Modulation of the ligand-independent traffic of Notch by Axin and Apc contributes to the activation of Armadillo in *Drosophila*

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SUMMARY

There is increasing evidence for close functional interactions between Wnt and Notch signalling. In many instances, these are mediated by convergence of the signalling events on common transcriptional targets, but there are other instances that cannot be accounted for in this manner. Studies in *Drosophila* have revealed that an activated form of Armadillo, the effector of Wnt signalling, interacts with, and is modulated by, the Notch receptor. Specifically, the ligand-independent traffic of Notch serves to set up a threshold for the amount of this form of Armadillo and therefore for Wnt signalling. In the current model of Wnt signalling, a complex assembled around Axin and Apc allows GSK3 (Shaggy) to phosphorylate Armadillo and target it for degradation. However, genetic experiments suggest that the loss of function of any of these three elements does not have the same effect as elevating the activity of β-catenin. Here, we show that Axin and Apc, but not GSK3, modulate the ligand-independent traffic of Notch. This finding helps to explain unexpected differences in the phenotypes obtained by different ways of activating Armadillo function and provides further support for the notion that Wnt and Notch signalling form a single functional module.

KEY WORDS: Apc, Axin, Ligand independent, Notch, Traffic, Armadillo/β-catenin, *Drosophila*

INTRODUCTION

Wnt signalling is a molecular device that modulates cell identity and behaviour during development and homeostasis (Clevers, 2006; Logan and Nusse, 2004; Reya and Clevers, 2005). The effects on cell identity rely on the regulation of the activity of β-catenin [Armadillo (Arm) in *Drosophila*], a modular protein that exists in two major pools: one associated with the adherens junctions and a second that is distributed between the cytoplasm and the nucleus and is involved in Wnt signalling (Daugherty and Gottardi, 2007). According to current views, in the absence of Wnt signalling, a cytoplasmic pool of β-catenin is recruited to a complex assembled around the scaffolding proteins Axin and Apc (Behrens et al., 1998; Fagotto et al., 1999; Hart et al., 1998; Kishida et al., 1999), where it is phosphorylated by Glycogen synthase 3 [GSK3; Shaggy (Sgg) in *Drosophila*] (Ikeda et al., 1998) and targeted for degradation via the proteasome (Aberle et al., 1997; Jiang and Struhl, 1998; Marikawa and Elinson, 1998; Orford et al., 1997). Wnt signalling promotes the disassembly of the degradation complex, leading to a rise in the soluble levels of dephosphorylated β-catenin, which enters the nucleus and promotes transcription (Daugherty and Gottardi, 2007; Logan and Nusse, 2004; Reya and Clevers, 2005). There is a good correlation between Wnt signalling and rises in the concentration of soluble β-catenin (Funayama et al., 1995; Korinek et al., 1997; Pai et al., 1997), but this is not the only factor that determines its activity (Brennan et al., 2004; Guger and Gumbiner, 2000; Hendriksen et al., 2008; Lawrence et al., 2001; Staal et al., 2002; Tolwinski et al., 2003). In particular, there is no simple correlation between rises in

the concentration of β-catenin and its transcriptional activity (Brennan et al., 2004; Guger and Gumbiner, 2000; Staal et al., 2002). Furthermore, genetic analysis has shown that Axin has a second function in controlling the activity of β-catenin that is independent of its role as a scaffold for GSK3 (Tolwinski, 2009; Tolwinski et al., 2003; Tolwinski and Wieschaus, 2001; Tolwinski and Wieschaus, 2004). These observations suggest that the activity of β-catenin is regulated not only through changes to its cytoplasmic concentration, but also through its cellular location and further protein modification (Hendriksen et al., 2008; Maher et al., 2010).

We have shown that, in *Drosophila*, one of these additional controls on the activity of β-catenin relies on Notch (Hayward et al., 2005; Lawrence et al., 2001; Sanders et al., 2009), a single-transmembrane receptor that acts as a membrane-tethered transcription factor in a ligand-dependent manner (Artavanis-Tsakonas et al., 1999; Hartenstein et al., 1992; Hayward et al., 2008; Schweisguth, 2004). In the absence of Notch, activated Arm promotes growth and alterations in cell-cell contact (Sanders et al., 2009). The effect of Notch on β-catenin is mediated by the ligand-independent traffic of the receptor (Hayward et al., 2005; Sanders et al., 2009). Furthermore, interactions between the two pathways are underscored by functional interactions between Axin and Notch (Hayward et al., 2006) and, significantly, by the observation that Dishevelled (Dsh), a major effector of Wnt signalling, interacts with Notch (Axelrod et al., 1996; Carmena et al., 2006; Muñoz-Descalzo et al., 2010; Ramain et al., 2001) and promotes its ligand-independent traffic (Muñoz-Descalzo et al., 2010).

Here, we have tested further the relationship between Notch and the Axin-based destruction complex. Our results show that whereas Axin and Apc are involved in the traffic of Notch, GSK3 is not, and provide further evidence to support the proposal that Notch and some components of Wnt signalling form a single functional device.

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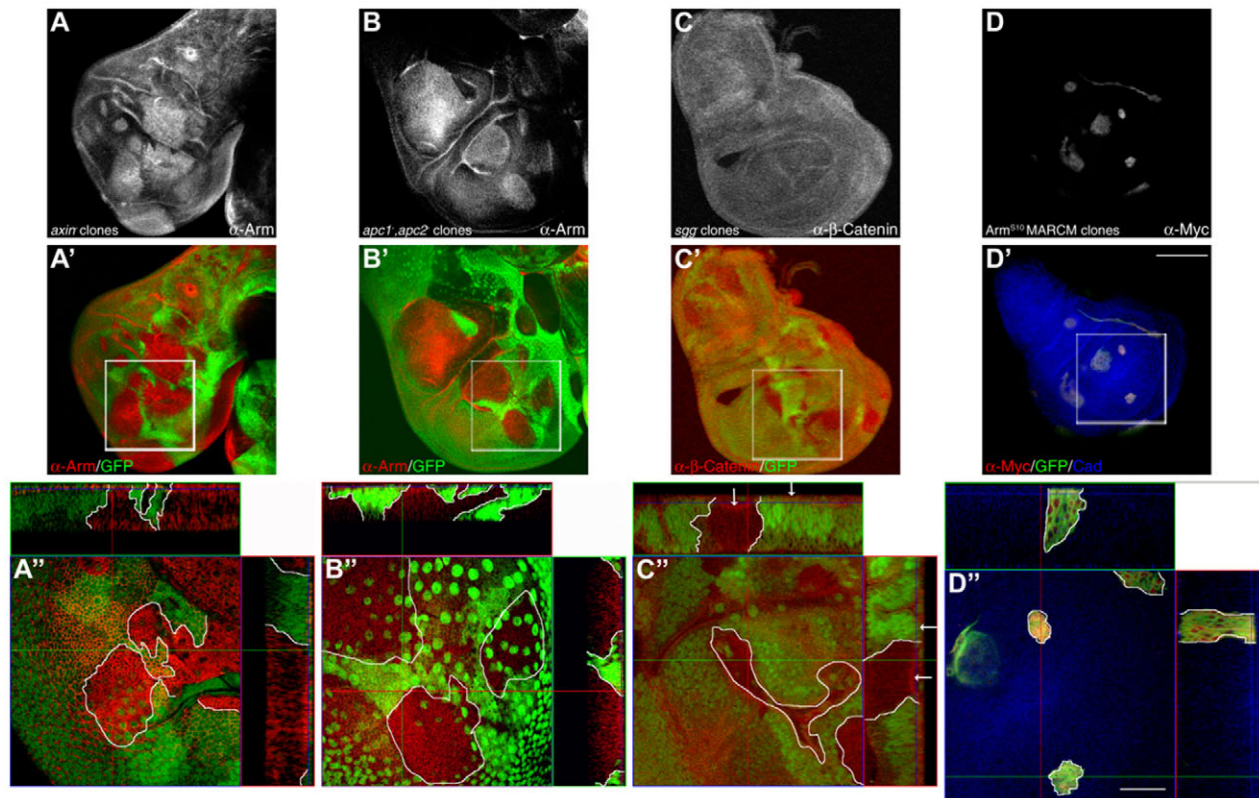
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Fig. 1. The absence of Axin, Apc or Sgg, but not the expression of Arm^{S10}, causes overgrowths in *Drosophila* wing imaginal discs.
(**A-D″**) Confocal images of third instar wing imaginal discs containing clones of cells lacking Axin (A-A″), Apc1 and Apc2 (B-B″) or Sgg (C-C″) (labelled by the absence of GFP, green in A′-C″), induced by *ptc*>FLP, and MARCM clones expressing Arm^{S10}, an activated form of Arm (D-D″; tagged with a Myc epitope and labelled by the presence of GFP, green in D′,D″). The red channel shows staining for Arm (A′,A″,B′,B″), total β-catenin (C′,C″) or Myc (D′,D″), whereas the blue channel (D′,D″) shows E-cadherin to outline the shape of the disc. A″-D″ show higher magnifications of the boxed regions in A′-D′, in which the pictures on the top and the right represent optical *z*-sections through the clones following the green and red lines, respectively, shown in the main image. Mutant clones are outlined (white lines). Note that the absence of Sgg causes invagination of the tissue (C″, arrows); this effect, which creates unevenness, is not observed in the other genetic backgrounds. Scale bars: 100 μm in A-D,A′-D′; 50 μm in A″-D″.

MATERIALS AND METHODS
Genetics
To generate the *Axin* and *Apc1,Apc2* clones, males (*w;;Axin^n*,FRT82B/TM3,Sb or *w;;*FRT82B,*Apc1^{Q8},Apc2^{d40}*/TM6B) were crossed to *f*,FLP122;*ptc*-Gal4,UAS-FLP;FRT82B,GFP/SM6a-TM6B females and clones identified by the loss of GFP. To generate *shaggy* mutant clones, *sgg^{m11},w,sn^3*,FRT14AB/FM6,*f* females were crossed to *y,w,*GFPx1,FRT14AB;*ptc*-Gal4;UAS-FLP,A101-lacZ/SM6a-TM6B males and, in the female offspring, the clones were recognised by the loss of GFP.

To generate clones of cells expressing Arm^{S10} using the MARCM system, FRT19A;UAS-Arm^{S10}/+ males were crossed to *w,*tubP-Gal80,FLP1,FRT19A;CyO/UAS-nucZ,UAS-CD8:GFP;*tubP*-Gal4/TM6B females. Clones were induced in larvae 48-72 hours after egg laying by applying a 1-hour heat shock at 37°C. In the female offspring, the clones were recognised by the presence of GFP and anti-Myc staining.

Clones of *Axin* mutant cells expressing CeN were generated by crossing *w;*UAS-CEN1a/CyO;*Axin^n*,FRT82B/TM6B males with *f*,FLP122;*ptc*-Gal4,UAS-FLP;FRT82B,GFP/SM6a-TM6B females. Discs containing clones and expressing CeN were recognised by the loss of cytoplasmic GFP and the presence of eGFP from the CeN molecule along the anteroposterior boundary. In the case of clones of *Axin* mutant cells expressing Notch^{RNAi} (Presente et al., 2002), Notch^{RNAi}/+;;*Axin^n*,FRT82B/+ females were crossed to *f*,FLP122;*ptc*-Gal4,UAS-FLP;FRT82B,GFP/SM6a-TM6B males. Discs with clones of Axin and a reduction in Notch levels were recognised by the absence of GFP and loss of Notch staining. The expression of Notch^{RNAi} using *ptc*-Gal4 reduces dramatically, but does not abolish, the expression of Notch protein; in particular, it eliminates the Notch located in vesicles and reduces its levels in the apical domain of the cell.

Details of the CeN chimera have been described previously (Hayward et al., 2006; Sanders et al., 2009). For the Axin overexpression experiments, w;UAS Axin (A1),myc/CyO males were crossed to w;*dpp*-Gal4 females.

Immunohistochemistry
The antibodies used in this study were mouse monoclonal antibody against the extracellular domain of Notch (C458.2H, DSHB); anti-NIC (Notch intracellular domain; sheep antisera generated in our lab); rat monoclonal against E-cadherin [Dcad2 (Shotgun), DSHB]; anti-Arm (N27A1, DSHB); anti-total β-catenin (C2206, Sigma; 1:500); and anti-Myc (ab9106, AbCam). Alexa-conjugated secondary antibodies were from Molecular probes. Fixed tissue stainings and pulse-chase experiments were performed as described (Sanders et al., 2009).

Image acquisition and preparation
Wing discs were examined under a Nikon Eclipse E800 microscope coupled to a Bio-Rad MRC1024 or Zeiss LSM 510-Meta confocal unit. The images of the pulse-chased wing discs at the different time points were acquired under the same conditions of laser, gain and iris and processed in exactly the same way using Photoshop (Adobe). The fluorescence intensity profiles were produced with ImageJ (NIH; using the Plot Profile option and RGB Profiler) and processed with Excel (Microsoft).
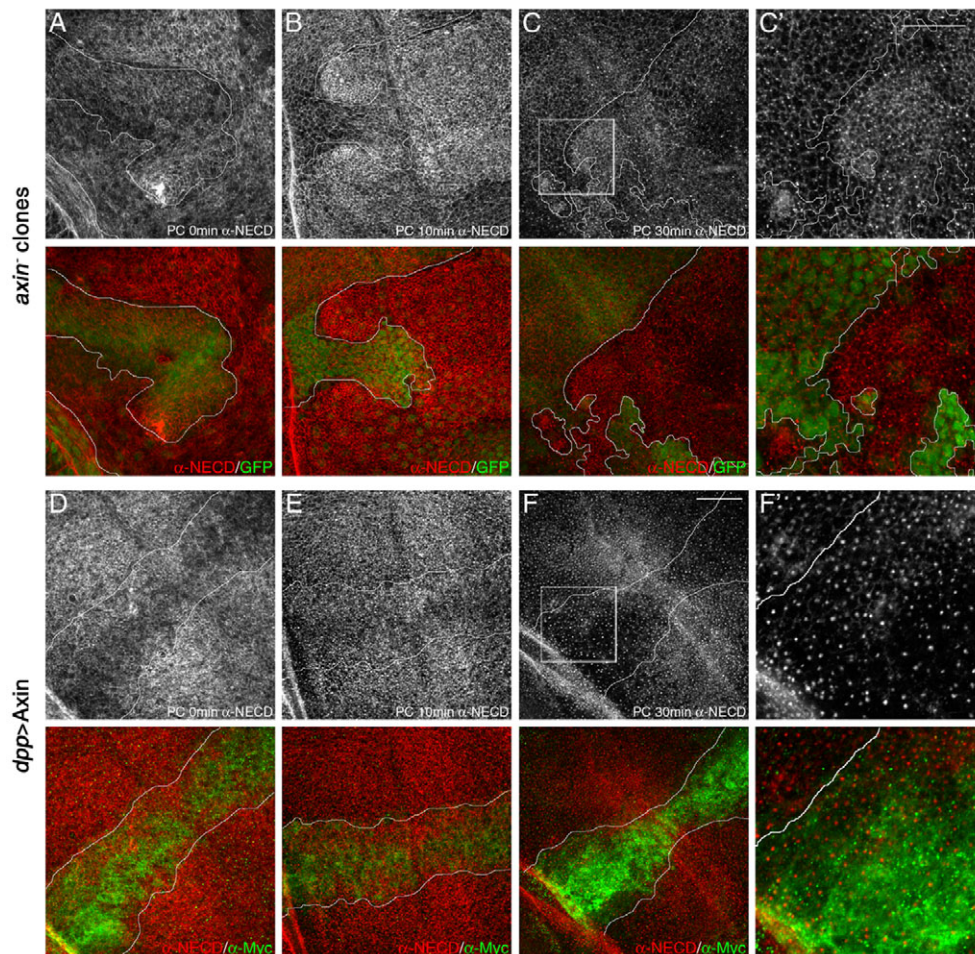
Fig. 2. Axin affects the dynamics of Notch endocytosis and traffic. (**A-F′**) Tracking of Notch in *Drosophila* wing discs with clones of *Axin* mutant cells (A-C) and overexpressing Axin, carrying a Myc tag, under the control of *dpp*-Gal4 (D-F) by pulsing with an antibody against the extracellular domain of Notch (red in merge) and chasing for 0 (A,D), 10 (B,E) and 30 (C,F) minutes. C′,F′ are higher magnifications of the boxed regions in C,F. The white lines indicate the localisation of *Axin* mutant clones (labelled by the absence of GFP, green channel in the lower panels) in A-F, and the domain of Axin overexpression (labelled by the anti-Myc staining, green channel in the lower panels) in D-F. In all cases, the maximum projection of an apical *z*-stack of confocal sections is shown. Proximal is to the left and posterior to the top. See Fig. S2 in the supplementary material for a quantification of the effect of the overexpression of Axin on Notch. Scale bars: 50 μm in F for A-F; 20 μm in C′ for C′,F′.

RESULTS AND DISCUSSION

Cells expressing Arm^{S10}, a form of Arm that is insensitive to phosphorylation by GSK3, do not overgrow and remain integrated in the epithelium (Sanders et al., 2009; Somorjai and Martinez-Arias, 2008). Clones of cells mutant for Axin, a central element of the Arm destruction complex, exhibit very high levels of Arm, some of which can be found in the nucleus (our unpublished observations), and exhibit overgrowths and round edges suggestive of defects in cellular recognition (Hayward et al., 2006) (Fig. 1A). These phenotypes are related to, but distinct from, those caused by expression of Arm^{S10} (Fig. 1D) and support the contention that Axin exerts controls on the activity of Arm that are additional to those mediated through its role as a scaffold for GSK3 (Tolwinski et al., 2003). The effects of Axin loss of function are reminiscent of those caused by expression of Arm^{S10} in cells with compromised Notch function (Sanders et al., 2009). As these effects are caused by the loss of the ligand-independent traffic of Notch, we tested whether Axin exerts some effect on the traffic of Notch.

Clones of cells mutant for *Axin* did not show alterations in ligand-dependent Notch signalling (see Fig. S1 in the supplementary material), although they exhibited a mild but reproducible increase in Notch protein on the apical side, and overexpression of Axin reduced the amount of Notch present at the cell surface (not shown). These observations suggest that Axin regulates the amount of Notch at the cell surface. To test whether this control is exerted by targeting the endocytosis and traffic of Notch, we performed label and chase experiments with Notch (see Materials and Methods). Under our experimental conditions and

focusing the analysis in the pouch of the wing imaginal disc, labelled Notch disappeared from the cell surface within 10 minutes of the chase and could be found in punctate intracellular structures, presumably vesicles associated with endocytic traffic (Muñoz-Descalzo et al., 2010; Sanders et al., 2009). Performing the same assay in the absence of Axin revealed that the endocytosis and traffic of Notch is impaired in *Axin* mutant cells, and after 30 minutes we could still detect a substantial amount of Notch on the cell surface (Fig. 2A-C). This suggests that Axin is involved in, or can influence, the traffic of Notch. Performing the same experiment in discs overexpressing Axin, we observed a decrease in the amount of Notch over time (Fig. 2D-F and see Fig. S2 in the supplementary material). Altogether, these results suggest that Axin contributes to the removal of Notch from the cell surface and to targeting it for degradation.

Regulation of the activity of Arm by Notch is mediated by its ligand-independent traffic as shown by the activity of chimeric receptors in which the extracellular domain of Notch has been substituted by the extracellular domain of CD8 (CeN) or Torso (TN; Tor – FlyBase) (Hayward et al., 2006; Hayward et al., 2005; Sanders et al., 2009). Since Wingless signalling promotes the traffic and degradation of these receptors (Muñoz-Descalzo et al., 2010) and cells lacking Axin have elevated levels of Wnt signalling, we examined what would happen to the stability of CeN in this situation. Surprisingly, the levels of CeN remained largely unchanged in clones of cells mutant for *Axin*, suggesting that in the absence of Axin, despite high levels of Wnt signalling, CeN cannot be degraded (Fig. 3A). This could be
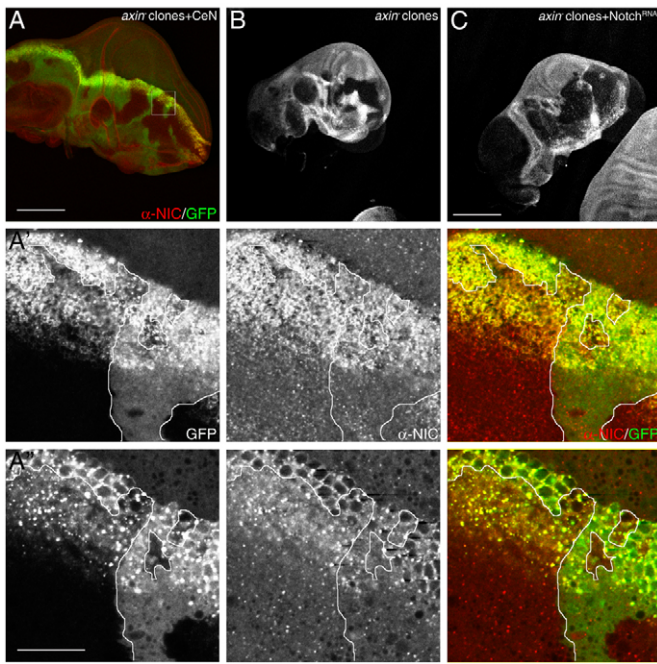
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Fig. 3. Functional and structural interactions between Axin and Notch. (**A-A″**) Confocal images of *Drosophila* third instar wing imaginal discs expressing CeN and containing clones of *Axin* mutant cells. CeN is a chimera in which the intracellular domain of Notch, tagged with eGFP, is tethered to the membrane with CD8 (see text and Materials and Methods for details). A′ and A″ are higher magnifications of the boxed region in A: A′ is an apical and A″ a basal image. There is a reduction in apically located CeN but not in the overall amount or appearance of the vesicles in the basal image. The clones are highlighted by the loss of cytoplasmic eGFP. The punctate eGFP pattern represents CeN and the discs are stained with an antibody against the intracellular domain of Notch (NIC; red); merge is yellow. (**B,C**) Confocal images of third instar wing imaginal discs containing *Axin* clones (B) or *Axin* clones expressing Notch^RNAi (C) labelled by the absence of GFP (black areas). Note the expansion in the size of the *Axin* mutant clones when the amount of Notch protein is reduced by expression of Notch^RNAi. Scale bars: 100 μm in A; 200 μm in C for B,C; 25 μm in A″ for A′,A″.

because Axin is required for the degradation of CeN or because this degradation is dependent on Wnt and Dsh but not on Axin. A contribution of Axin is favoured by the observations that overexpression of Axin reduces, and Axin loss of function increases, Notch levels.

A functional relationship between Axin and Notch is also highlighted by the observation that, in tissue culture, simultaneous reductions of Notch and Axin induce very high levels of Arm activity (Hayward et al., 2006). However, in vivo, simultaneous loss of both Notch and Axin leads to a suppression of the growth
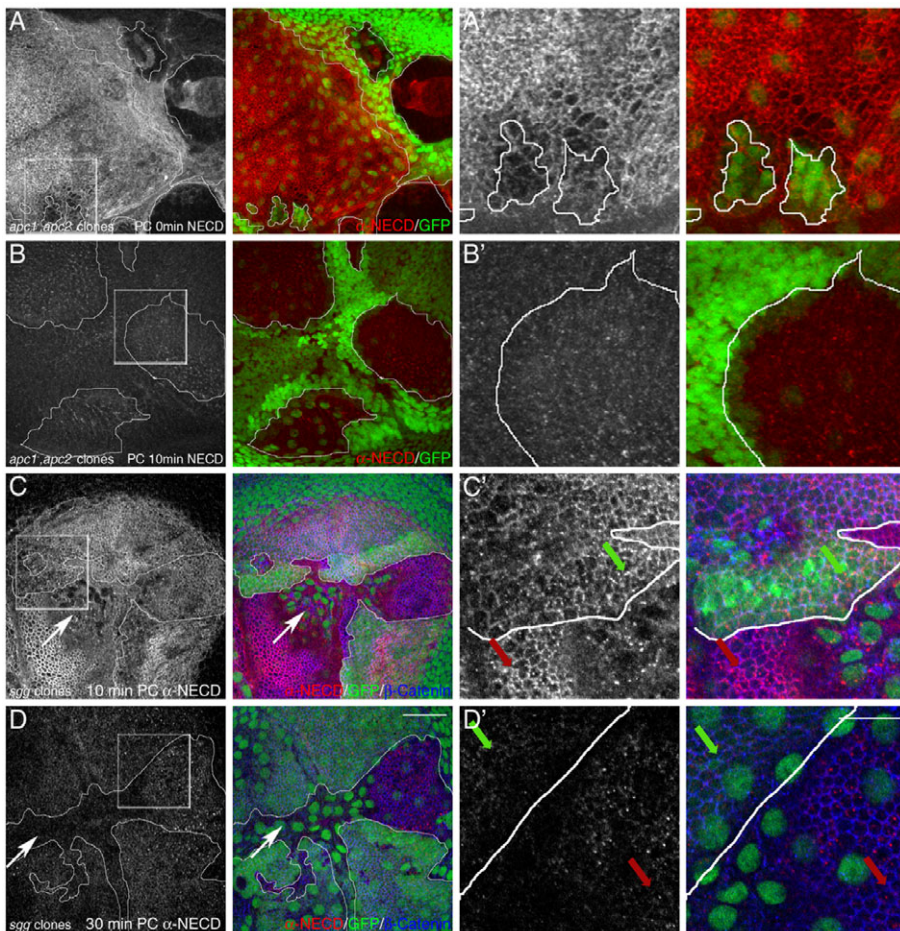

Fig. 4. Reduction of Apc1 and Apc2 increases the levels of Notch, whereas the reduction of Sgg does not. (**A-D′**) Tracking of Notch in *Drosophila* wing discs with clones of *Apc1* and *Apc2* (A-B′) or *sgg* (C-D′) mutant cells by pulsing with an antibody against the extracellular domain of Notch (red in merges) and chasing for 0 (A), 10 (B,C) or 30 (D) minutes. A′-D′ are higher magnifications of the boxed regions in A-D. The green and red arrows (C′,D′) point to wild-type and mutant regions of the discs, respectively. Mutant clones are outlined (white lines), labelled by the absence of GFP (green in the merges). Within the *Apc1,Apc2* clones, the traffic of Notch is impaired as indicated by the presence of higher levels of Notch (particularly in B), but this is not the case in the *sgg* mutant clones (in some instances, there is the appearance of a difference in Notch protein but this is an optical effect associated with the unevenness of the tissue that characterises these *sgg* mutant clones). Note that the removal of *sgg* causes changes in the organisation of the epithelium that result in cells being in different planes (white arrows and see Fig. 1C). The blue channel in the merge shows total β-catenin, used to label the adherens junctions. In all cases, the maximum projection of an apical z-stack of confocal sections is shown. Proximal is to the left and posterior to the top. Scale bars: 50 μm in D for A-D; 20 μm in D′ for A′-D′.

induced by the loss of Axin alone, a phenotype that is associated with extensive cell death (Hayward et al., 2006) and perhaps reflects a synergy of the roles of each protein in apoptosis (Liu et al., 2007; Neo et al., 2000; Quillard et al., 2009). For this reason, to test the synergy between the two proteins in determining Arm activity in vivo, we expressed a Notch[RNAi] construct that reduces, but does not abolish, Notch function (Presente et al., 2002) in clones of cells mutant for *Axin* (Fig. 3B,C). Under these conditions, there is no apoptosis (data not shown) and we observed larger outgrowths than those promoted by the loss of Axin alone. These phenotypes indicate a synergistic effect of the mutations and suggest that Axin is involved in the modulation of Notch while it traffics through the cell.

Apc, a second element of the Arm destruction complex, is encoded in *Drosophila* by *Apc1* (*Apc* – FlyBase) and *Apc2*, which play redundant roles in the regulation of Wnt signalling (Ahmed et al., 2002; Akong et al., 2002a; Akong et al., 2002b). In order to test whether Apc is also involved in the traffic of Notch, we generated clones of cells mutant for *Apc1* and *Apc2* in wing imaginal discs and assessed the traffic of Notch. In these clones, cells exhibited very similar phenotypes to those of *Axin* mutants in terms of growth, overall shape and levels of Arm (Fig. 1B). In addition, they exhibited altered traffic of Notch (Fig. 4A,B). However, instead of being clearly localised in vesicles or in the cell membranes, as in the case of *Axin* mutant cells, Notch protein appeared as a 'fuzzy' stain throughout the cytoplasm of the *Apc1/2* mutant cells that was not associated with any subcellular structure. Axin and Apc have been shown to play functionally related, but distinct, roles in the regulation of Arm/β-catenin (Tolwinski, 2009) and these differences might extend to their effects on Notch.

The function of Axin and Apc is to provide a scaffold for the phosphorylation of Arm/β-catenin by Sgg/GSK3. Since, in mammalian systems, GSK3 has been shown to phosphorylate Notch (Espinosa et al., 2003; Foltz et al., 2002) and there are reports of interactions between Notch and Sgg in *Drosophila* (Heitzler and Simpson, 1991; Ruel et al., 1993), we tested whether Sgg has an effect on the traffic of Notch. Clones of cells mutant for *sgg* displayed elevated levels of Arm (Fig. 1C) but no discernible effects on the endocytosis and traffic of Notch (Fig. 4C,D). This is consistent with the observation that Sgg is not required for the effects of Notch on Wnt signalling (Hayward et al., 2005).

In addition to their interactions with Wnt signalling, Axin and Apc display interactions with other signalling pathways (Luo and Lin, 2004; Salahshor and Woodgett, 2005) and, in the case of Apc, with the cytoskeleton (Bahmanyar et al., 2009; Etienne-Manneville, 2009). These additional interactions might contribute to the differences between the effects of activated Arm and the loss of function of Axin and Apc. Notwithstanding this, our results reveal a function of Axin and Apc in the traffic of Notch. Previous studies have shown that compromising the traffic of Notch elevates the activity of an activated form of Arm (Sanders et al., 2009). In *Axin* or *Apc1,Apc2* mutant clones, in addition to the elevation of active Arm, the traffic of Notch is compromised and probably contributes to the increase in Arm activity. In this situation, the levels and activity of Arm would be higher than those resulting from the expression of an activated form of Arm alone (see Fig. S3 in the supplementary material). There is evidence that Axin functions in the regulation of Arm activity in a manner that is independent of its role as a scaffold for GSK3 (Tolwinski et al., 2003). Some of these effects could be mediated through its role in the endocytosis and traffic of Notch, which also could traffic with a GSK3-independent form of Arm.

Our results underscore the inadequacy of the notion that Wnt signalling flows through a linear pathway to target the destruction complex and promote β-catenin transcriptional activity (Daugherty and Gottardi, 2007; Logan and Nusse, 2004; Reya and Clevers, 2005). Although this framework helps to explain some of the effects associated with Wnt signalling, it is inconsistent with the observation that, in many instances, changes in the concentration of Arm/β-catenin are insufficient to promote transcriptional activity (Brennan et al., 2004; Guger and Gumbiner, 2000; Lawrence et al., 2001; Staal et al., 2002; Tolwinski et al., 2003). While the axis Wnt-Dsh-Axin/Apc-β-catenin is the backbone of Wnt signalling, it is clear that there are additional elements that are not simply modulatory add-ons. In this regard, the interactions between Wnt and Notch signalling are a recurrent theme in developmental biology and disease and might not reflect a simple functional convergence in specific processes at the transcriptional level (Hayward et al., 2008). The results presented here reinforce the notion that Wnt and Notch configure a molecular device (Wntch), in which the mutual control of their activities serves to regulate the assignation of cell fates with the effect of Notch providing a buffer to fluctuations in the resting levels of Arm (Hayward et al., 2006; Sanders et al., 2009).

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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