Development 128, 2107-2117 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV4538

Armadillo nuclear import is regulated by cytoplasmic anchor Axin and nuclear anchor dTCF/Pan

Nicholas S. Tolwinski and Eric Wieschaus

Howard Hughes Medical Institute, Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA
*Author for correspondence (e-mail: ewieschaus@molbio.princeton.edu)

Accepted 12 March 2001

SUMMARY

Drosophila melanogaster **Armadillo plays two distinct roles during development. It is a component of adherens junctions, and functions as a transcriptional activator in response to Wingless signaling. In the current model, Wingless signal causes stabilization of cytoplasmic Armadillo allowing it to enter the nucleus where it can activate transcription. However, the mechanism of nuclear import and export remains to be elucidated. In this study, we show that two gain-of-function alleles of Armadillo activate Wingless signaling by different mechanisms. The S10 allele was previously found to localize to the nucleus, where it activates transcription. In contrast, the ΔArm allele localizes to the plasma membrane, and forces endogenous Arm into the nucleus. Therefore, ΔArm is dependent on the presence of a functional endogenous allele of *arm* to activate transcription. We show that ΔArm may function by titrating Axin protein to the membrane, suggesting that it acts as a cytoplasmic anchor keeping Arm out of the nucleus. In *axin* mutants, Arm is localized to the nuclei. We find that nuclear retention is dependent on dTCF/Pangolin. This suggests that cellular distribution of Arm is controlled by an anchoring system, where various nuclear and cytoplasmic binding partners determine its localization.**

Key words: Wnt/Wingless, Armadillo, Nuclear import/export, *Drosophila melanogaster*

INTRODUCTION

Studies in insects and vertebrates have established an essential role for Wnt/*wingless* signaling during development. *Drosophila wg* is critical for development of many tissues including the embryonic cuticle (Nüsslein-Volard and Wieschaus, 1980). Segmentation of the embryonic epidermis depends on cell fate choices made by the epidermal cells in response to *wg* and other signals (reviewed by Wodarz and Nusse, 1998). Armadillo (Arm)/β-catenin is the key mediator of Wnt/*wg*. In response to Wnt/*wg*, its degradation is inhibited allowing it to translocate to the nucleus where it functions as a transcriptional activator. Its activity is controlled by a large number of binding partners that affect its stability and localization. Mutation of β-catenin or other components of the Wnt pathway leads to oncogenic transformation (Polakis, 1999). However, Armadillo/β-catenin is not only a signaling molecule. β-catenin was first isolated as a component of adherens junctions. It binds both E-cadherin and α-catenin linking adherens junctions to actin polymers, effectively linking a transmembrane receptor to the cytoskeleton (McCrea et al., 1991; Peifer and Wieschaus, 1990).

In the developing embryo, Arm plays a central role in the Wg-dependent transcriptional induction of naked cuticle cell fate. Wg binds to Frizzled (Fz) family receptors which in turn activate Disheveled (Dsh). Dsh inactivates Zeste white 3 (Zw3/GSK3β), a kinase responsible for phosphorylation of Arm. Arm phosphorylation targets it for degradation; consequently, in the absence of Wg signal Arm protein is rapidly degraded. This process requires the scaffold protein Axin (Hamada et al., 1999; Willert et al., 1999) and the tumor suppressor APC (Ahmed et al., 1998; Salic et al., 2000). This so called 'destruction complex' keeps cytoplasmic Arm levels low. Wg inactivates the destruction complex leading to Arm stabilization, cytoplasmic accumulation, and nuclear translocation. In the nucleus, Arm binds to dTCF directly activating transcription (van de Wetering et al., 1997).

Although it is clear that Arm must enter the nucleus to affect transcription, the mechanism remains obscure. It has been proposed that simply increasing levels of Arm protein may account for nuclear entry (Peifer et al., 1994b). This view is compatible with the diffuse cytoplasmic and nuclear staining observed in Wg responding cells (Peifer and Wieschaus, 1990), and the failure to identify specific nuclear localization in *Drosophila*. However, in vertebrates specific nuclear localization has been observed in some cell types (reviewed by Wodarz and Nusse, 1998). Studies using tissue culture have shown that β-catenin is constitutively nuclear in a cell free assay (Fagotto et al., 1998). Another study showed that import and export are highly dynamic, but the preferred state is nuclear (Yokoya et al., 1999). Both these studies suggest that in the absence of an inhibitory effect of cytoplasm, Arm would be

constitutively nuclear. They suggest that nuclear levels of β-catenin may be regulated in part by cytoplasmic and nuclear retention.

Here we examine intracellular localization of Arm. We show that overexpression of a membrane tethered, gain-of-function product of an *arm* allele drives endogenous Arm protein into the nucleus. This nuclear localization is not due to an increase in protein levels as in *zw3* mutants, but affects a second mechanism downstream of stability. We show that elimination of *axin* leads to nuclear Arm accumulation, suggesting a cytoplasmic anchoring role for *axin*. Furthermore, we find that expression of a dominant negative form of *dTCF* leads to loss of nuclear Arm. We propose a model of Arm nuclear import and export based on nuclear and cytoplasmic anchoring.

MATERIALS AND METHODS

Fly strains

The wild-type stock used was Oregon R. UAS-ΔArm expresses an allele of *arm* the product of which has the first 128 amino acids deleted, but it has an N-terminal HA tag and a consensus myristoylation site (Zecca et al., 1996). UAS-S10 expresses an allele, the product of which is deleted in amino acids 34-87, and contains a c-Myc tag in the C terminus (Pai et al., 1997). UAS-Arm full-length expresses the wild-type form of Arm (White et al., 1998). arm^{043A01} is an EMS-induced allele creating a stop codon eliminating repeats 11-12 and the entire C terminus, almost identical to arm^{XP33} (Cox et al., 1996). The alleles arm^{XM19}, $arm^{H8.6}$ delete the entire C terminus and half of the C terminus respectively (Peifer and Wieschaus, 1990). $zw3^{M11-1}$ (Siegfried et al., 1992) is a null allele. $axn^{S044230}$ is a P-element insertion eliminating gene product (Hamada et al., 1999). UAS-Axin expresses the full length allele (Willert et al., 1999). UAS-dTCFΔN expresses a 31 amino acid N-terminal truncation of *dTCF* (van de Wetering et al., 1997). UAS-dTCF expresses full-length dTCF (van de Wetering et al., 1997). The ArmGAL4 line containing GAL4 under the control of the zygotic Armadillo promoter was a gift from J. P. Vincent (National Institute for Medical Research, MRC, London, UK). The 67.15 stock containing second and third chromosomal inserts of GAL4-VP16 under the control of the maternal α-tubulin promoter was a gift from D. St. Johnston (Cambridge University, UK).

Crosses and generation of germline glones

Germline clones were generated to produce germlines and embryos depleted of maternal product and containing arm^{043A01}, arm^{XM19}, $arm^{H8.6}$, $zw3^{M11-1}$ and $axn^{S044230}$ mutant products only by using the FLP recombinase-dominant female sterile technique (Chou and Perrimon, 1992). The stocks, XXyf/ovo^{D1} FRT101; hs-flp 38 and *yw* hs-flp 38/FM7; FRT 2059 ovo^{D1}/TM3, are as described previously (Chou and Perrimon 1992).

For the generation of *arm* and *zw3* germline clones expressing UAS alleles, second and third instar larvae generated from the cross between *arm* FRT101/FM6; ArmGAL4 or *zw3* FRT101/FM6; ArmGAL4 females and ovo^{D1} FRT101; hs-flp 38 males were heat shocked in an incubator at 37°C for 3 hours. This induces site-specific homologous mitotic recombination at FRT sequences. Owing to the presence of the ovo^{D1} female sterile mutation, which allows only germ cells homozygous for the *arm* or *zw3* mutation to develop, the only fertile females hatching from this cross will have mutant germlines. These females are essentially *arm*/*arm*; ArmGAL4/+ or *zw3*/*zw3*; ArmGAL4/+ so when crossed to *yw*; ΔArm or *yw*; S10 males, they lay embryos which at a frequency of 25% are maternally and zygotically mutant, and express the UAS allele. Female embryos receive a zygotic, paternal copy of arm^{+} or $zw3^{+}$. Only half of the embryos receive the ArmGAL4 driver.

axn germline clones were generated by essentially the same technique. *yw* hs-flp; FRT 2059 ovo^{D1}/TM3 males were crossed to FRT $axn^{S044230}$/TM3 so that the only non-balancer females that are fertile must contain a germline homozygous for $axn^{S044230}$. These females were then crossed to $axn^{S044230}$/TM3 males to produce embryos maternally and zygotically $axn^{S044230}$ at a frequency of 50%.

Immunofluorescence

Embryos were dechorionated in bleach, and fixed for 30 minutes at the interface of a heptane/4% formaldehyde in PBS fix solution. For Armadillo staining PBS was substituted by PEM-NP40 (0.1 M Pipes pH 6.9, 1 mM EGTA, 2 mM $MgSO_4$, 1% Nonidet P-40). The aqueous phase was removed and an equal amount of methanol was added to devitellinize embryos. Antibody stainings were done in PBT (PBS, 0.1% Triton X-100, 1% bovine serum albumin, 0.1% Azide). The following antibodies were used: anti-Engrailed (mAb 4D9 from the Developmental Studies Hybridoma Bank, University of Iowa, Des Moines, IA), anti-Armadillo (mAb N2 7A1 from the Developmental Studies Hybridoma Bank), anti-Armadillo (rAb N2, Peifer et al., 1994b), anti-Hemagglutinin (mAb HA.11 16B12, BabCo), anti-Hemagglutinin (ratAb HA 3F10, Roche), anti-c-Myc (mAb 9E10, Santa Cruz Biotechnology), anti-β-tubulin (mAb E7 from the Developmental Studies Hybridoma Bank), and anti-Sex lethal (mAb, M-14 from the Developmental Studies Hybridoma Bank). Alexa 488-and alexa 546-conjugated anti-mouse, anti-rabbit, or anti-rat secondary antibodies were used (Molecular Probes, Inc.). For triple stainings, a biotin-conjugated secondary antibody was used followed by strepavidin-Cy5 (Jackson Laboratories, Inc.). DNA was detected by Hoechst DNA dye (Sigma). Embryos were mounted in Aquapolymount® (Polysciences, Inc.). Images were obtained on an inverted Zeiss LSM510 confocal microscope. All images were processed using Adobe Photoshop® and Illustrator® software.

Cuticle preparations

Embryos collected overnight and aged 24 hours were dechorionated in bleach and mounted in Hoyers' medium followed by an overnight incubation at 60°C.

Western blotting

Heat fixed embryos (described by Peifer et al., 1994b) were selected to be of similar stage. Embryos were lysed, the extracts were separated on 8% SDS-PAGE, and blotted as described by Peifer et al. (Peifer et al., 1992). Bands were quantitated using NIH Image software.

RESULTS

ΔArm drives endogenous Arm into the nucleus

Expression of stabilized forms of Arm (Pai et al., 1997) causes a change in patterning to naked cuticle cell fates, a phenotype similar to that produced by uniform Wg activation. To address the mechanism that causes these cell transformations, we used two different constitutively active alleles of *arm*, ΔArm and S10. ΔArm is a stabilized form of Arm due to a large amino-terminal deletion that removes Zw3 phosphorylation sites, disrupts the α-catenin binding site, and substitutes in a myristoylation site (Zecca et al., 1996, see Materials and Methods for details on *arm* alleles). The S10 allele of Arm is also stabilized, but through a smaller deletion that removes the Zw3 phosphorylation sites and leaves the α-catenin binding site intact (Pai et al., 1997). We expressed both alleles using the GAL4/UAS system (Brand and Perrimon, 1993), and looked at the cuticles of first instar larvae. As is shown in Fig. 1A, both ΔArm and S10 lead to the complete absence of

denticles from the cuticle. This is the expected phenotype for activated Wg signaling.

We next examined the intracellular localization of both the expressed alleles and the endogenous Arm protein through immunofluorescence. Although the two forms produce identical cuticle phenotypes, ΔArm localizes to the membrane (Fig. 1B), whereas S10 localizes to the nucleus (Fig. 1B and Pai et al., 1997). The surface localization of ΔArm may be due to myristoylation, but this raises the question of how ΔArm produces a cell fate transformation to naked cuticle if it doesn't enter the nucleus. We did, however, observe a striking nuclear accumulation of endogenous Arm. Endogenous Arm protein is localized overwhelmingly to the nuclei of cells expressing ΔArm. This is readily apparent in the large cells of the amnio serosa (Fig. 1B). Using an earlier driver (67.15 GAL4), we observe that ΔArm can affect the nuclear localization of endogenous Arm in the epidermal cells of the embryo as early as stage 9 (Fig. 2C), specifically at a stage when Wg establishes segment polarity. In contrast, overexpression of a full-length form of Arm did not lead to nuclear accumulation (Fig. 1B; Orsulic et al., 1996). Consequently, we concluded that both alleles activate Wg signaling, but based on their intracellular localization, and the distribution of endogenous protein, the mechanisms by which this is achieved are probably quite different.

ΔArm is dependent on endogenous Arm to activate Wg signaling

The nuclear localization of endogenous Arm in ΔArm-expressing embryos suggests that ΔArm may require functional endogenous protein to activate transcription. Alternatively, some ΔArm might enter the nucleus to activate signaling independently of endogenous protein. To test this, we made germline clones to generate embryos that express ΔArm, but contain only mutant forms of endogenous *arm*. We used the strong allele arm^{043A01} (see Materials and Methods for allele and cross details) which retains some function in cellular adhesion, but cannot signal. To assay the effects of ectopic Arm, we used Engrailed staining, a convenient marker for Wg signaling activity in the epidermis (Martinez Arias et al., 1988). We used anti-Sex lethal and anti-HA staining to determine the genotype of the embryos. Germline clone embryos expressing ΔArm that receive a wild-type allele from their fathers (*arm*/+) show expanded En stripes characteristic of Wg activation (Fig. 2A top panel), whereas *arm*/+ embryos without ΔArm show the wild-type En expression pattern of one or two cell stripes (Fig. 2A, second panel). In contrast, *arm/arm* embryos expressing ΔArm show only nervous system En expression (Fig. 2A, fourth panel), similar to *arm/arm* embryos without ΔArm (Fig. 2A third panel). To confirm that this effect is more general and not linked to En expression alone, we repeated both the ΔArm and S10 (see below) experiments using anti-Wg immunofluorescence as a marker for signaling, with similar results (data not shown). These results suggest that ΔArm activation of signaling is dependent on the presence of functional endogenous Arm protein.

S10 localizes directly to the nucleus, and does not seem to affect the localization of endogenous protein. We used the same approach to ascertain whether S10 functions

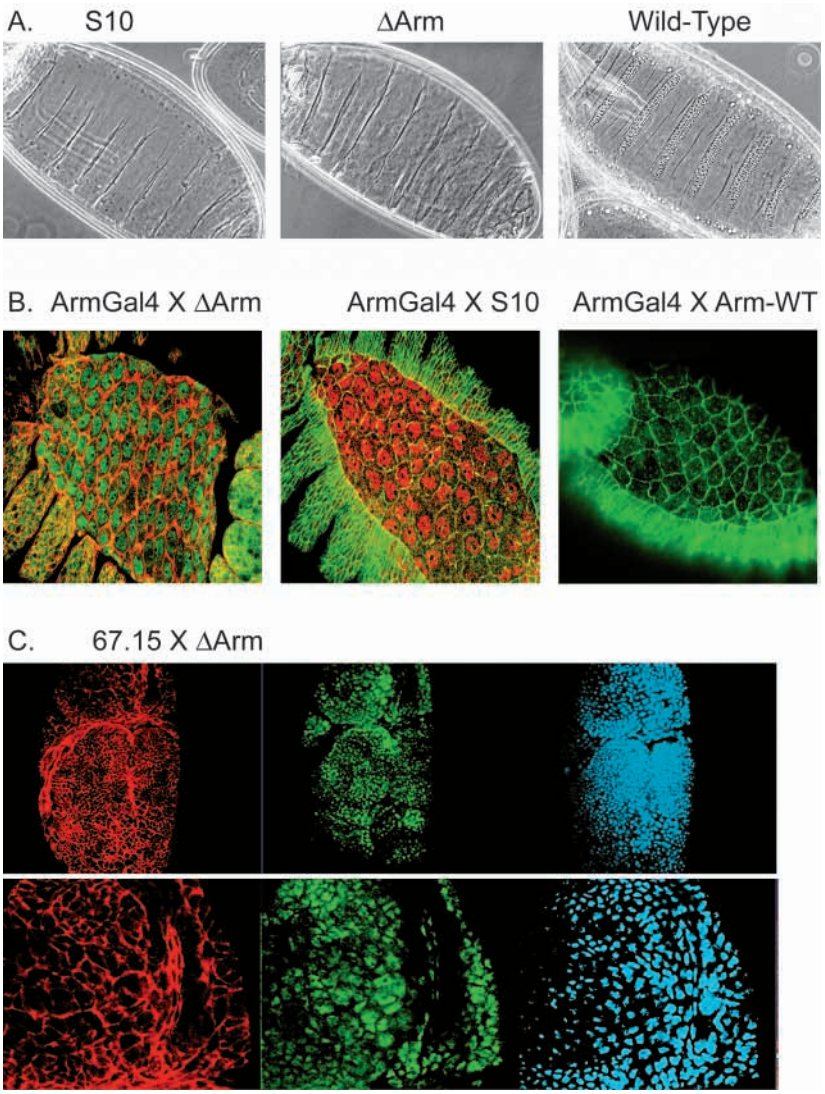

Fig. 1. Overexpression of Arm alleles in embryos using the GAL4/UAS system. The two alleles, ΔArm and S10, both produce the same activated *wg* signaling phenotype in first instar larval cuticles, but ΔArm localizes to the membrane while S10 localizes to the nucleus. (A) Phase contrast images of cuticle preparations of ArmGAL4 crossed to either ΔArm, or S10. A wild-type cuticle is shown for comparison. The ventral side of all embryos is shown. (B) Confocal microscope images of embryos using the ArmGAL4 driver to drive expression ΔArm (α-HA, red), S10 (α-c-Myc, red), and UAS-full-length-Arm (rAb, green). (C) Using the maternal GAL4 driver, 67.15, expression of ΔArm (α-HA, red), endogenous Arm protein (rAb, green), and nuclei (Hoechst, blue) is shown. Lower panel shows a close up of the same embryo. The amino-terminal deletions in S10 and ΔArm delete the epitope used to make the Arm antibody allowing staining of expressed protein and endogenous protein without cross-reactivity. Overexpressed full-length Arm shows both endogenous and expressed protein.

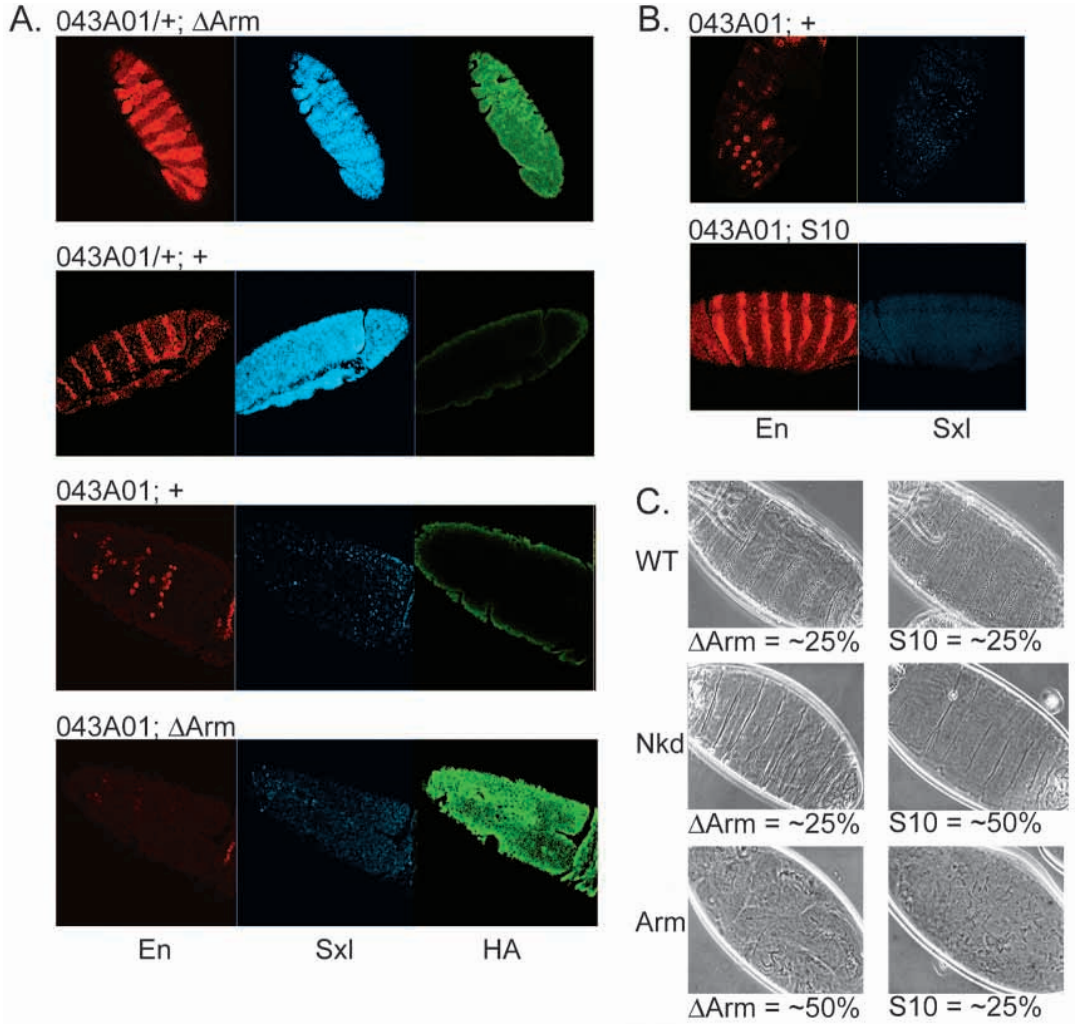

Fig. 2. ΔArm is dependent on endogenous Arm to activate Wg signaling, while S10 is not. a*rm*^{043A01} germline clones made using the FLP recombinase system expressing either ΔArm or S10 using the GAL4/UAS system. (A) Confocal images of the four embryos obtained when a*rm*^{043A01}; ArmGAL4/+ germline clones were crossed to ΔArm. En stripes are shown in red. Sxl antibodies were used to detect female *arm*/+ embryos (blue). ΔArm expression is shown by α-HA staining (green). (B) Confocal images of embryos (a*rm*^{043A01}; ArmGAL4/+ crossed to S10) showing the En stripes (α-En, red), and lack of Sxl expression in these male embryos (*arm*/Y, blue). (C) Phase contrast images of a*rm*^{043A01}; ArmGAL4/+ crossed to either S10 or ΔArm. The percentages are the approximate frequency with which the phenotype was observed over the course of three independent experiments. Cuticle preparations from the *arm*^{043A01}/ΔArm cross show approx. 25% naked (*n*=43), approx. 25% wild-type (*n*=36), and approx. 50% severe *arm* phenotype (*n*=78), consistent with *arm*/+; ΔArm/ArmGAL4 leading to naked cuticle, *arm*/+; ΔArm/+ leading to wild-type cuticle, *arm*/Y; ΔArm/ArmGAL4 and *arm*/Y; ΔArm/+ both leading to the severe *arm* phenotype. Cuticle preparations from the *arm*^{043A01}/S10 cross show ~50% naked (*n*=105), ~25% wild-type (*n*=57), and ~25% severe *arm* phenotype (*n*=51), consistent with *arm*/+; S10/ArmGAL4 and *arm*/Y; S10/ArmGAL4 both leading to naked cuticle, *arm*/+; S10/+ leading to wild-type cuticle, *arm*/Y; S10/+ leading to the severe *arm* phenotype.

independently of *arm*^{043A01}. As shown in Fig. 2B, expression of S10 in *arm*^{043A01}/*arm*^{043A01} germline clone embryos leads to ectopic stripes of En (Fig. 2B) and Wg (not shown) indicating that S10 does not require functional endogenous Arm protein to activate signaling.

Germline clone embryos of the stronger *arm* alleles such as *arm*^{043A01} and *arm*^{XP33} ultimately show very severe defects in adhesion and cuticle formation similar to *shotgun*/Ecadherin mutants (Oda et al., 1994; Müller and Wieschaus, 1996). In contrast, expression of ΔArm or S10 in otherwise wild-type embryos leads to a naked cuticle. Therefore, we investigated which cuticle defect would be dominant to the other. Assuming that ΔArm is dependent on endogenous Arm, then the *arm*^{043A01} phenotype should supersede the naked cuticle of ΔArm. However, since S10 is independent of endogenous Arm, the naked cuticle phenotype should overcome the *arm*^{043A01} phenotype. Indeed, as shown in Fig. 2C, the adhesion defect of *arm*^{043A01} is not rescued by ΔArm expression, but is rescued by S10 expression. This is consistent with ΔArm being defective in α-catenin binding making it unable to function in adherens junctions (Zecca et al., 1996). In contrast, the S10 rescue of the *arm*^{043A01} phenotype suggests that it can not only activate signaling, but also rescue the junction defects of this *arm* allele (similar results were observed for S10 by Pai et al., 1997 with the *arm*^{XP33} allele).

Taken together, these experiments show that ΔArm is

dependent on functional endogenous Arm protein to activate signaling. Expression of ΔArm leads to ectopic activation of Wg signaling, but it can only do so through nuclear localization of endogenous Arm. S10, in contrast, does not require endogenous Arm, and can substitute for all the required functions of Arm protein rescuing junctions as well as signaling.

ΔArm can force a moderate *arm* allele to signal

It has recently been demonstrated that products of moderate and weak loss-of-function *arm* alleles can be induced to signal by the expression of a membrane-tethered, wild-type form of Arm (Cox et al., 1999b) leading to a wild-type cuticle and hatching. From this result, it would appear that expression of a membrane-tethered allele that cannot be degraded should lead to a naked cuticle in embryos expressing only hypomorphic alleles of *arm*. Membrane-tethered wild-type Arm is still subject to Wg control and leads to normal segmentation, whereas a gain-of-function allele is independent and causes naked cell fate transformations throughout the cuticle. We used the same technique described above to engineer embryos maternally and zygotically arm^{XM19}, which also express ΔArm from the ArmGAL4 driver. We observed the expected small abnormally shaped, denticle covered cuticles (characteristic of arm^{XM19}), naked cuticles (characteristic of activated Arm), wild-type cuticles (Fig. 3A panels 1-3), and a fourth, new phenotype where the embryo shows the cell transformations to naked cuticle, but is small and abnormally shaped (Fig. 3A panel 4). This phenotype appears to be intermediate between the naked and *arm* phenotypes, because though there are no denticles the embryo is small and shaped much like that of the arm^{XM19} germline clone. This suggests that the ΔArm activated arm^{XM19} reaches the nucleus to cause naked cuticle cell transformations, but is unable to rescue the morphological defects. In contrast, when we performed the experiment with S10 as the expressed allele, we observed only three phenotypes, with half the embryos displaying the naked cuticle phenotype (data not shown and Pai et al., 1997), confirming that that S10 functions independently of endogenous protein. To extend these results we repeated both the ΔArm and S10 experiments using the weaker allele, $arm^{H8.6}$, with similar results (data not shown).

To test the ability of arm^{XM19} to signal more directly, we assayed En expression in arm^{XM19} germline clones expressing ΔArm. The crosses were essentially the same as those detailed in the previous section for arm^{043A01}, substituting arm^{XM19} as the *arm* allele used. Embryos maternally and zygotically arm^{XM19} expressing ΔArm showed ectopic En stripes in the embryonic epidermis (Fig. 3B). arm^{XM19} embryos not expressing ΔArm showed little or no En expression. Expression of S10 in these embryos also leads to ectopic En stripes (Fig. 3B).

Taken together these results suggest that arm^{XM19} can be induced to signal by the expression of ΔArm. The presence of ΔArm must cause arm^{XM19} protein to bypass the degradation machinery and enter the nucleus where it activates transcription. ΔArm is not subject to Wg control, therefore arm^{XM19} is induced to signal in all cells leading to a naked cuticle phenotype. This is similar to the results reported by Cox et al. (Cox et al., 1999), although they used a wild-type allele to induce arm^{XM19} to signal. However, we find that the ΔArm activated arm^{XM19} does not rescue the morphological defects, either through inadequate junctional activity, or by activating only a sub-set of Wg targets. Also, ΔArm appears to be functioning through a different mechanism than the tethered wild-type allele, since arm^{XM19} protein is no longer subject to Wg control (see Discussion).

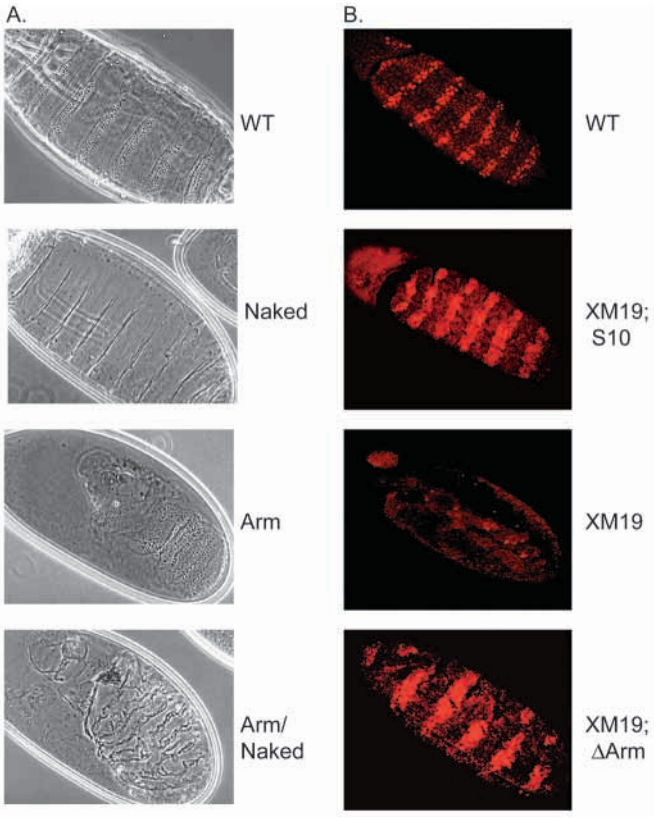

Fig. 3. ΔArm expression can activate the transcriptional activity of a moderate loss-of-function allele of *arm*. arm^{XM19} germline clones made using the FLP recombinase system expressing either ΔArm or S10 using the GAL4/UAS system. (A) Phase contrast images of (arm^{XM19}; ArmGAL4/+ crossed to ΔArm) first instar larvae show approx. 25% naked (*n*=32), approx. 25% wild-type (*n*=26), approx. 25% *arm* phenotype (*n*=27), and approx. 25% naked/*arm* phenotype (*n*=35), consistent with *arm*/+; ΔArm/ArmGAL4 leading to naked cuticle, *arm*/+; ΔArm/+ leading to wild-type cuticle, *arm*/Y; ΔArm/+ leading to *arm* cuticle, and *arm*/Y; ΔArm/ArmGAL4 leading to the naked/*arm* cuticle. (B) Confocal images of embryos (arm^{XM19}; ArmGAL4/+ crossed to either ΔArm or S10) showing the En stripes (α-En, red). An embryo with wild-type Engrailed stripes is shown for comparison. All panels were also stained with Sxl (not shown) to separate out the arm^{XM19}/+ embryos.

ΔArm function is independent of protein levels

We next addressed whether the nuclear accumulation of Arm in ΔArm-expressing embryos functions through an increase in protein levels. According to the standard model of Wg signaling, Arm stabilization, or the increase in Arm protein levels, leads to transcriptional activation (Salic et al., 2000). ΔArm could simply stabilize endogenous protein to affect the naked cuticle phenotype. To assay this, we compared protein levels by western blot analysis. As an internal control, we used embryos from germline clones homozygous for *zw3* that

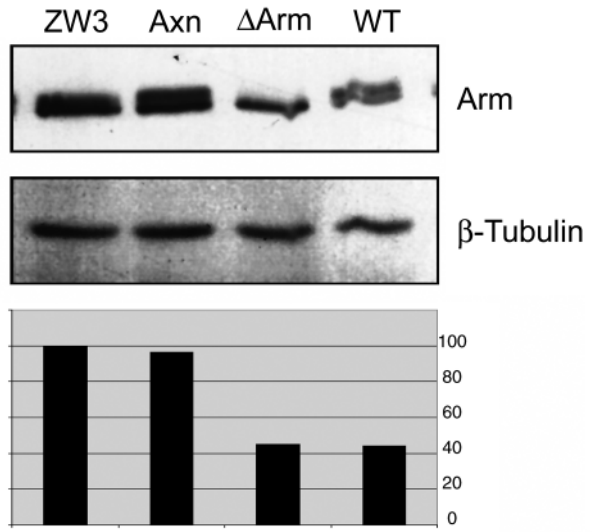

Fig. 4. (Top) Protein levels in ΔArm-expressing embryos are not significantly higher than in wild-type embryos, but much lower than in *zw3* or *axin* germline clones. The *zw3* lane contains extracts from four embryos from $zw3^{M11\text{-}1}$; ArmGAL4/+ crossed to wild type. Half the embryos will receive a paternal wild-type copy of *zw3*, reducing the number of true mutant embryos per lane. The *axin* lane contains extracts from four embryos from *axn* germline clone crossed to *axn*/TM3, therefore half the embryos will receive a zygotic wild-type copy of *axn* reducing the number of true mutant embryos. However, though the *axin* and *zw3* lanes mix mutant and non-mutant embryos, they still show a significant increase in endogenous Arm protein levels. The ΔArm lane contains extracts from four embryos where the 67.15 GAL4 driver was crossed to ΔArm, therefore all embryos express ΔArm. The wild-type lane contains extracts from four OreR embryos. All embryos were selected to be at similar stages (stage 11 to 12). (Middle) β-tubulin was used as a loading control, and shows that all lanes were loaded equally. (Bottom) Arm bands were quantitated using NIH Image, and the results graphed. The units are arbitrary.

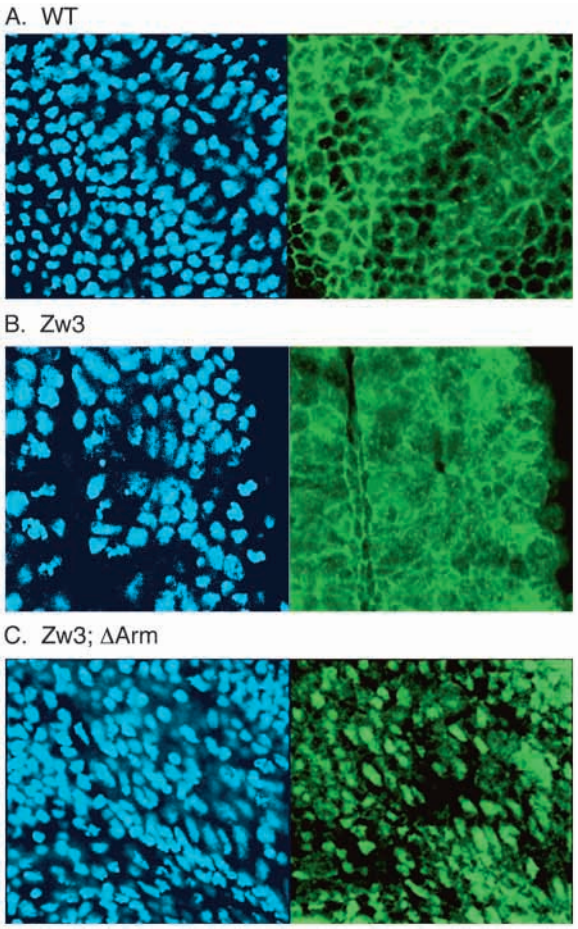

Fig. 5. ΔArm acts downstream of *zw3*, and is independent of the increased levels present in *zw3* germline clones. Confocal microscope images of stage 9 embryos showing Arm protein (N2, green) and nuclei (Hoechst, blue). (A) A wild-type embryo, OreR. (B) $zw3^{M11\text{-}1}$;ArmGAL4/+ crossed to wild type. The embryo was also stained with α-Sxl to separate out the females (not shown). (C) $zw3^{M11\text{-}1}$; ArmGAL4/+ crossed to ΔArm. The embryo was also stained with α-HA to find embryos expressing ΔArm, and with α-Sxl to separate out the females (not shown).

showed cuticular phenotypes similar to ΔArm and S10, and eliminated the kinase which phosphorylates and targets Arm for degradation (Peifer et al., 1994b; reviewed by Wodarz and Nusse, 1998). In Fig. 4, a representative blot is shown where extracts from four embryos were loaded per lane. As is apparent from the quantitation of the bands, *zw3* germline clone embryos show a much increased level of Arm protein compared to wild-type and ΔArm lanes. However, the total level of endogenous Arm protein does not differ significantly between ΔArm and wild-type lanes, although the two Arm bands in the wild-type lane are collapsed into a single band in the ΔArm lane. These data suggest that ΔArm does not affect protein levels, but acts through a separate mechanism which affects intracellular localization. Though we do not know either the nature or the reason for the mobility shift observed for endogenous Arm in ΔArm-expressing embryos, it may suggest that different intracellular localization of Arm may be associated with different post-translational modifications (some evidence for this was previously observed by Peifer et al., 1994a).

Wg is expressed in stripes, which in turn lead to stripes of Arm in the embryonic epidermis. The Arm stripes do not show a specific nuclear localization, but instead show a diffuse distribution throughout the cells (Peifer and Wieschaus, 1990). In *zw3* embryos, stripes are not seen; all cells have increased Arm, not just those responding to Wg (Peifer et al., 1994b). Both wild-type Arm stripes and Arm in *zw3* mutant embryos show a similar subcellular distribution of Arm protein, namely a diffuse pattern throughout the cytoplasm and nucleus as well as cell surface localization (Fig. 5A,B). Since ΔArm appears to act by a mechanism independent of protein levels, we investigated whether it is independent of *zw3* as well. We expressed ΔArm in *zw3* germline clone embryos. As shown in Fig. 5C, expression of ΔArm leads to the nuclear localization of Arm, demonstrating that ΔArm can force even the much increased Arm levels found in *zw3* germline clones into the nucleus. This suggests that ΔArm functions independently of Arm protein levels to promote nuclear localization of endogenous Arm. ΔArm appears to act downstream of *zw3* by affecting a second step, which retains endogenous Arm protein in the cytoplasm.

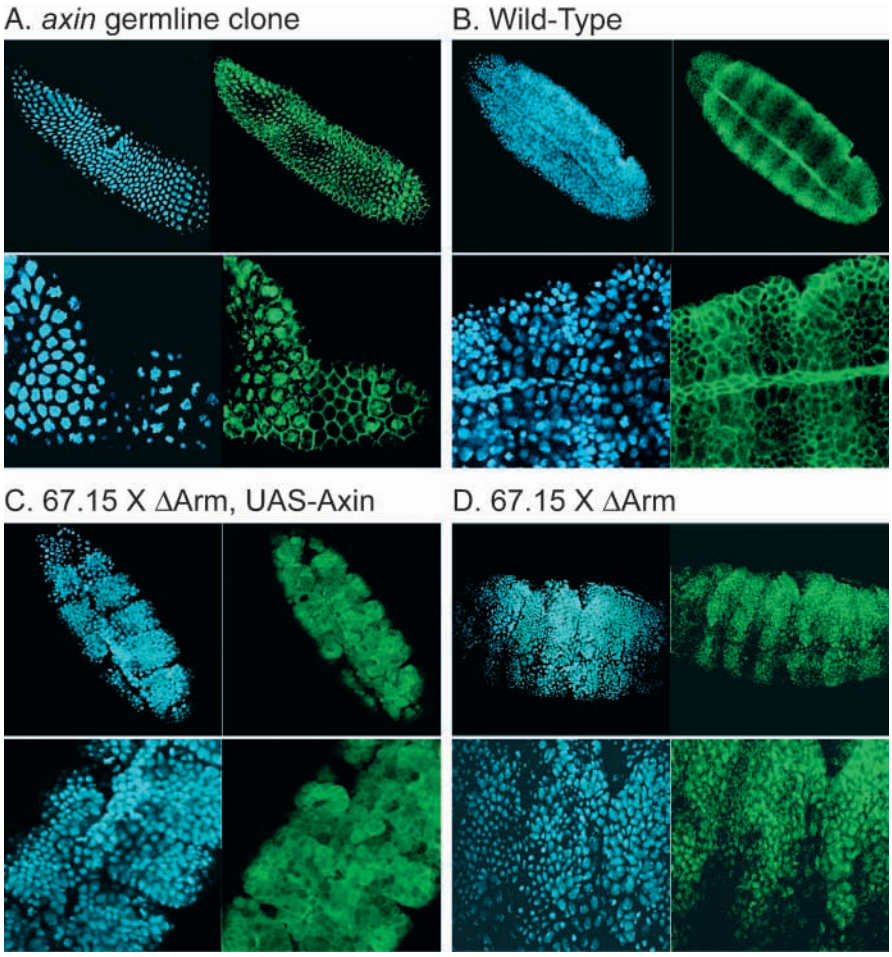

Fig. 6. Axin functions in retaining Arm in the cytoplasm. (A) Confocal microscope images of embryos showing endogenous Arm protein (N2, green) and nuclei (Hoechst, Blue). (A) *axin* germline clone embryos show very specific nuclear and plasma membrane localization, and a lack of cytoplasmic staining. (B) Wild-type embryo shown for comparison where diffuse staining is observed throughout the cell. (C) 67.15 crossed to ΔArm, UAS-Axin. This embryo was also stained with α-HA to confirm expression of ΔArm (not shown). Diffuse staining is observed throughout the cell with no obvious preference for the nucleus. (D) 67.15 crossed to ΔArm alone shown for comparison. Nuclear localization of endogenous Arm is observed.

Axin may function as a cytoplasmic anchor for Arm

The above results suggest that nuclear import of Arm may be controlled by another mechanism in addition to degradation. It has been suggested that nuclear import of β-catenin is controlled by a cytoplasmic anchor (Cox et al., 1999a; Fagotto et al., 1998; Yokoya et al., 1999). A likely candidate for this function is Axin. Axin is a scaffold protein required for efficient Arm degradation in the cytoplasm (Hamada et al., 1999; Willert et al., 1999). Axin localizes to the cytoplasm and plasma membrane (Fagotto et al., 1999). It does not have catalytic properties, but it does facilitate the formation of the cytoplasmic destruction complex (Salic et al., 2000). To test the involvement of *axin* in cytoplasmic anchoring, we made germline clone embryos with a null *axin* allele (see Materials and Methods for details of cross and alleles). Removing *axin* results in increased Arm protein levels comparable to those observed on the western blot for *zw3* germline clones (Fig. 4). In contrast to the results with *zw3*, Arm protein in an *axin* germline clone localizes to the nucleus as well as the plasma membrane (Fig. 6A). Little or no Arm is detected in the cytoplasm as compared to wild-type embryo Arm stripes (Fig. 6B). The lack of cytoplasmic localization is striking in comparison to a *zw3* germline clone (Fig. 5B) and reminiscent of ΔArm expression (Figs 6D, 1). Although both *axin* and *zw3* are required for activation of the destruction box, they differ in their effect on localization.

One possibility for how ΔArm drives endogenous Arm into the nucleus is that ΔArm titrates something that would normally keep endogenous Arm in the cytoplasm. The germline clone experiment suggests that this might be Axin. If this were true, one might expect that the effect of ΔArm could be suppressed by overexpression of Axin. Therefore, we expressed both ΔArm and UAS-Axin in embryos. As shown in Fig. 6C, combined expression leads to diffuse staining throughout the cell, and not mostly in the nucleus as observed with ΔArm alone (Fig. 6D). Also, coexpression leads to a partial suppression of the naked phenotype induced by ΔArm expression alone with partial denticle belts reappearing (data not shown). Taken together, these results show that removal of *axin* causes nuclear accumulation and overexpression prevents it. This is consistent with *axin* playing a role in cytoplasmic anchoring of Arm.

dTCF may function in nuclear retention of Arm

Since import and export of β-catenin have been reported to be dynamic processes (Yokoya et al., 1999), it is likely that Arm must be retained in the nucleus as it is in the cytoplasm. There must be a nuclear anchor to prevent export. Therefore, we also tested the possible involvement of a downstream pathway component, the transcription factor *dTCF/pan* (van de Wetering et al., 1997; Brunner et al., 1997; reviewed by Bienz, 1998). *dTCF* provides the DNA binding activity that Arm requires in order to activate transcription. To test whether *dTCF* acts as the nuclear anchor, we used a dominant negative form,

dTCFΔN, which as the result of an amino-terminal deletion, no longer binds Arm, but retains its ability to bind DNA. When expressed in embryos, this protein blocks Wg signaling (van de Wetering et al., 1997). We simultaneously overexpressed ΔArm and dTCFΔN together in embryos from the 67.15 driver. As shown in Fig. 7, coexpression of dTCFΔN and ΔArm appears to block the nuclear accumulation of endogenous Arm observed in embryos expressing ΔArm alone. dTCFΔN is completely epistatic to ΔArm and S10 leading to *wg*-like cuticle phenotypes (data not shown and van de Wetering et al., 1997). Expression of dTCFΔN by itself does not appear to affect Arm distribution (Fig. 7B). Neither does expression of full-length dTCF (Fig. 7C). These results are consistent with a role for dTCF as a nuclear retention factor, a possibility suggested previously (Fagotto et al., 1998 and Yokoya et al., 1999), but under normal conditions, dTCF levels do not themselves confer nuclear import.

DISCUSSION

ΔArm titrates Axin to drive endogenous Arm into the nucleus

The gain-of-function *arm* allele used in this study (ΔArm) is membrane tethered, presumably by a consensus myristoylation site in its amino terminus. Although there is no reason to assume that this myristoylation would be sufficient to keep all the protein out of the nucleus, we have shown that ΔArm has no effect in embryos where the only endogenous Arm is signaling deficient. This suggests that the cell fate transformation associated with ΔArm depends on its ability to drive endogenous Arm into the nucleus. Alternatively, the larger deletion in ΔArm may somehow behave differently from the smaller deletion in S10, leading to nuclear accumulation of endogenous Arm. This seems unlikely, however, since a similar large, untethered, amino-terminal deletion behaved similarly to S10 (Pai et al., 1996 and 1997). Also, deletion of just the α-catenin binding domain confers the wild-type Wg signaling phenotype to an otherwise signaling-deficient *arm* mutant (Orsulic et al., 1996), suggesting that the ability to bind α-catenin (the main difference between ΔArm and S10) does not confer ectopic signaling defects.

Using ΔArm, we were able to compare transcriptional activity of C-terminal truncations of Armadillo that normally do not accumulate in the nucleus. We find that ΔArm can activate signaling through arm^{XM19}, but not through arm^{043A01}. The former is truncated after repeat 12, whereas the truncation in arm^{043A01} occurs in repeat 10. Our results agree with Cox et al. (Cox et al., 1999b), who used a tethered form of wild-type Arm in similar experiments. They proposed that arm^{XM19} lacks signaling activity, both because of intrinsic defects in signaling and because of its reduced levels. Our results, however, differ from those of Cox et al., in that the tethered, full-length Arm they used restores arm^{XM19} germline clone embryos to a wild-type cuticle and hatching (Cox et al., 1999b). ΔArm by contrast causes a fully penetrant cell fate transformations to naked cuticle, but fails to rescue the size and shape defects of arm^{XM19} germline clone embryos. Their results point to the fact that ΔArm affects endogenous protein in a different manner than that proposed for wild-type, tethered Arm (Cox et al., 1999b). ΔArm affects intracellular localization, a step downstream of stability, leading to nuclear accumulation of endogenous Arm and ectopic Wg signaling activation. In contrast, overexpression of wild-type, tethered Arm makes more arm^{XM19} protein available for signaling. This protein remains sensitive to Wg control leading to a wild-type cuticle.

However, under these conditions if arm^{XM19} retained all wild-type functions, one would expect the ΔArm phenotype in arm^{XM19} germline clone embryos to be similar to that observed when ΔArm is expressed in a wild-type background. Instead, the morphological defects we observe point to some deficiency in arm^{XM19} protein. They may reflect quantitative differences in the levels of arm^{XM19} and wild-type protein, or the inability of arm^{XM19} to activate all Wg transcriptional targets. Both these models assume that arm^{XM19} is not fully competent as a transcriptional activator, either through low levels or through some partial loss of transactivation function. Alternatively, the abnormal morphology of arm^{XM19} ΔArm embryos might reflect a direct effect on cell junctions. Although arm^{XM19} protein contains all regions required for junction formation (Orsulic et al., 1996), its low levels may make those junctions more sensitive to disruption. ΔArm may titrate limiting arm^{XM19} protein from junctions, but unlike the full-length wild-type protein, it cannot itself substitute for the released arm^{XM19} since it lacks the α-catenin binding region, making it incapable of participating in junctions.

Overexpression of tethered β-catenin was originally shown to activate signaling in *Xenopus* where it leads to embryonic axis duplication. Miller and Moon (Miller and Moon, 1997) proposed that tethered β-catenin titrates out APC, leading to a stabilization of endogenous β-catenin and ectopic Wnt signaling. Merriam et al. (Merriam et al., 1997) proposed that tethered plakoglobin (a paralog of β-catenin) titrated out negative regulators. Here we provide further evidence for the titration model, but focus on potential cytoplasmic anchors that retain β-catenin/Arm in the cytoplasm. We show that endogenous Arm accumulates in the nucleus in response to expression of ΔArm, and that the underlying mechanism appears to be independent of protein levels. We show that ΔArm functions downstream of *zw3*, and does not increase endogenous protein levels appreciably. These results point to a mechanism by which ΔArm affects some component of the cytoplasmic retention machinery. We show that *axin* may be this component, since its mutation leads to nuclear Arm accumulation, and its overexpression prevents it. Axin appears to be amenable to a titration model, because its function is highly dose dependent. Only maternal mutation of *axin* leads to a naked cuticle with a partial rescue by a paternal copy. Zygotic mutation doesn't produce an embryonic phenotype (Hamada et al., 1999). Overexpression leads to a *wg* phenotype only if expressed very early (Willert et al., 1999). Observations in tissue culture show that Axin is localized to the cytoplasmic membrane and the cytoplasm, but is excluded from the nucleus (Fagotto et al., 1999; Torres and Nelson, 2000). Also, mutant forms of Arm lacking repeats which are required for Axin binding localize to the nucleus (Orsulic et al., 1996). Therefore, we favor a model where ΔArm directly titrates out Axin, leading to nuclear localization of endogenous Arm. ΔArm retains arm repeats 3 through 8, shown to be required for Axin binding (Willert et al., 1999), and may sequester Axin away from endogenous Arm. This suggests a dual role for Axin, both as a scaffold for

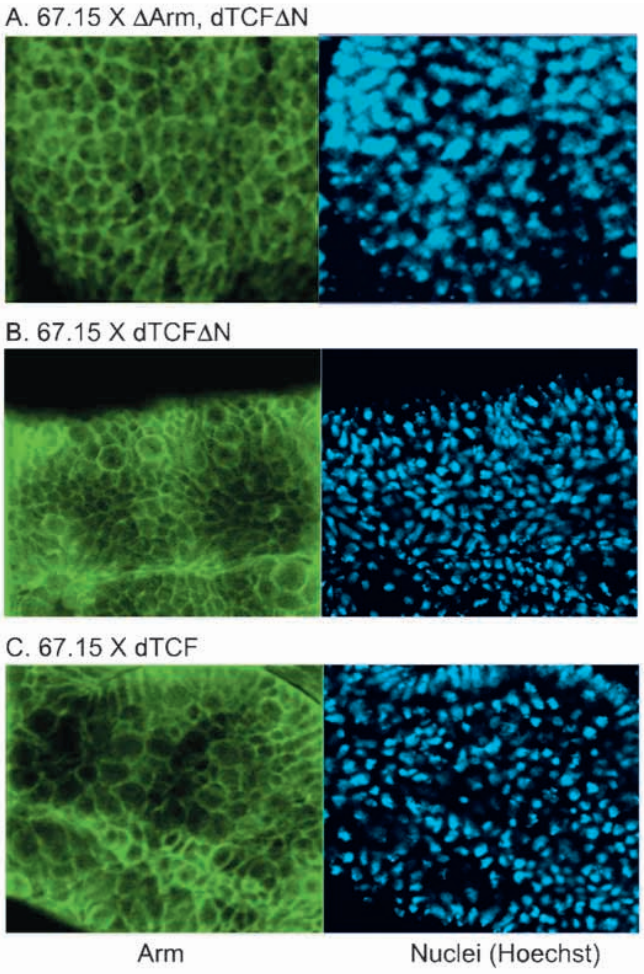

Fig. 7. *Tcf/pan* may function as a nuclear anchor for Arm. Confocal microscope images of embryos showing endogenous Arm protein (N2, green) and nuclei (Hoechst, Blue). (A) 67.15; ΔArm, dTCFΔN embryos show a lack of nuclear accumulation of endogenous Arm expected from ΔArm alone. (B) 67.15; dTCFΔN embryos also show a lack of nuclear accumulation. (C) 67.15; dTCF (full length) embryos do not show nuclear accumulation of endogenous Arm protein.

degradation and as a component of the cytoplasmic retention machinery.

UAS driven expression of full-length Arm does not cause cell fate transformations, Wg activation (Orsulic et al., 1996), or accumulation of Arm in the nucleus. Though one might expect increased Arm levels to titrate Axin leading to Wg activation, this is not observed. Our results suggest that the expression levels are not high enough to overcome the degradation machinery, because both endogenous Arm and UAS-expressed full-length Arm continue to be degraded, and Wg signaling is not activated. However, the same expression system driving ΔArm does cause Wg activation. The intrinsic stability of ΔArm and its potential myristoylation might lead to longer interaction with Axin, and its localization to the membrane. This may allow some endogenous Arm to bypass cytoplasmic anchoring and destruction, and accumulate in the nucleus. As our western analyses indicate, the bypass of degradation is not high compared to *axin* and *zw3* mutants, but must be significant enough to cause Wg activation.

Nuclear import of Arm

Nuclear import of Armadillo/β-catenin is crucial for activation of the transcriptional response to Wg signaling. Wg stabilizes cytoplasmic pools of Arm/β-catenin that must subsequently be imported into the nucleus to activate Wg targets. The mechanism of Arm/β-catenin stabilization has been studied extensively (Salic et al., 2000; reviewed by Wodarz and Nusse, 1998; Peifer and Polakis, 2000), but the understanding of nuclear import of Arm/β-catenin remains vague. Studies have shown that β-catenin nuclear import is independent of importinβ/β-karyopherin, instead it depends on the direct interaction of the central Armadillo (Arm) repeats to the nuclear pore complex. β-catenin contains 12 tandem Arm repeats which are necessary and sufficient for nuclear accumulation (Funayama et al., 1995). Arm repeats are fundamentally similar to the HEAT repeats of importinβ/β-karyopherin (Malik et al., 1997), suggesting that β-catenin may interact directly with the pore complex as importinβ/β-karyopherin does. Indeed, Fagotto et al. (Fagotto et al., 1998) found that β-catenin binds directly to a yeast nucleoporin, Nup1. These studies suggest that β-catenin does not use the standard NLS/importin dependent import pathway (reviewed by Mattaj and Englmeier, 1998), but instead supplies an importin-like activity itself.

Two studies have found that β-catenin import is constitutive (Fagotto et al., 1998 and Yokoya et al., 1999). They suggest a system of cytoplasmic and nuclear anchors that control the flow of β-catenin into and out of the nucleus. However, prevention of import by cytoplasmic anchoring may be the regulated step, since export is probably controlled by APC (see below). In resting cells, β-catenin is observed mostly at the cell membrane, therefore it seems likely that localization of β-catenin to this compartment prevents it from entering the nucleus. Axin has been observed to localize to the plasma membrane, as well as the cytoplasm (Fagotto et al., 1999), and is thus well positioned to function as an anchor. We observed a strong nuclear localization of Arm in experiments where no Axin protein was present. In contrast, overexpressed Axin prevented the nuclear accumulation of Arm normally associated with ΔArm expression.

Since Arm import and export have been reported to be highly dynamic (Yokoya et al., 1999), a second mechanism must be in place to retain the imported Arm within the nucleus. One possibility is that dTCF/Pan anchors nuclear Arm to the DNA. By expressing a dominant negative form of TCF that interacts with DNA but no longer binds Arm, we were able to block the nuclear accumulation observed following ΔArm expression alone. Overexpressed dTCFΔN may occupy many of the DNA binding sites that Arm normally uses to stay in the nucleus, making it susceptible to export. Expression of dTCFΔN did not lead to complete exclusion of endogenous Arm from the nucleus, suggesting that there may be more relevant nuclear factors, possibly *groucho* (Cavallo et al., 1998) or CBP (Waltzer and Bienz, 1998). Overexpression of full-length dTCF did not lead to nuclear accumulation of endogenous Arm, suggesting that dTCF levels are not limiting. This is consistent with overexpression of dTCF having only a very subtle cuticle phenotype (van de Wetering et al., 1997). Overexpression of LEF-1 (a mammalian homologue of dTCF) in tissue culture cells, however, does lead to nuclear accumulation of β-catenin (Huber et al., 1996). We do not

observe this in *Drosophila* embryos, suggesting that limiting levels of nuclear anchor may be a feature of specific cell types that we have yet to observe in *Drosophila*.

We favor a model where the dynamic import and export of Arm is controlled by binding partners in the cytoplasm and the nucleus. Axin is involved in cytoplasmic anchoring, and dTCF/Pan is involved in nuclear retention. Arm retained in the cytoplasm is degraded unless it enters adherens junctions. In response to Wg, degradation stops, and Arm accumulates in the cytoplasm bound to Axin. Some Arm enters the nucleus where it binds dTCF/Pan. As a result of active import and export, and inactive degradation an equilibrium is reached. This is the situation in Arm stripes where diffuse staining throughout the cell is observed. However, the existence of anchoring offers a second level of signaling control that could induce a rapid and concentrated nuclear accumulation of Arm with no change in levels. Specific nuclear accumulation has been observed in *Xenopus* (Schneider et al., 1996) and sea urchin (Logan et al., 1999). Though levels were not measured, the striking lack of cytoplasmic β-catenin is suggestive of a lack of cytoplasmic anchoring. Another response of this type may be what is observed in the epithelial to mesenchyme transition. Here, ILK was overexpressed in epithelial cells resulting in very high nuclear accumulation of β-catenin without an increase in levels, suggesting the possibility of inhibition of cytoplasmic anchoring (Novak et al., 1998).

Recently, two studies have suggested that APC is involved in the nuclear export of Arm/β-catenin (Rosin-Arbesfeld et al., 2000; Henderson, 2000). They found that APC contains a nuclear export signal (NES) which is required for efficient export of β-catenin from the nucleus. Combining this result with our data, we propose that there are at least two levels of control of Arm/β-catenin localization involving cytoplasmic anchoring and active export. APC may play a role in preventing Arm/β-catenin from accumulating in the nucleus due to dTCF binding. Both controls must be overcome to accumulate enough Arm/β-catenin to activate transcription. We are currently undertaking studies to ascertain the role of APC and its control in our ΔArm system for nuclear transport.

We thank all the members of the Wieschaus and Schupbach labs for helpful discussions. We are grateful to K. Willert, R. Nusse, J. P. Vincent, D. St. Johnson, M. Peifer, K. Bassler, T. Akiyama, and the Developmental Studies Hybridoma Bank for providing stocks and reagents. We thank R. Hoang, J. Thomas, Y. Ahmed, and A. Nouri for critical reading of the manuscript, J. Goodhouse for help with confocal microscopy, and R. Samanta for technical assistance. This work was supported by National Institutes of Health grants 5RO1HD22780 and PO1CA41086 to E. W.

REFERENCES

Ahmed, Y., Hayashi, S., Levine, A. and Weischaus, E. (1998). Regulation of Armadillo by a *Drosophila* APC inhibits neuronal apoptosis during retinal development. *Cell*. **93**, 1171-1182.

Bienz, M. 1998. TCF: transcriptional activator or repressor? *Curr. Opin. Cell Biol*. **10**, 366-372.

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

Brunner, E., Peter, O., Schweizer, L. and Basler, K. (1997). *pangolin* encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in *Drosophila*. *Nature* **385**, 829-833.

Cavallo, R. A., Cox, R. T., Moline, M. M., Roose, J., Polevoy, G. A., Clevers, H., Peifer, M. and Bejsovec, A. (1998). Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature*. **395**, 604-608.

Chou, T. B. and Perrimon, N. (1992). Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. *Genetics.* **131**, 643-653.

Cox, R. T., Pai, L. M., Miller, J. R., Orsulic, S., Stein, J., McCormick, C. A., Audeh, Y., Wang, W., Moon, R. T. and Peifer, M. (1999a). Membrane-tethered *Drosophila* Armadillo cannot transduce Wingless signal on its own. *Development.* **126**, 1327-1335.

Cox, R. T., Kirkpatrick, C. and Peifer, M. (1996). Armadillo is required for adherens junction assembly, cell polarity, and morphogenesis during *Drosophila* embryogenesis. *J. Cell Biol.* **134**, 133-148.

Cox, R. T., Pai, L. M., Kirkpatrick, C., Stein, J. and Peifer, M. (1999b). Roles of the C terminus of Armadillo in Wingless signaling in *Drosophila*. *Genetics.* **153**, 319-332.

Fagotto, F., Glück, U. and Gumbiner, B. (1998). Nuclear localization signal-independent and importin/karyopherin-independent nuclear import of β-catenin. *Curr. Biol.* **8**, 181-190.

Fagotto, F., Jho, Eh., Zeng, L., Kurth, T., Joos, T., Kaufmann, C. and Costantini, F. (1999). Domains of axin involved in protein-protein interactions, Wnt pathway inhibition, and intracellular localization. *J. Cell Biol.* **145**, 741-756.

Funayama, N., Fagotto, F., McCrea, P. and Gumbiner, B. M. (1995). Embryonic axis induction by the armadillo repeat domain of beta-catenin: evidence for intracellular signaling. *J. Cell Biol.* **128**, 959-968.

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

Henderson, B. R. (2000). Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover. *Nature Cell Biol.* **2**, 653-660.

Huber, O., Korn, R., McLaughlin, J., Ohsugi, M., Herrmann, B.G. and Kemler, R. (1996). Nuclear localization of beta-catenin by interaction with transcription factor LEF-1. *Mech. Dev*. **59**, 3-10.

Logan, C. Y., Miller, J. R., Ferkowicz, M. J. and McClay, D. R. (1999). Nuclear beta-catenin is required to specify vegetal cell fates in the sea urchin embryo. *Development* **126**, 345-357.

McCrea, P. D., Turck, C. W. and Gumbiner, B. (1991). A homolog of the Armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science.* **254**, 1359-1361.

Malik, H. S., Eickbush, T. H. and Goldfarb, D. S. (1997). Evolutionary specialization of the nuclear targeting apparatus. *Proc. Natl. Acad. Sci. USA* **94**, 13738-13742.

Martinez Arias, A., Baker, N. and Ingham, P. W. (1988). Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103**, 157-170.

Mattaj, I. W. and Englmeier, L. (1998). Nucleocytoplasmic transport: the soluble phase. *An. Rev. Biochem.* **67**, 265-306.

Merriam, J. M., Rubenstein, A. B. and Klymkowsky, M. W. (1997). Cytoplasmically anchored plakoglobin induces a WNT-like phenotype in Xenopus. *Dev. Biol*. **185**, 67-81.

Miller, J. R. and Moon, R. T. (1997). Analysis of the signaling activities of localization mutants of beta-catenin during axis specification in Xenopus. *J. Cell Biol.* **139**, 229-43.

Müller, H. J. and Wieschaus, E. (1996). *armadillo*, *bazooka*, and *stardust* are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in *Drosophila*. *J. Cell Biol.* **134**, 149-163.

Novak, A., Hsu, S.C., Leung-Hagesteijn, C., Radeva, G., Papkoff, J., Montesano, R., Roskelley, C., Grosschedl, R. and Dedhar, S. (1998). Cell adhesion and the integrin-linked kinase regulate the LEF-1 and beta-catenin signaling pathways. *Proc. Natl. Acad. Sci. USA* **95**, 4374-4379.

Nüsslein-Volhard, C. and Wieschaus, E. (1980). Mutations affecting segment number and polarity in *Drosophila*. *Nature* **287**, 795-801.

Oda, H., Uemura, T., Harada, Y., Iwai, Y. and Takeichi, M. (1994). A *Drosophila* homologue of Cadherin associated with Armadillo and essential for embryonic cell-cell adhesion. *Dev. Biol.* **165**, 716-726.

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.

Pai, L. M., Kirkpatrick, C., Blanton, J., Oda, H., Takeichi, M. and Peifer, M. (1996). Drosophila alpha-catenin and E-cadherin bind to distinct regions of Drosophila Armadillo. *J. Biol. Chem*. **271**, 32411-32420.

Pai, L. M., Orsulic, S., Bejsovec, A. and Peifer, M. (1997). Negative regulation of Armadillo, a Wingless effector in *Drosophila*. *Development* **124**, 2255-2266.

Peifer, M. and Wieschaus, E. (1990). The segment polarity gene *armadillo* encodes a functionally modular protein that is the *Drosophila* homolog of human plakoglobin. *Cell* **63**, 1167-1178.

Peifer, M., McCrea, P. D., Green, K. J., Wieschaus, E. and Gumbiner, B. M. (1992). The vertebrate adhesive junction proteins beta-catenin and plakoglobin and the *Drosophila* segment polarity gene armadillo form a multigene family with similar properties. *J. Cell Biol.* **118**, 681-691.

Peifer, M., Pai, L. M. and Casey, M. (1994a). Phosphorylation of the Drosophila adherens junction protein Armadillo: roles for wingless signal and zeste-white 3 kinase. *Dev. Biol.* **166**, 543-556.

Peifer, M., Sweeton, D., Casey, M. and Wieschaus, E. (1994b). *wingless* signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. *Development.* **120**, 369-380.

Peifer, M. and Polakis, P. (2000). Wnt signaling in oncogenesis and embryogenesis–a look outside the nucleus. *Science.* **287**, 1606-1609

Polakis, P. (1999). The oncogenic activation of β-catenin. *Curr. Opin. Gen. Dev.* **9**, 15-21.

Rosin-Arbesfeld, R., Townsley, F. and Bienz, M. (2000). The APC tumour suppressor has a nuclear export function. *Nature.* **406**, 1009-1012.

Salic, A., Lee, E., Mayer, L. and Kirschner, M. W. (2000). Control of beta-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in Xenopus egg extracts. *Mol. Cell.* **5**, 523-532.

Schneider, S., Steinbeisser, H., Warga, R. M. and Hausen, P. (1996). Beta-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. *Mech. Dev.* **57**, 191-198.

Siegfried, E., Chou, T. B. and Perrimon, N. (1992). *wingless* signaling acts through *zeste-white 3*, the Drosophila homolog of glycogen synthase kinase-3, to regulate *engrailed* and establish cell fate. *Cell*. **71**, 1167-1179.

Torres, M. A. and Nelson, W. J. (2000). Colocalization and redistribution of dishevelled and actin during Wnt-induced mesenchymal morphogenesis. *J. Cell. Biol.* **149**, 1433-1442.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., Peifer, M., Mortin, M. and Clevers, H. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. *Cell* **88**, 789-799.

Waltzer, L. and Bienz, M. (1998). Drosophila CBP represses the transcription factor TCF to antagonize Wingless signalling. *Nature* **395**, 521-525.

White, P., Aberle, H. and Vincent, J.-P. (1998). Signaling and adhesion activites of mammalian β-catenin and Plakoglobin in Drosophila. *J. Cell Biol.* **140**, 183-195.

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.

Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Ann. Rev. Cell Dev. Biol.* **14**, 59-88.

Yokoya, F., Imamoto, N., Tachibana, T. and Yoshihiro, Y. (1999). β-catenin can be transported into the nucleus in a Ran-unassisted manner. *Mol. Biol. Cell* **10**, 1119-1131.

Zecca, M., Basler, K. and Struhl, G. (1996). Direct and long-range action of a Wingless morphogen gradient. *Cell* **87**, 833-844.