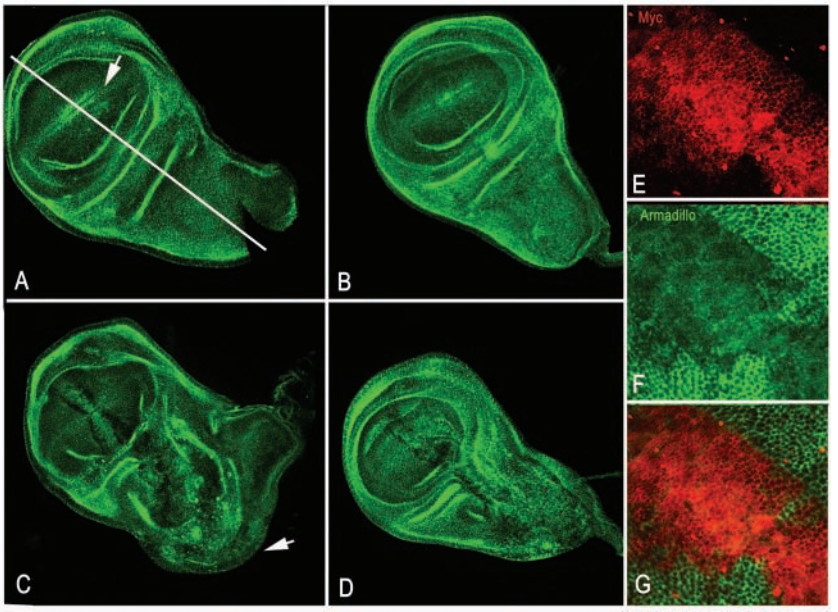

Fig. 1. Notch modulates the activity of an activated form of Armadillo. (A-D) Apical sections through third larval instar wing discs stained for endogenous Armadillo (N27A1) and expressing different signalling molecules under the control of *dpp* GAL4. (A) Wild-type wing disc. Notice elevated levels of Armadillo around the dorsal-ventral boundary (arrow) which coincide with high levels of Wingless signalling. The white line indicates the region of ectopic expression in experimental situations. (B) Disc expressing TNotch. Slight differences in the pattern of endogenous Armadillo, particularly at the DV boundary are observed. (C) Wing disc expressing Armadillo^{s10} (Pai et al., 1997). This molecule lacks the epitope recognised by the monoclonal antibody N27A1. The domain of expression of Armadillo^{S10} expression is demarcated by changes in the concentration and subcellular location of the endogenous Armadillo. Also note the alterations of growth in the notum (arrowhead) (see Pai et al., 1997). (D) Co-expression of TNotch with Armadillo^{S10} suppresses significantly the effects of Armadillo^{S10} both on the shape of the disc and on the altered distribution of endogenous Armadillo (compare to C, similar focal plane, also see Fig. S1 in supplementary material). (E-G) Wing disc expressing Armadillo^{S10}, apical section at the level of the adherens junctions; posterior to right and anterior to left. (E) Armadillo^{S10} detected with anti-myc antibody. Notice its association with the adherens junctions. (F) Endogenous Armadillo (N27A1 antibody) is excluded from the adherens junctions over the domain of Armadillo^{S10} expression. The shadows correspond to the cells from the peripodial membrane. (G) Merged image of E and F.

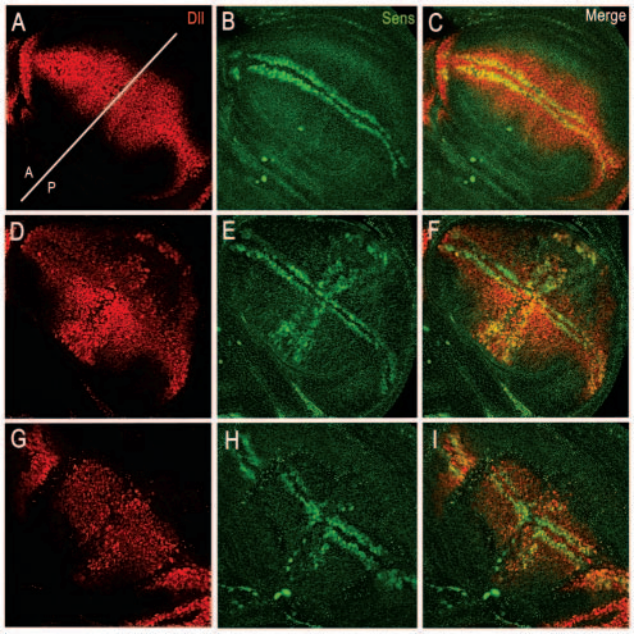

Fig. 2. Notch suppresses the activity of Armadillo^{S10}. Wing pouch region of third larval instar wing discs showing the response of high (Senseless, Sens) and low (Distalless, Dll) threshold targets of Wingless signalling to normal or ectopic activity of Armadillo. (A-C) Wild type. (A) Distalless expression; notice a slight elevation of the expression at the DV boundary. The white line indicates the approximate domain of *dpp* expression. (B) Senseless expression highlighting neural precursors that develop in response to high levels of Wingless signalling (Couso et al., 1993). (C) Merged image of A and B. (D-F) Wing pouch of a disc expressing Armadillo^{S10} under the control of *dpp*GAL4. (D) Distalless expression is now elevated and expanded over the AP boundary. (E) Senseless can be detected over a new domain along the AP boundary. (F) Merged image of D, E. (G-I) Wing pouch of a disc expressing Armadillo^{S10} and TNotch under the control of *dpp*GAL4. (G) The effects of Armadillo^{S10} on Distalless are suppressed by TNotch, a reduction of wild-type levels is also observed. (H) The ectopic expression of Senseless induced by Armadillo^{S10} is suppressed by TNotch. Notice also the reduction in the endogenous expression over the domain of TNotch expression. (I) Merged image of G and H.

denticles and a 'naked' posterior region, devoid of denticles (Pai et al., 1997) (see Fig. S2A in supplementary material). The extent of the 'naked' region depends on the level of Wingless signalling, and ubiquitous Wingless signalling associated with strong expression of Armadillo^{s10} results in cuticles all devoid of denticles (Pai et al., 1997). By modulating the levels of expression of Armadillo^{s10} it is possible to modulate the extent of denticle loss: weak expression leads to a patchy loss of denticles (see Fig. S2D in supplementary material) in contrast, strong expression results in ventral cuticles completely devoid of denticles (see Fig. S2B in supplementary material). Expression of TNotch modulates the effects that Armadillo^{s10} has on the pattern of the cuticle: while strong effects of Armadillo^{s10} are often suppressed (see Fig. S2C in supplementary material), weak effects are very easily suppressed (see Fig. S2E in supplementary material). This observation confirms that Notch exerts a negative modulation on Wnt signalling and suggests that this might be a general phenomenon.

Altogether these observations suggest that there is an activity of Notch, independent of Su(H), which modulates the Wingless signalling pathway at or below the level of Armadillo.

Torso-Notch modulates the levels of Armadillo

The effects of Notch on the activity of Armadillo^{s10} could be due to a squelching of GAL4 by the UASTNotch construct reducing the expression of other constructs co-expressed with it. However in situ experiments demonstrate that UASTNotch transcription does not affect UASArmadillo^{s10} expression (P.H., unpublished). This suggests that the effects of Notch on the activity of Armadillo result either from a parallel input on Wingless target gene expression or from an effect on Armadillo itself. In order to test this we have analysed the effects of Notch on the levels, state and localisation of the Armadillo protein.

In the epithelium of the wing disc, Armadillo is preferentially localised at the level of the adherens junctions (Fig. 1E,F) and exists in at least two different phosphorylation states (Fig. 3F) that have been correlated with function (Peifer et al., 1994a): a hypophosphorylated form that has been associated with nuclear activity (Staal et al., 2002) and a hyperphosphorylated form that is predominantly restricted to the adherens junctions (Peifer et al., 1994b). In our experiments, when Armadillo^{s10} is expressed it becomes preferentially localised to the adherens junctions (Fig. 1E). The expression of Armadillo^{s10} has a significant effect on the endogenous Armadillo, which is displaced from the adherens junctions and accumulates in the cytoplasm (Fig. 1C,F). In western blots this is translated into a rise in endogenous Armadillo levels and is correlated with an increase in the proportion of the hypophosphorylated form (Fig. 3E,F). These effects are likely to be associated with the enhanced stability of Armadillo^{s10} and its ability to interact with and titrate the activity of components of the Armadillo destruction complex (Cox et al., 1999; Pai et al., 1997).

To provide a measure of the effects of Armadillo^{s10} we performed western blot analysis on its steady state levels and those of endogenous Armadillo, in the presence or absence of various forms of the Notch receptor. These experiments were performed with three different Gal4 lines which direct expression of effector genes in different but overlapping domains: the whole wing pouch (C5Gal4; Fig. 3F), the Hh signalling domain (*dpp*Gal4; Fig. 3E) and a domain around the DV boundary (C96Gal4; P.H. and P.S., unpublished data). Consistent with what we observe in the disc epithelium, expression of Armadillo^{s10} elevates the overall levels of endogenous Armadillo with a pronounced increase in the hypophosphorylated form (lower band of doublet, Fig. 3F). Expression of both TNotch and full length Notch can reduce the levels of all forms of Armadillo, but the extent depends on the expression domain. Expression of TNotch under the control of *dpp*Gal4 results in a large reduction of both endogenous Armadillo and Armadillo^{s10}, whereas the effects of full length Notch are limited to endogenous Armadillo. Under the control of C5Gal4 expression of both TNotch and full length Notch regulates the levels of Armadillo^{s10}. Under these conditions the hypophosphorylated form of endogenous Armadillo is also

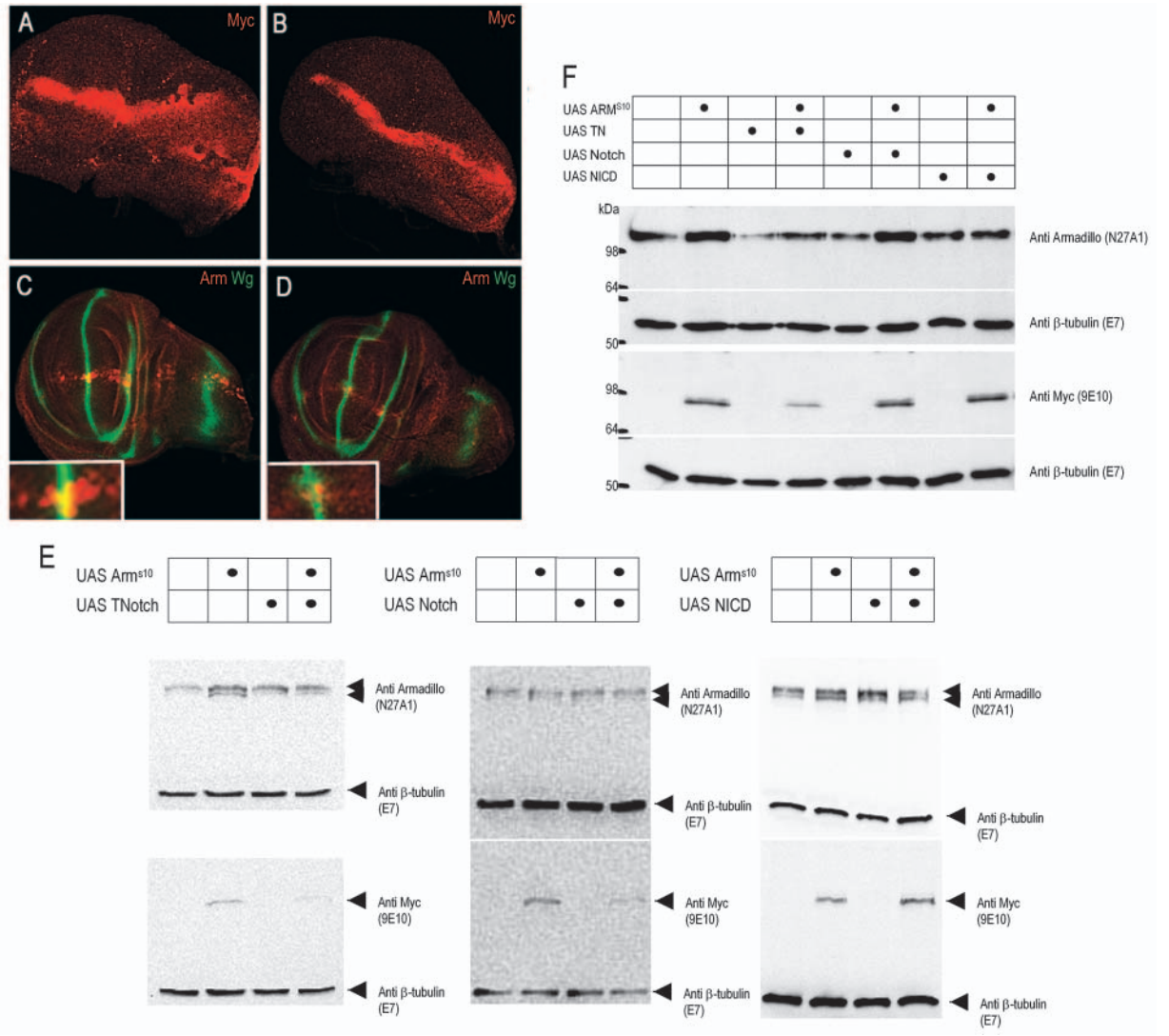

Fig. 3. Notch affects the levels of Armadillo and of Armadillo^{S10}. (A-D) Effects of TNotch on the stability of Armadillo^{S10} and wild-type Armadillo in third instar wing discs (anterior down and posterior up); expression is under the control of *dpp*GAL4 at 22°C. (A) Expression of Armadillo^{S10}. (B) Expression of TNotch with Armadillo^{S10}; notice the reduction in the overall amount of Armadillo^{S10}. (C) Expression of wild-type Armadillo. Armadillo (red) is very unstable and is only stabilised in the presence of Wingless (green; see also Fig. S3 in supplementary material). (D) Expression of wild-type Armadillo with TNotch. The ectopic Armadillo has been eliminated except for a small amount in the neighbourhood of the Wingless-expressing cells (see inset) and this is not associated with a loss of Wingless expression. (E,F) Western blots showing the concentration of endogenous Armadillo and Armadillo^{S10} in the presence or absence of various forms of Notch, expression is under the control of *dpp*Gal4 (F) or C5Gal4 (E). In the presence of Armadillo^{s10} an elevation of endogenous Armadillo levels (E) or an increase in the hypophosphorylated form (lower band of doublet, F) is observed compared to wild type. Expression of TNotch under the control of *dpp*Gal4 results in a marked decrease of both Armadillo and Armadillo^{s10} (due to insufficient separation Armadillo doublet is visualised as one band in E), whereas expression of TNotch under the control of C5Gal4 results in a reduction of hypophosphorylated form of endogenous Armadillo and of Armadillo^{s10} (F). The effects of full length Notch (Notch) are less marked, under the control of *dpp*Gal4 Notch expression results in a decrease of endogenous Armadillo (E); and with C5Gal4 a reduction in Armadillo^{s10} is apparent. (F). Expression of NICD results in small increases in the amounts of Armadillo^{s10} (E,F).

reduced in the presence of TNotch. In contrast, NICD expression results in an increased accumulation of Armadillo^{s10} and no obvious effect on endogenous Armadillo levels (Fig. 3E,F). This is likely to be due to the ectopic expression of Wingless induced by NICD (Diaz-Benjumea and Cohen, 1995), which will lead to an increased stabilisation of Armadillo.

To test further the effects of Notch on Armadillo we over-expressed full length Armadillo together with TNotch. When Armadillo is overexpressed on its own, it accumulates to very high levels in the cytoplasm of the cells (Marygold and Vincent, 2003) in a manner that is strictly dependent on Wingless signalling and other less characterised factors (see Fig. S3 in supplementary material). This accumulation is significantly reduced in the presence of TNotch (Fig. 3C,D).

This data demonstrates that in the imaginal wing disc the activity and levels of both Armadillo and Armadillo^{s10}, a form that mimics oncogenic forms of β-catenin, are the subject of regulation by the Notch receptor.

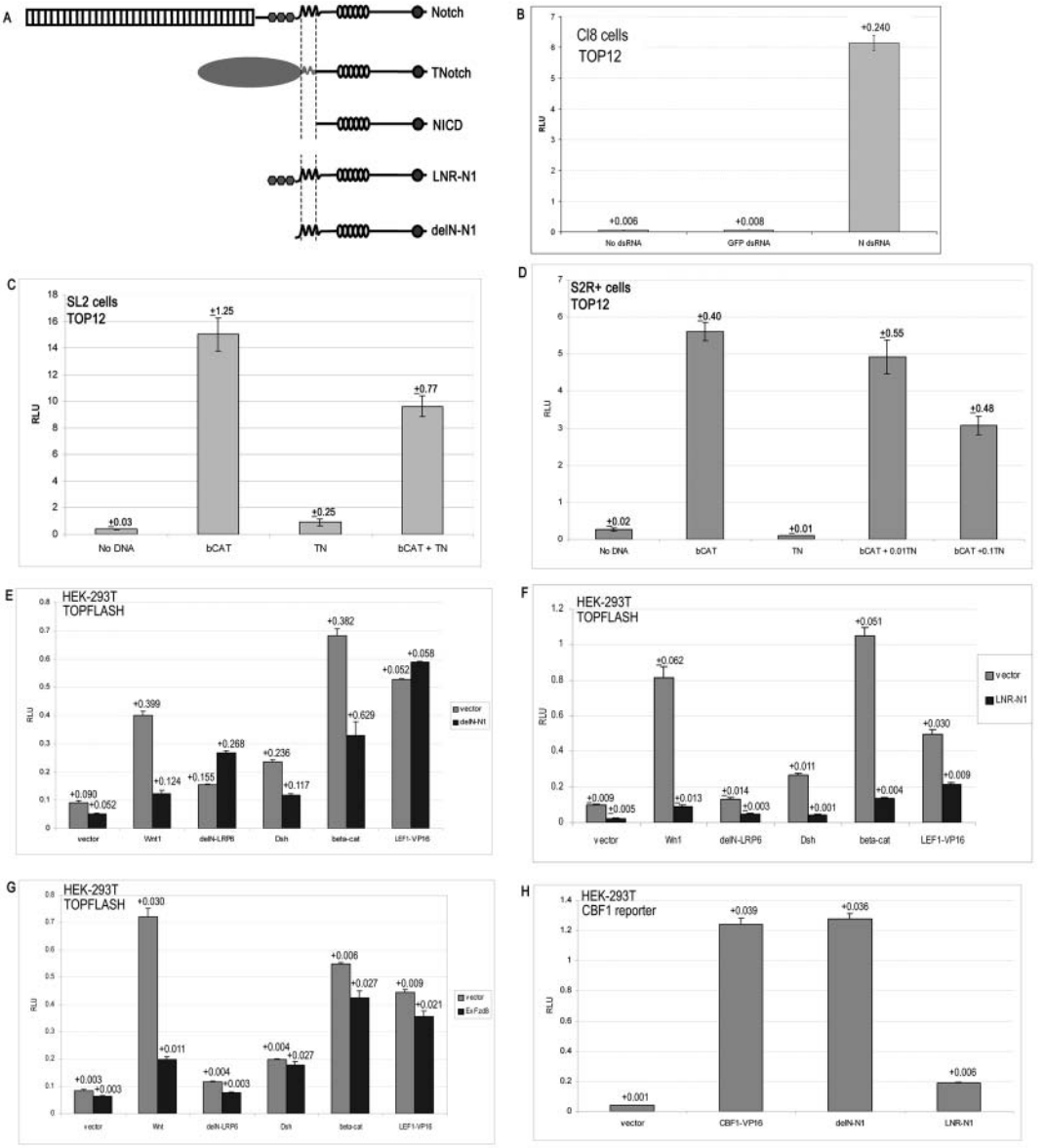

Fig. 4. Notch modulates Wnt pathway transcriptional activity in both *Drosophila* and vertebrate cells. (A) Diagram of the Notch molecules used. (B) Ectopic activation of the Wnt signalling pathway was observed in *Drosophila* clone8 (cl8) cells in the presence of Notch dsRNA (104-fold activation compared to no dsRNA), but not GFP dsRNA (1.1-fold activation). (C,D) In *Drosophila* SL2 (C), or S2R$^+$ cells (D) Wnt signalling was induced with an oncogenic form of β-catenin, S37A β-catenin (Schweizer and Varmus, 2003), the presence of a membrane tethered form of Notch (TNotch) significantly reduced the level of ectopic Wnt signalling (C,D). (E-H) ΔN-N1 (delN-N1) cleaves spontaneously to release the NICD domain of Notch1 as shown by the strong activation of the CBF1 reporter (H), whereas LNR-N1 rarely cleaves as shown by the weak activation of the CBF1 reporter (H) (Mumm et al., 2000). A further inhibitor of Wnt signalling ExFz8 acts by titrating Wnt (Brennan et al., 2004). Ectopic Wnt signalling was induced with Wnt1, delN-LRP6, Dishevelled, activated β-catenin or LEF1-VP16 in HEK-293T cells. Both forms of Notch are capable of significantly repressing ectopic Wnt signalling induced by Wnt1, Dsh, and activated β-catenin (E,F), LNR-N1 effects extended to ectopic Wnt signalling induced by delN-LRP6 and LEF1-VP16. Whereas, ExFz8 repressed ectopic Wnt signalling induced by Wnt1, some small effects on the intracellular mediators of Wnt signalling were observed, such effects have previously been reported (Suzuki et al., 2004) (G).

Notch modulates the transcriptional activity of Armadillo

These results demonstrate a regulatory effect of Notch on the concentration and transcriptional effects of an activated form of Armadillo in vivo. Although our observations suggest a direct effect of Notch on the activity of Armadillo, the complexity of the in vivo regulatory networks could conceivably create situations that would produce the observed effects indirectly. To rule this out, and analyse the interaction further, we studied the effects of Notch loss and gain of function on Wnt signalling in *Drosophila* cells in culture by measuring the effects of Notch on the activity of a Wnt reporter (TOP12).

If gain of function of Notch suppresses Wnt signalling in the wing disc, we asked what would happen to Wnt signalling in the absence of Notch. Earlier experiments in vivo have shown that removal of Notch results in ectopic activity of a Wnt reporter (Lawrence et al., 2001). We tested this in culture using Clone8 cells (cl8), a diploid cell population derived from wing imaginal discs that have been used for a variety of assays of Wnt activity (van Leeuwen et al., 1994). The TOP12 reporter is functional in these cells and is activated by Wnt signalling in a dose-dependent manner (R.G., unpublished data). Strong activation of the reporter is also observed in these cells when Notch signalling is reduced by targeted RNA interference (RNAi) of the *Notch* gene (Fig. 4B). In these experiments, four different dsRNA molecules directed against the coding region of the intracellular domain of Notch resulted in a quantitatively different but qualitatively similar effect (P.H., unpublished data). These results confirm that Notch exerts a negative effect on Wnt signalling.

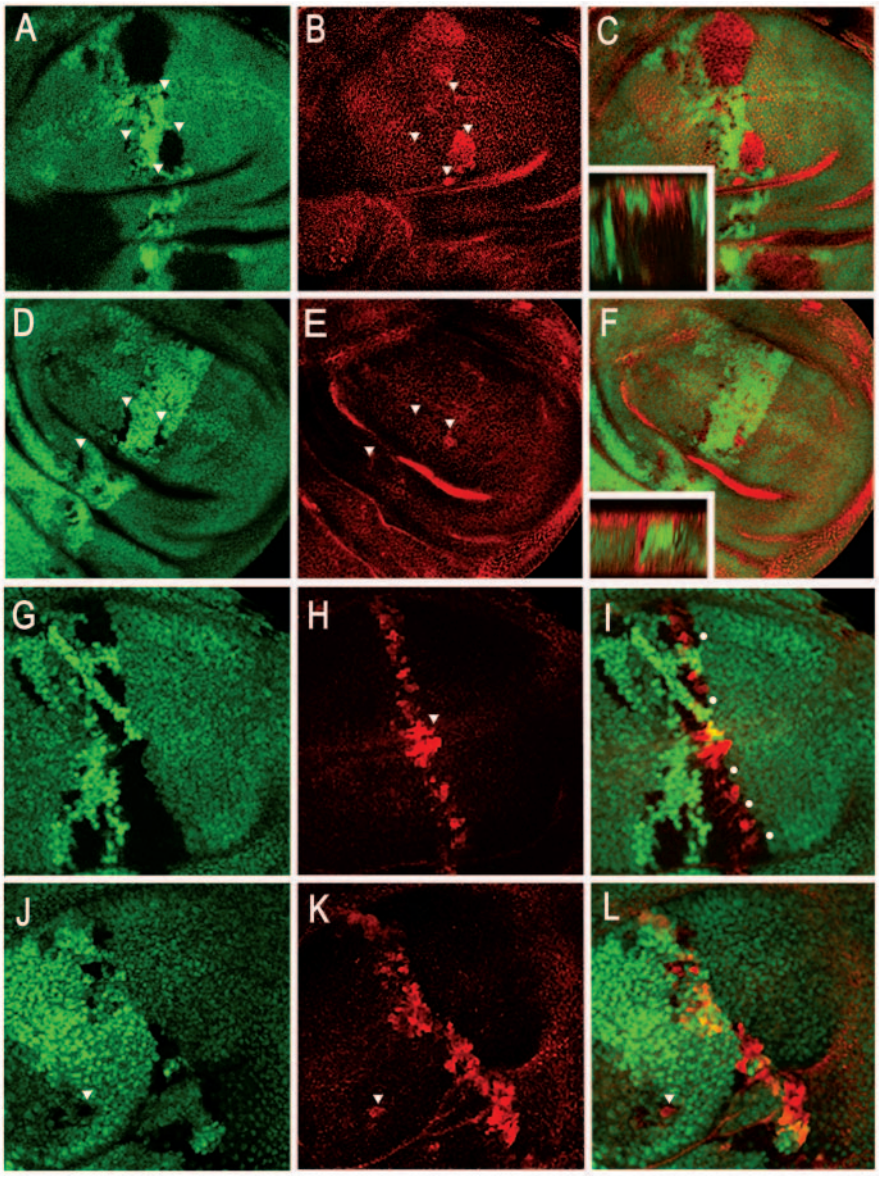

Fig. 5. Effects of loss of function of *Notch* and *shaggy* on the stability of Armadillo. (A,D,G,J) GFP, green; (B,E,H,K) endogenous Armadillo, red; (C,F,I,L) merge of GFP and Armadillo images. (A-C) Loss of function of *shaggy* (loss of GFP) results in a cell autonomous elevation of the levels of Armadillo, which remains largely associated with adherens junctions (inset C, apical is up). Notice that only clones of a certain size (> about five cells) show the elevated levels of Armadillo (arrowheads in A,B); this is probably due to the long perdurance of Shaggy. The epithelium looks very thick (compare to inset F) because the loss of *shaggy* affects the epithelial organisation of the cells (A.M.A., unpublished data). (D-F) Simultaneous loss of *Notch* and *shaggy* results in very elevated levels of Armadillo that appear delocalised within the cytoplasm (inset F). The clones are small. Loss of *Notch* function affects cell proliferation (de Celis and Garcia-Bellido, 1994). (G-I) Expression of wild-type Armadillo under the control of *ptc*Gal4 results in a Wingless-dependent stabilisation of Armadillo (see also Fig. S3 and Fig. S4 in supplementary material) in a narrow band at the AP border and, in particular, at the intersection with the dorsoventral boundary (arrowhead in H) where levels of Wingless are highest. Clones of wild-type cells (loss of GFP) do not change the instability of the ectopic Armadillo (dots indicate regions in clones where Armadillo has not accumulated). (J-L) Wing disc heterozygous for *Notch* ($Df(1)N^{81k}$/+). The *ptc*Gal4-driven expression of Armadillo is broader and contains more cells maintaining higher levels of Armadillo than in wild type. Furthermore, within the *Notch* mutant clones an increased number of cells have high levels of Armadillo (compare with G-I). Notice some clones, that lie far from that AP boundary (arrowhead in J-L) maintain high levels of Armadillo. N.B. In general we do not observe changes in the levels of Armadillo as a result of loss of *Notch* function alone, but in some experiments we observe an elevation in the levels of Armadillo. This elevation is always observed in the neighbourhood of the DV boundary (A.M.A., unpublished data). Unfortunately this effect is not reproducible and therefore should remain anecdotal.

To study the effects of activation of Notch signalling we made use of SL2 cells and S2R+ cells (both derived from the *Drosophila* S2 cell line); the former lack DFz2 (Nagao et al., 1996; Yanagawa et al., 1998). In these cells, transfection of an oncogenic form of vertebrate β-catenin, S37A β-catenin, which signals constitutively, results in a robust and significant activity of the TOP12 reporter (Fig. 4C,D). Co-transfection of TNotch or full length Notch with S37A β-catenin, results in a decrease in the activity of the reporter (Fig. 4C,D and P.H., unpublished data). The reduction in activity is related to the amount of Notch molecules in the assay (Fig. 4D).

These results confirm and extend our observations in vivo and support the notion that the effects of Notch on Wnt signalling are mediated through a direct negative regulation of the activity of Armadillo. To test whether these effects are restricted to *Drosophila* Notch we have tested the ability of mouse Notch1 to modulate Wnt signalling in HEK-293T cells. A previous report has indicated that Notch1 NICD can suppress β-catenin-mediated Wnt signalling in *Notch1* mutant keratinocytes (Nicolas et al., 2003). We have tested the ability of two different forms of membrane tethered Notch1 to modulate Wnt signalling (Fig. 4E,F). One form ΔN–N1, a version of ΔE that removes all but 13 amino acids of the extracellular domain (Mumm et al., 2000) (Fig. 4A), can undergo spontaneous cleavage and activate a CBF reporter (Fig. 4H). This form can also suppress β-catenin activity (Fig. 4E). A second membrane-tethered form LNR-N1 (Fig. 4A), a version of N^{LNR} is rarely cleaved (Mumm et al., 2000) and only activates the CBF reporter very weakly (Fig. 4H), but still strongly suppresses the activity of β-catenin (Fig. 4F).

These observations, together with the observation that Notch cannot inhibit Wnt reporter activity driven by a LEF1-VP16

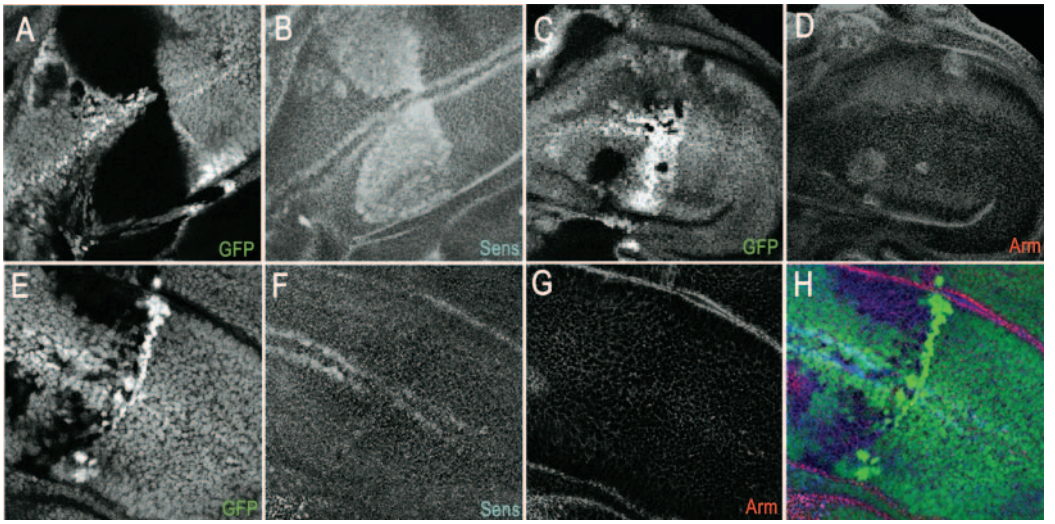

Fig. 6. TNotch can suppress Wingless signalling induced by the loss of function of GSK3β/Shaggy. (A-D) Wild-type wing disc harbouring clones of cells mutant for *shaggy*. (A,B) Loss of *shaggy* function (black in A,C) leads to ectopic expression of the high threshold target of Wingless signalling Senseless (B) and ectopic elevation of Armadillo (D). (E-H) Wing discs expressing TNotch under the control of *dpp*Gal4 and containing clones of cells lacking *shaggy*. TNotch reduces the ectopic expression of Senseless (F) and the elevation of Armadillo (G). H is a merged colour image of E-G. The effect on Senseless is fully penetrant but that on Armadillo can be variable (A.M.A., unpublished data). Anterior is to the left and posterior to the right.

fusion protein confirm and extend the results from *Drosophila* that indicate that Notch has an ability to interfere with the activity of β-catenin. They also support the notion that this effect might not require the cleavage of Notch or its ability to activate transcription. The effects of Notch on the activity of β-catenin contrast with those of a soluble form of Frizzled8 (ExFz8) which, as shown previously are effective in suppressing Wnt-induced Wnt signalling (Brennan et al., 2004) but are not able to suppress β-catenin-induced Wnt signalling (Fig. 4G).

Notch can regulate Armadillo independently of Sgg/GSK3β

The results described above show that Notch modulates the amount and the activity of Armadillo and that this effect is different from that mediated by NICD. To explore these relationships further we have analysed the effects of loss of *Notch* function on the stability of Armadillo.

In the imaginal discs, cells lacking *shaggy* function exhibit elevated levels of Armadillo that is enriched in the neighbourhood of the adherens junctions (Fig. 5A-C). In contrast, loss of *Notch* function does not alter the levels of endogenous Armadillo in a reproducible manner, although some times we have observed an increased accumulation of Armadillo in the neighbourhood of the DV boundary (see legend to Fig. 5). However, simultaneous loss of *shaggy* and *Notch* function results in small clones of cells in which Armadillo is not restricted to the adherens junctions as it is in *shaggy* mutants, but it is now distributed throughout the cytoplasm (Fig. 5D-F).

We were surprised to observe that removal of *Notch* function has no reproducible effects on the levels of Armadillo that can be detected in the presence of Shaggy. We reasoned that perhaps this is due to the fact that the hypophosphorylated form of Armadillo is in very small amounts because of the efficiency of the Armadillo destruction machinery (Tolwinski and Wieschaus, 2001) and therefore, in order to see the effects of *Notch* loss of function, the amounts of soluble Armadillo have to be above a certain level, as in the case of *shaggy* mutant cells. Therefore we saturated the levels of Armadillo by over-expressing high levels of full length Armadillo and observed the effect of loss of *Notch* function on these saturating amounts (Fig. 5G-L). Over-expression of Armadillo leads to its accumulation in a 'salt and pepper' pattern which reveals a requirement for the cell cycle (Marygold and Vincent, 2003) and highlights its dependence on Wingless signalling (Fig. 5G-I and see Fig. S4 in supplementary material). In the absence of Notch, the added Armadillo is consistently stabilised (Fig. 5J-L) and this effect can be shown to be independent of Wingless (see Fig. S4 in supplementary material; A.M.A., unpublished data). This observation mirrors the fact that gain of function of Notch eliminates any excess added Armadillo (Fig. 3C,D) and indicates that Notch can regulate the stability of Armadillo and have effects on the equilibria of the different pools.

The relationships that we have observed between Notch and Armadillo, as well as between Notch and a form of Armadillo that is resistant to Shaggy-mediated degradation, led us to enquire whether Notch could reverse the effects of removal of Shaggy/GSK3β. To do this we expressed TNotch in clones of cells that had lost *shaggy* in the developing wing disc. Loss of *shaggy* generates large clones with cell autonomous high levels of Wnt signalling, as revealed by high levels of Armadillo and ectopic expression of targets of Wingless signalling, e.g. *senseless* (Fig. 6A-D). When TNotch is expressed in cells that have lost *shaggy*, Armadillo is returned to wild-type levels and the transcriptional response is abolished (Fig. 6E-H).

These results support the observation that TNotch can regulate the activity of Armadillo^{s10}, which is resistant to Shaggy-mediated regulation, and indicate that the effects of Notch on Armadillo are independent of, and acting on, the Wingless pathway downstream of Shaggy/GSK3β.

Armadillo associates with Notch in *Drosophila*

Our observations indicate a close functional association between Armadillo and Notch. One possibility is that the effects of Notch are indirect and are mediated by some proteins associated with a Su(H)-independent activity. Although this may be the case, it is also possible that Armadillo is part of

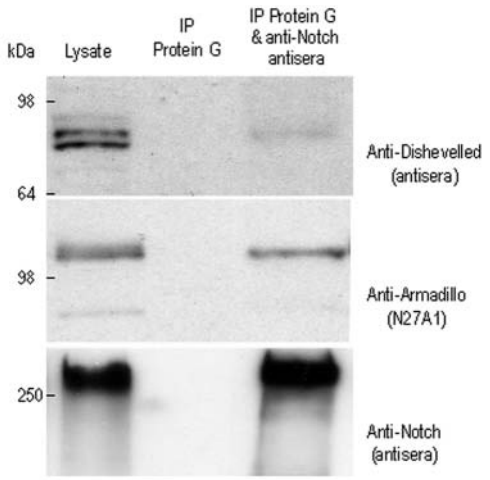

Fig. 7. Armadillo associates with Notch in *Drosophila* embryos. Notch protein was immunoprecipitated from wild-type embryos and the presence of associated proteins was assessed by western blot. The majority of Notch protein present in embryo lysate and immunoprecipitated was the full-length protein (>250 kDa) which, in *Drosophila* is the predominant form of Notch at the cell surface (Kidd and Lieber, 2002). Detected in association with immunoprecipitated Notch were both Armadillo (middle panel) and Dishevelled (upper panel) protein. The lane labelled 'lysate' represents a fifth of the total protein added to the immunoprecipitation reaction. Immunoprecipitated Notch reflects about 5-10% of the total Notch and associated with this is 0.5-3% of the total Armadillo and less than 0.5% of the total Dishevelled. Given that the pool of Armadillo associated with Notch is not associated with E-cadherin it is not surprising that this Notch-associated fraction of Armadillo is only a small proportion of the total cellular Armadillo.

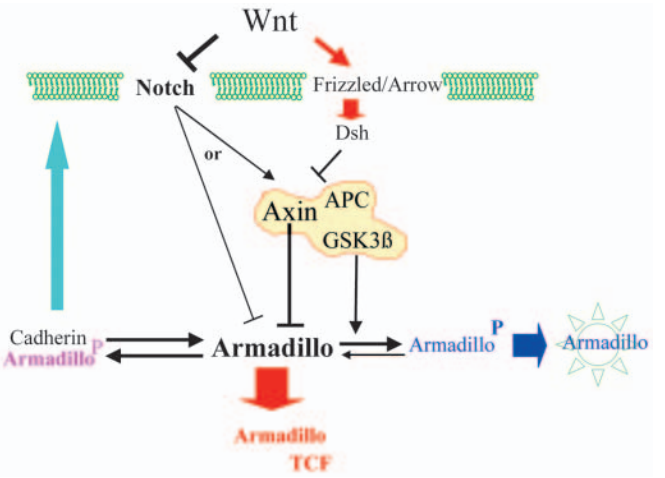

Fig. 8. Modulation of Wnt signalling by Notch in *Drosophila* (see text for details of interactions). In the steady state, Armadillo exists in a number of molecularly distinct pools which appear to be in equilibrium. Armadillo associates readily with cadherin. Also it, associates with a complex, which includes Axin and APC, leading to its phosphorylation on the N terminus by GSK3β (Shaggy) and the delivery of the phosphorylated form to the proteasome, where it is degraded. In addition, Axin can prevent the formation of its active complex with TCF in a GSK3β-independent manner. Our results indicate that Notch modulates the activity and amounts of hypophosphorylated Armadillo either by targeting the GSK3β-independent activity of Axin or via an independent mechanism. The net effect of the inactivation of Notch and the Axin-based complex results in an efficient accumulation of Armadillo in the nucleus and its interaction with TCF.

this complex. This possibility is suggested by the observation that Armadillo and Notch show a high degree of co-localisation at the adherens junction of the epidermal cells of the wing disc (Fehon et al., 1991; Lamb et al., 1998). To test whether Notch and Armadillo are associated in the cell, we immunoprecipitated Notch from developing embryos and searched for Armadillo amongst the co-immunoprecipitated proteins. Two different anti-Notch antibodies were used and in both cases Armadillo protein was detected in the same protein complex as the immunoprecipitated Notch protein (Fig. 7). Interestingly, the predominant form of Notch protein detected in these assays is unprocessed and uncleaved (Kidd and Lieber, 2002), suggesting that this complex is membrane associated. The reverse experiment, in which Armadillo protein is immunoprecipitated, was also undertaken; here an unprocessed and uncleaved form of Notch was found to be associated with Armadillo (P.H., unpublished data). Previous experiments have indicated that Dishevelled, another element of Wnt signalling, can associate with Notch in a yeast two-hybrid assay. We have confirmed this and further shown this association in the same immunoprecipitates from embryos in which we find the complex between Notch and Armadillo (Fig. 7). Other proteins such as E-cadherin and nuclear lamin were not detected in the immunoprecipitates (see Fig. S5 in supplementary material).

These results indicate that the intracellular domain of Notch and a proportion of the Armadillo protein of the cell are associated in the same protein complex. Preliminary data suggests that this association is preferentially mediated by the region C-terminal to the cdc10/ANK repeats (P.S., unpublished data) and such an interaction might be an element in the functional interactions that we have described above.

Discussion

Wnt signalling plays crucial and diverse roles in normal and pathological situations and therefore it is not surprising that the activity of its key effector, Armadillo/β-catenin is tightly regulated (Giles et al., 2003; Polakis, 2000; Wodarz and Nusse, 1998). The precise mechanism whereby Wnt proteins elicit the activity of β-catenin is still under scrutiny but it is generally agreed that the stability and amount of cytoplasmic Armadillo/β-catenin are rate-limiting steps in the signalling event (Gottardi and Gumbiner, 2001; Lee et al., 2003). This pool of Armadillo/β-catenin is under very tight control by a destruction complex assembled on Axin, which together with Shaggy/GSK3β are the main targets of Wnt signalling (Wodarz and Nusse, 1998). However, there is increasing evidence that high levels of cytoplasmic Armadillo/β-catenin are not sufficient to promote Wnt signalling (Brennan et al., 2004; Guger and Gumbiner, 2000; Lawrence et al., 2001; Staal et al., 2002). Recently emphasis has been placed on the observation that Axin can regulate the activity of Armadillo/β-catenin in a Shaggy/GSK3β-independent manner (Tolwinski et al., 2003; Tolwinski and Wieschaus, 2004b). This has led to the

conclusion that Wnt regulates the activities of Shaggy/GSK3β and Axin co-ordinately and that there might be other factors contributing to the control of Armadillo/β-catenin activity. Consistent with this possibility it has been reported that Wnt signalling can regulate the activity of stable oncogenic forms of β-catenin (Suzuki et al., 2004).

Here we have shown that Notch signalling provides an important input into Wnt signalling in *Drosophila* by associating with Armadillo and regulating its levels and activity during Wingless signalling (Fig. 8). This activity of Notch, which is different and probably independent of that which mediates CBF1/Su(H)-dependent signalling, lies functionally downstream of Shaggy/GSK3β and targets the concentration and activity of the hypophosphorylated form of Armadillo. It can also modulate the activity of an oncogenic form of vertebrate β-catenin and we have demonstrated that this functional interaction between Notch and Armadillo extends to the vertebrate system, with mNotch1 regulating the activity of β-catenin in tissue culture cells.

A role for Notch in the modulation of Wnt signalling has been inferred from genetic analysis. However, although these results indicate that Notch antagonises Wnt signalling, alone they do not provide insights into the mechanism of the interaction. Our work does, and it is likely that the molecular interactions that we report underpin the observed modulation of Wnt signalling by Notch (Martinez Arias, 2002). Wingless signalling can be activated in vivo in the absence of Notch and this activation does not require Dishevelled (Brennan et al., 1999a; Lawrence et al., 2001). Our observations that removal of Notch in cl8 cells leads to activation of a synthetic Wnt reporter confirm this and suggest a direct regulatory effect of Notch on the mechanism of Wnt signalling. Furthermore, the effects of Notch on the activated form of Armadillo offer an explanation for why removal of Notch can bypass a requirement for Dishevelled. It may well be that even under steady state conditions there is a small amount of hypophosphorylated, active Armadillo/β-catenin which escapes the Axin/GSK3β mediated degradation. Given the high specific activity of this molecule (Lee et al., 2001), it is not surprising that there might be further mechanisms that control it. Notch appears to be an essential part of these mechanisms and in its absence this active form of Armadillo would operate even in the absence of Dishevelled. Axin is also likely to be involved in the regulation of the active form (Tolwinski et al., 2003) and we have observed that Axin can also suppress the effects of an activated form of Armadillo (A.M.A., unpublished data). It will be of interest to explore the relationships between Notch and Axin.

Previous studies have implicated Deltex and Dishevelled as important elements of the interaction between Notch and Wingless signalling (Axelrod et al., 1996; Martinez Arias et al., 2002; Ramain et al., 2001). Both proteins bind Notch, but they do so in different places. Deltex binds to the cdc10/ANK repeats (Matsuno et al., 1995) and promotes Su(H)-independent Notch signalling. Whereas, Dishevelled binds within a broad region C-terminal to this domain and reduces the Su(H)-independent activity of Notch (Axelrod et al., 1996; Ramain et al., 2001; Zecchini et al., 1999). Here we have shown that Armadillo also interacts with Notch, probably through the same broad region that binds Dishevelled. Mutations in Notch that impair this domain result in Notch receptors that interfere with Wnt signalling (Ramain et al., 2001) and we have observed that its deletion reduces the efficiency with which the intracellular domain of Notch affects the levels and activity of Armadillo (P.H., unpublished data). Together these observations underscore the role of this region of Notch in mediating interactions between Notch and Wnt signalling by targeting the active form of Armadillo/β-catenin.

The relationship between Notch and Armadillo in *Drosophila* extends to their vertebrate homologues, Notch1 and β-catenin. This interaction, rather than an interaction of Dishevelled with Notch/CBF signalling, might reflect the functional relationships between the two signalling systems that have been reported during the development of the skin (Lowell et al., 2000; Nicolas et al., 2003; Zhu and Watt, 1999), the immune system (Radtke et al., 1999; Reya et al., 2000) and in somitogenesis (Aulehla et al., 2003; Dale et al., 2003; Pourquie, 2003). In these instances Wnt and Notch drive alternative fates (skin and immune system) or act antagonistically (somites) perhaps by a combination of their individual pathways and the modulatory interaction that we have described here. One consequence of this modulatory interaction might also be the observed tumour suppressor function of Notch1 in the mouse skin where removal of *Notch1* results in the generation of tumours associated with an increase in the levels of active β-catenin and Wnt signalling (Nicolas et al., 2003). Whilst some of the elevation of β-catenin in these cells might be a secondary consequence of activation of Wnt signalling, our observations suggests that the loss of *Notch1* can also contribute to this increase by allowing the activation of β-catenin. In a different study carboxyl-terminal deletions in *Notch1*, which include the region that binds Dishevelled and Armadillo, enhanced the oncogenic effects of a chimeric E2A-PBX1 protein (Feldman et al., 2000). It is possible that some of this effect is due to misregulation of β-catenin in the tumours.

In summary, we have shown that Notch provides a modulatory input in the activity of Armadillo/β-catenin (Fig. 8). This modulation provides two functions: it establishes a threshold for Wnt signalling that is likely to play an important role in the patterning of tissues and the assignation of cell fates during development (Martinez Arias, 2002) and, in addition it provides a stringent regulation of the activated form of Armadillo/β-catenin. The second function might be crucial in pathological situations and might contribute to the understanding of Notch as a tumour suppressor (Radtke and Raj, 2003).

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/8/1819/DC1

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