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

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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-Arm 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 (Zeca 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). armH8.6 is an EMS-induced allele creating a stop codon eliminating repeats (Hamada et al., 1999). The wild-type stock used was Oregon R. UAS-dTCF expresses full-length dTCF (van de Wetering et al., 1997). The ArmGAL4 line containing GAL4-VP16 under the control of the maternal Armadillo promoter was a gift from D. St. Johnston (Cambridge University, UK). The 67.15 stock containing second and third chromosomal inserts of GAL4-VP16 under the control of the zygotic Armadillo promoter was a gift from (van de Wetering et al., 1997). UAS-dTCF expresses full-length dTCF (van de Wetering et al., 1997). A cross was constructed to produce germlines and embryos for a dominant negative form of dTCF, which acts as a transactivator to drive expression of a dominant negative form of dTCF. Crosses and generation of germline clones were generated to produce germlines and embryos from which UAS alleles could be crossed to FRT armH8.6;TM3 so that the only non-balancer females that are fertile must contain a germline homozygous for armH8.6. These females were then crossed to armH8.6;TM3 males to produce embryos maternally and zygotically armH8.6 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 MgSO4, 1% Nonidet P-40). The aqueous phase was removed and an equal amount of methanol was added to demethylize 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 lyzed, the extracts were separated on 8% SDS-PAGE, and blotted as described by Peifer et al. (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.

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 (Zeca 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 these 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, DArm localizes to the membrane (Fig. 1B), whereas S10 localizes to the nucleus (Fig. 1B and Pai et al., 1997). The surface localization of DArm may be due to myristoylation, but this raises the question of how DArm 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 DArm. This is readily apparent in the large cells of the amnio serosa (Fig. 1B). Using an earlier driver (67.15 GAL4), we observe that DArm 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 DArm, but contain only mutant forms of endogenous arm. We used the strong allele arm043A01 (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 DArm 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 DArm show the wild-type En expression pattern of one or two cell stripes (Fig. 2A, second panel). In contrast, arm/arm embryos expressing DArm show only nervous system En expression (Fig. 2A, fourth panel), similar to arm/arm embryos without DArm (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...
independently of arm043A01. As shown in Fig. 2B, expression of S10 in arm043A01; arm043A01 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 arm043A01; 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 (arm043A01; 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 arm043A01; 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 arm043A01/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 arm043A01/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.

Fig. 2. ΔArm is dependent on endogenous Arm to activate Wg signaling, while S10 is not. arm043A01 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 arm043A01; 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 (arm043A01; 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 arm043A01; 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 arm043A01/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 arm043A01/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.
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 H8.8, 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).

**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 germine clones homozygous for zw3 that
showed cuticular phenotypes similar to ∆Arm 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 ∆Arm lanes. However, the total level of endogenous Arm protein does not differ significantly between ∆Arm and wild-type lanes, although the two Arm bands in the wild-type lane are collapsed into a single band in the ∆Arm lane. These data suggest that ∆Arm 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 ∆Arm-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 ∆Arm appears to act by a mechanism independent of protein levels, we investigated whether it is independent of zw3 as well. We expressed ∆Arm in zw3 germline clone embryos. As shown in Fig. 5C, expression of ∆Arm leads to the nuclear localization of Arm, demonstrating that ∆Arm can force even the much increased Arm levels found in zw3 germline clones into the nucleus. This suggests that ∆Arm functions independently of Arm protein levels to promote nuclear localization of endogenous Arm. ∆Arm appears to act downstream of zw3 by affecting a second step, which retains endogenous Arm protein in the cytoplasm.

Fig. 4. (Top) Protein levels in ∆Arm-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 zw3M11; 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 axin germline clone crossed to axin/TM3, therefore half the embryos will receive a zygotic wild-type copy of axin 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 ∆Arm lane contains extracts from four embryos where the 67.15 GAL4 driver was crossed to ∆Arm, therefore all embryos express ∆Arm. 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. ∆Arm 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) zw3M11; ArmGAL4/+ crossed to wild type. The embryo was also stained with α-Sxl to separate out the females (not shown). (C) zw3M11; ArmGAL4/+ crossed to ∆Arm. The embryo was also stained with α-HA to find embryos expressing ∆Arm, and with α-Sxl to separate out the females (not shown).
Axin and dTCF/Pan regulate Arm localization

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. dTCFAN 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 dTCFAN 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 armXM19, but not through armO043A01. The former is truncated after repeat 12, whereas the truncation in armO043A01 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 armXM19 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 armXM19 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 armXM19 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 armXM19 protein available for signaling. This protein remains sensitive to Wg control leading to a wild-type cuticle.

However, under these conditions if armXM19 retained all wild-type functions, one would expect the ΔArm phenotype in armXM19 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 armXM19 protein. They may reflect quantitative differences in the levels of armXM19 and wild-type protein, or the inability of armXM19 to activate all Wg transcriptional targets. Both these models assume that armXM19 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 armXM19 ΔArm embryos might reflect a direct effect on cell junctions. Although armXM19 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 armXM19 protein from junctions, but unlike the full-length wild-type protein, it cannot itself substitute for the released armXM19 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
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/importinophilin, 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/importinophilin (Malik et al., 1997), suggesting that β-catenin may interact directly with the pore complex as importinβ/importinophilin 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 dTCFαN may occupy many of the DNA binding sites that Arm normally uses to stay in the nucleus, making it susceptible to export. Expression of dTCFαN did not lead to complete exclusion of endogenous Arm from the nucleus, suggesting that there may be more relevant nuclear factors, possibly groucho (Cavallaro 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

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.

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, dTCFαN embryos show a lack of nuclear accumulation of endogenous Arm expected from ΔArm alone. (B) 67.15; dTCFαN embryos also show a lack of nuclear accumulation. (C) 67.15; dTCF (full length) embryos do not show nuclear accumulation of endogenous Arm protein.
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.

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**REFERENCES**


Axin and dTCF/Pan regulate Arm localization


