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Membrane-tethered *Drosophila* Armadillo cannot transduce Wingless signal on its own

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SUMMARY

***Drosophila* Armadillo and its vertebrate homolog β-catenin are key effectors of Wingless/Wnt signaling. In the current model, Wingless/Wnt signal stabilizes Armadillo/β-catenin, which then accumulates in nuclei and binds TCF/LEF family proteins, forming bipartite transcription factors which activate transcription of Wingless/Wnt responsive genes. This model was recently challenged. Overexpression in *Xenopus* of membrane-tethered β-catenin or its paralog plakoglobin activates Wnt signaling, suggesting that nuclear localization of Armadillo/β-catenin is not essential for signaling. Tethered plakoglobin or β-catenin might signal on their own or might act indirectly by elevating levels of endogenous β-catenin. We tested these hypotheses in *Drosophila* by removing endogenous Armadillo. We generated a series of mutant Armadillo proteins with altered intracellular localizations, and expressed these in wild-type and *armadillo* mutant backgrounds. We found that membrane-tethered Armadillo cannot signal on its own; however it can function in adherens junctions. We also created mutant forms of Armadillo carrying heterologous nuclear localization or nuclear export signals. Although these signals alter the subcellular localization of Arm when overexpressed in *Xenopus,* in *Drosophila* they have little effect on localization and only subtle effects on signaling. This supports a model in which Armadillo's nuclear localization is key for signaling, but in which Armadillo intracellular localization is controlled by the availability and affinity of its binding partners.**

Key words: Armadillo, β-catenin, Wingless, Wnt, Drosophila

INTRODUCTION

Normal embryonic development unfolds in a series of remarkable events. Among these is the process by which individual cells, descended from a common cellular ancestor, differentiate into the many cell types of the adult. A relatively small number of intercellular signaling molecules direct an inordinate number of cell fate decisions. This led to an intense focus on these ligands and the proteins which receive and transduce their signals. For example, signaling via Wingless (Wg)/Wnt glycoproteins is critical for normal development in both insects and mammals, while inappropriate activation of their signal transduction pathway contributes to human cancer. Several biological systems have made critical contributions to our understanding of this pathway. Many components were identified in *Drosophila* by virtue of mutations that altered cell fate decisions in the embryonic epidermis. In *Xenopus*, mammals and *C. elegans* novel proteins involved in signaling have also been identified.

We can assemble much of this data into a working model for how Wg/Wnt1 signals alter cell fates (reviewed by Cox and Peifer, 1998; Gumbiner, 1998). The pivotal molecule in Wg/Wnt signaling is Armadillo (Arm; vertebrate β-catenin (βcat)), the stability of which is regulated by signaling. In the absence of Wg/Wnt signal Arm/βcat is either assembled into cell-cell adherens junctions, where it supports cell adhesion, or is rapidly destroyed. Destruction requires Zeste-white3 kinase (vertebrate glycogen synthase kinase-3β), the tumor suppressor adenomatous polyposis coli (APC), and Axin. By an unknown mechanism, these proteins direct ubiquitination and destruction of Arm/β-cat, perhaps via the F-box protein *slimb* (Jiang and Struhl, 1998). Wg/Wnt ligands bind Frizzled family receptors, which act via Dishevelled to inactivate the destruction machinery. As a result, levels of Arm/βcat outside adherens junctions rise, allowing free Arm/βcat to enter nuclei and bind DNA-binding proteins of the TCF/LEF family. Together they activate expression of Wg/Wnt responsive genes.

While this model explains much of the data, other issues remain unresolved. For example, APC's role is controversial (reviewed by Cox and Peifer, 1998). Biochemical studies in mammalian cells strongly support a role for APC as a negative regulator of signaling; likewise, *Drosophila* APC negatively regulates Arm, though perhaps only in a few tissues (Ahmed et al., 1998). In contrast, data from *C. elegans* and *Xenopus*

suggest that APC positively promotes signaling. Likewise, TCF proteins appear to play opposite roles in flies and *C. elegans*.

Here we focus on a different discrepancy: the necessity of Arm/βcat nuclear localization for signaling. Our working model in *Drosophila* suggests that Arm influences cell fate by acting together with dTCF in the nucleus. This was called into question, however, by provocative experiments in *Xenopus* showing that membrane-tethered versions of βcat or its paralog plakoglobin could activate Wnt signaling. Wnt signaling in *Xenopus* is assayed by the ability of injected mRNA to induce an ectopic embryonic dorsal-ventral axis. Injection of βcat or plakoglobin mRNA leads to axis duplication (Funayama et al., 1995; Karnovsky and Klymkowsky, 1995). Merriam et al. (1997) created membrane-tethered forms of plakoglobin, preventing nuclear translocation; it was expected that this would prevent signaling. Surprisingly, membrane-tethered plakoglobin caused axis duplication (Merriam et al., 1997), as did membrane-tethered βcat (Miller and Moon, 1997). Several possibilities could explain this: (1) nuclear localization might not be critical for Arm/βcat signaling, (2) signaling may occur by different mechanisms in flies and frogs, or (3) tethered plakoglobin/βcat might bind APC and block destruction of endogenous βcat, which thus signals. The two studies came to different conclusions: Miller and Moon (1997) conclude that tethered βcat prevents destruction of endogenous βcat while Merriam et al. (1997) conclude that tethered plakoglobin binds TCFs, preventing them from repressing Wnt responsive genes. This latter model received further support from genetic data suggesting that the *C. elegans* Arm relative antagonizes the *C. elegans* TCF relative (reviewed by Cox and Peifer, 1998) and from data supporting TCF's role as a repressor of Wg/Wnt responsive genes (Riese et al., 1997; Brannon et al., 1997; Cavallo et al., 1998; Roose et al., 1998). To distinguish between these two models, we altered the nuclear localization of Arm in *Drosophila*, where we can remove endogenous Arm and thus test the ability of mutant proteins to function on their own.

MATERIALS AND METHODS

Mutants

Arm-TM, -NLS, and -NES were generated by using PCR to add targeting sequences to Arm's N terminus: Arm-TM, the *Xenopus* N-cadherin transmembrane domain (Detrick et al., 1990); Arm-NLS, the nuclear localization sequence of SV40 large T-antigen (Kalderon et al., 1984); Arm-NES, the nuclear export sequence of rabbit pKI (Wen et al., 1995). All carry a 5× c-myc epitope at Arm's C terminus; the control is wild-type Arm with a similar tag (Arm-WT). All were cloned into pUAST (Brand and Perrimon, 1993). Arm-CAAX was in an *arm* minigene with a c-myc tag in the C-terminal domain (Orsulic and Peifer, 1996). The 18 C-terminal amino acids of human KRas4B were added to Arm's C terminus as a PCR-derived fragment flanked by *Bgl*II sites cloned into a *Bgl*II site introduced just before the stop codon. The *y w* stock (arm^{+}) was used for microinjection and as a control.

Biochemistry

GAL4 stocks were from the Bloomington *Drosophila* stock center. To analyze protein expression, embryos from crosses of transformant lines to *e22c*-GAL4 */CyO* were collected at various times after egg laying, protein extracts were made and analyzed by immunoblotting with anti-Arm and ECL detection (Amersham). To detect phosphoisoforms we used alkaline phosphatase-coupled secondary Ab, NBT and BCIP (Promega). Cell fractionation of embryo extracts was as described by Peifer (1993). The soluble fraction was concentrated using a 10 kDa Millipore membrane (Millipore). Immunoprecipitation with anti-c-myc was as described by Orsulic and Peifer (1996). Dilutions for immunoblotting: monoclonal anti-c-myc (9E10; undiluted), monoclonal anti-Arm 7A1 (Peifer et al., 1994a; 1:500), monoclonal anti-BicD (Suter and Steward, 1991; 1:30), monoclonal anti-α-catenin (Oda et al., 1993,1:100), and monoclonal anti-E-cadherin (Oda et al., 1994,1:20).

Immunofluorescence

Embryo collection, antibody incubations and washes were as described by Peifer et al. (1993). Primary antibody incubation was for 1 hour at 25°C or overnight at 4°C. Secondary antibody treatment was as described by Cox et al. (1996). Fixation conditions: anti-β-galactosidase antibody (1:200; Cappel), 4% formaldehyde in PEM+1%NP-40, and methanol dechorionation; monoclonal c-myc antibody (affinity purified; 1:100), Arm-TM and Arm-NES, fixation as described by Orsulic and Peifer (1996); monoclonal anti-c-myc, Arm-NLS and Arm-WT and monoclonal anti-Arm 7A1 (1:200); 5 minutes in 37% formaldehyde and hand devitellinization.

Xenopus experiments

Immunofluorescence of Arm constructs in *Xenopus* was as described by Miller and Moon (1997). To assess ability to induce Wnt-responsive genes, two-cell *Xenopus* embryos were injected at the animal pole with 2 ng synthetic RNA encoding GFP, Arm-WT, Arm-NES, Arm-NLS and Arm-TM. Animal cap explants were cut at stage 8 and RNA preparation and RT-PCR was carried out as described by Cui et al. (1995).

Genetics

arm^{YD35}, $arm^{H8.6}$, arm^{XM19}, arm^{XP33} and arm^{S10} are described by Peifer et al. (1993, 1994a), Cox et al. (1996) and Pai et al. (1997). Genetic tests were carried out at 25°C with two or more independent lines of each mutant (*UAS-arm-X*). Eggs were collected for 24 hours and hatch rates determined, and cuticle preparations were made of both hatched larvae and unhatched embryos. Tests: (1) Dominant phenotypes. Cross *e22c*-GAL4*/CyO* females × *UAS-arm-X* homozygous males. (2) Rescue of the zygotic null arm^{YD35}. Cross arm^{YD35}*/FM7; e22c*-GAL4*/CyO* females to *UAS-arm-X* homozygous males. (3) Rescue of animals maternally and zygotically arm^{XM19} mutant. Generate germline clones of arm^{XM19} (Peifer et al., 1993) and cross *e22c*-GAL4/+ females carrying such germline clones to *UAS-arm-X* homozygous males. Tests of *arm-CAAX* were similar, but did not include a GAL4 driver. For *arm-CAAX* we also generated germline clones of arm^{XP33} crossed these females to *arm-CAAX* homozygous males.

RESULTS

Mutant Arm proteins

We designed a series of mutant *arm* genes which should alter Arm subcellular localization (Fig. 1A; see Methods for details) to address two questions: (1) is Arm nuclear localization critical for signaling, (2) if not, by what mechanism might membrane-tethered Arm alter signaling? Two constructs tethered Arm to the membrane. Arm-CAAX carries the ras lipid modification signal, addition of which drove membrane localization of other proteins (e.g., Quilliam et al., 1994). Arm-TM carries the *Xenopus* N-cadherin transmembrane domain, and thus is identical in design to βcat-TM, which activates Wnt

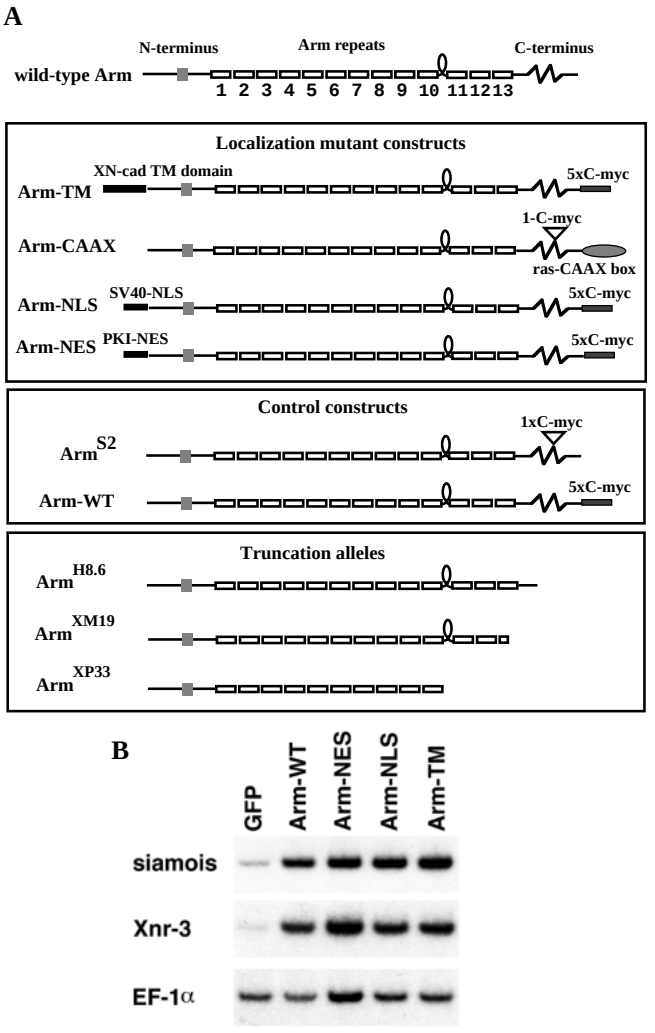

Fig. 1. Arm mutants used in this work. (A) Diagrams of the structure of mutant constructs used in this work. (B) Overexpression of Armadillo localization mutants activates expression of Wnt responsive genes in *Xenopus.* RNAs encoding GFP (control), Arm-WT, Arm-NES, Arm-NLS and Arm-TM were injected at the animal pole of 2-cell stage embryos and the expression of the Wnt responsive genes *siamois* and *Xnr-3* were assayed in animal cap explants by RT-PCR. Injection of GFP results in very low levels of *siamois* and *Xnr-3* expression while injection of Arm-WT, Arm-NES, Arm-NLS and Arm-TM drove high levels of expression of both genes. EF-1α serves as a control for the reverse transcriptase reaction and gel loading.

signaling in *Xenopus* (Miller and Moon, 1997). As a control we created a wild-type Arm construct, Arm-WT, with a similar five-fold myc-epitope tag. We tested both Arm-TM and Arm-WT in *Xenopus* for ability to activate Wnt signaling upon injection of RNA; both drove expression of the Wnt-responsive genes *siamois* and *Xnr-3* (Fig. 1B; Arm-CAAX was made as a minigene with *arm* introns and thus could not be tested in this way). Two other constructs were designed to alter the fraction of nuclear Arm. Arm-NLS carries a heterologous nuclear localization signal (NLS) while Arm-NES carries a heterologous nuclear export signal (NES). Arm-NLS and Arm-NES parallel the βcat-NLS and βcat-NES constructs we previously tested in *Xenopus* (Miller and Moon, 1997). We also tested Arm-NLS and Arm-NES for their ability to activate Wnt signaling in *Xenopus* upon injection of RNA; like their βcat counterparts (Miller and Moon, 1997), these Arm constructs could activate Wnt responsive genes (Fig. 1B). In addition to these new Arm constructs, we also used a set of pre-existing *arm* mutants (Fig. 1A).

To circumvent dominant lethal effects, we used the inducible GAL4-UAS system (Brand and Perrimon, 1993) with which mutants can be introduced into *Drosophila* in a silent state and later activated in specific temporal and spatial patterns. We used the GAL4 driver *e22c*-GAL4, which is expressed relatively ubiquitously from embryonic stage 10 (Fig. 2A,B). We repeated certain experiments with *arm*-GAL4 (White et al., 1998), which produced overlapping or slightly stronger phenotypes (data not shown). While use of the GAL4-UAS system circumvents dominant lethality, it has a disadvantage. The lack of maternal GAL4 expression and the delay before sufficient GAL4 accumulates to drive transcription of UAS constructs mean that activation with GAL4 drivers begins later in development than expression of endogenous *arm*. Arm-CAAX was expressed under the control of the *arm* promoter; fortunately it did not have dominant effects.

Tethered Arm cannot signal on its own

We examined the subcellular distribution of Arm-CAAX and Arm-TM both in situ and by subcellular fractionation. Arm-CAAX is expressed both maternally and zygotically from the beginning of development. Prior to and during cellularization, both wild-type Arm (Fig. 2D) and Arm-CAAX (Fig. 2C) are excluded from nuclei. Arm-CAAX is somewhat enriched at the cortex and is punctate, perhaps vesicular in the cytoplasm (Fig. 2C). Later in development, Arm-CAAX (Fig. 2E) is greatly enriched at the plasma membrane relative to wild-type Arm (Fig. 2F). However, Arm-CAAX is also found within cells in a punctate distribution (Fig. 2E), perhaps reflecting association with internal cell membranes. Like wild-type Arm, Arm-CAAX accumulates in a segmentally striped pattern, suggesting that it can be recognized by the destruction machinery that regulates levels of wild-type Arm.

When βcat-TM was expressed in *Xenopus*, it accumulated in the perinuclear region of each cell (Miller and Moon, 1997). When we expressed Arm-TM in *Xenopus* embryos, it also localized to a perinuclear region and was excluded from nuclei (Fig. 2J). Despite this dramatic mis-localization, both βcat-TM (Miller and Moon, 1997) and Arm-TM (Fig. 1B) activate Wnt-responsive genes in *Xenopus*. When we expressed Arm-TM driven by *e22c*- GAL4 in *Drosophila* embryos, it was first detected in mid-germ band extension, when it accumulates at high levels in a perinuclear area of most cells; this may represent the ER and/or the Golgi apparatus (Fig. 2G-I). In some cells Arm-TM accumulates to a level higher than that of endogenous Arm during its peak stage of accumulation (stage 9; Fig. 2I). The cell-cell variation in the level of Arm-TM accumulation may reflect the patchiness of the *e22c* -GAL4 driver (Fig. 2A,B).

These localization data were consistent with membrane-tethering, but could not resolve protein bound to internal membranes from protein free in the cytoplasm. To distinguish this, we fractionated embryonic cells into soluble (S100) and membrane-associated (P100) fractions and compared the subcellular distribution of Arm-CAAX and Arm-TM to that of

wild-type Arm. Wild-type Arm is enriched in the membrane-associated fraction (Fig. 3A; M=P100), reflecting its interaction with cadherins (Fig. 3B; Peifer, 1993), but if one concentrates the soluble fraction, wild-type Arm is also easily detectable (Fig. 3A; S=S100). In contrast, both Arm-CAAX and Arm-TM were exclusively in the membrane fraction (Fig. 3A), demonstrating the efficacy of membrane-targeting. Arm-CAAX also interacts with Arm's normal junctional partners. We used the myc-tag on Arm-CAAX to specifically immunoprecipitate mutant protein, and found that both DE-cadherin and α-catenin co-immunoprecipitate (Fig. 3B). The apparent molecular mass of Arm-CAAX shifts by much more than can be accounted for by the 31 additional amino acids, consistent with lipid modification.

Membrane-tethered plakoglobin (Merriam et al., 1997), βcat (Miller and Moon, 1997) and Arm (Fig. 1B) all activate Wnt signaling in wild-type *Xenopus*. In contrast, neither Arm-CAAX nor Arm-TM disrupted cell fate choices in wild-type *Drosophila*. Arm-CAAX lines are adult viable and fertile, and thus Arm-CAAX has no significant effect on Wg-signaling or cell adhesion in a wild-type background. While two of three Arm-TM lines tested caused embryonic lethality, they did so without altering Wg-dependent cell-fate choices. Embryos expressing Arm-TM had defects in head development and occasionally failed to retract their germband (Fig. 4B), defects reminiscent of weak DE-cadherin mutants (Uemura et al., 1996; Tepass et al., 1996) which fail during late morphogenetic movements. Arm-TM may thus have weak dominant negative effects on adherens junctions. The third Arm-TM line had no effect on embryogenesis.

We tested the ability of tethered Arm-CAAX to signal by expressing it in a background with virtually no functional Arm. We used embryos maternally and zygotically mutant for the nearly null allele arm^{XP33}, which retains very little function in either junctions or signaling. In such embryos the embryonic epithelium is disrupted due to loss of functional adherens junctions, leading to a fragmented cuticle (Fig. 4C; Cox et al., 1996). Arm-CAAX completely rescued the adherens junction function of arm^{XP33} mutants; cuticle integrity is fully restored (Fig. 4D). In contrast, Arm-CAAX completely failed to rescue the Wg signaling defect of arm^{XP33} (Fig. 4D); the embryos had a *wg* null-like phenotype, similar to that of embryos maternally and zygotically mutant for arm^{XM19} (Fig. 4E). At the same level of expression, wild-type Arm on a similar transgene fully rescues the arm^{XP33} patterning defects (Cox et al., 1996). Thus in the near complete absence of endogenous Arm, tethered Arm cannot signal.

e22c-GAL4 and other ubiquitous GAL4 drivers initiate expression too late in development to rescue arm^{XP33}, even when driving Arm-WT (Pai et al., 1997 and unpublished data), and thus we could not test Arm-TM in this context. Instead, we tested it in embryos expressing only the C-terminally truncated *arm* mutant Arm^{XM19}, which

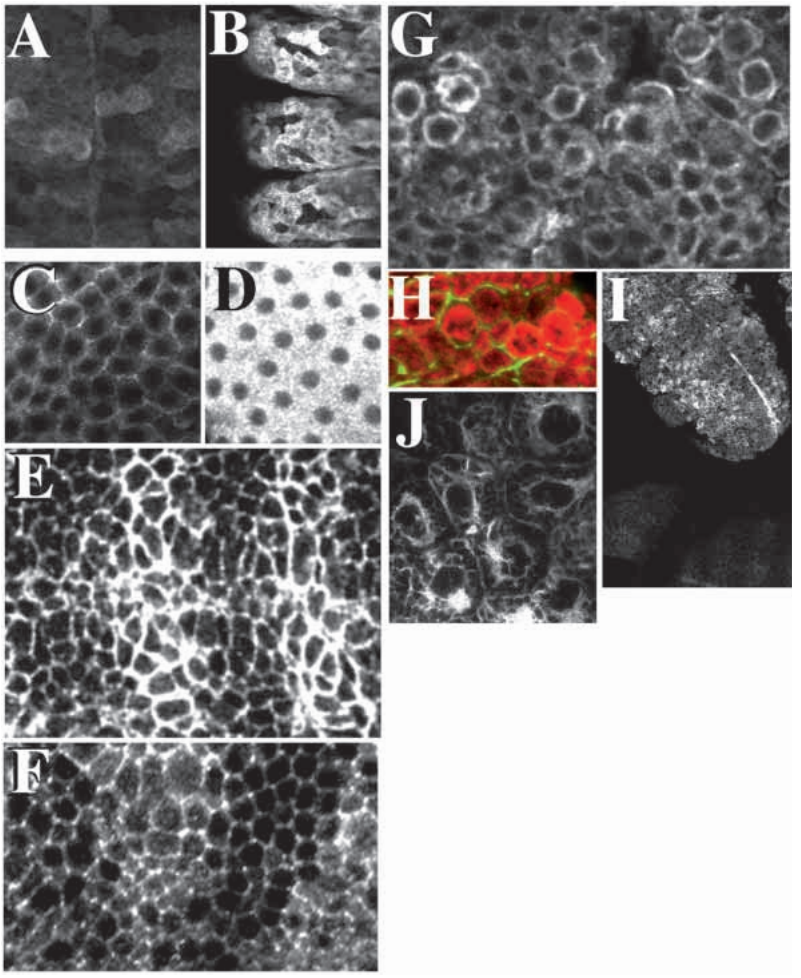

Fig. 2. Arm-CAAX and Arm-TM localization. (A,B) UAS-βgal expression driven by the *e22c*-GAL4 driver. Expression begins in stage 10 (A) and increases through dorsal closure (B). Differences in expression between cells become more pronounced as development proceeds. (C-F) Localization of Arm-CAAX versus wild-type Arm. In C-E, embryos were stained with anti-myc, recognizing Arm-CAAX; F was stained with anti-Arm, recognizing endogenous wild-type Arm. (C) Arm-CAAX and (D) wild-type Arm (Arm^{S2}) localization in pre-blastoderm embryos. Arm-CAAX is enriched at membranes and also accumulates in a punctate fashion inside cells. Arm^{S2} is excluded from nuclei and accumulates diffusely in the cytoplasm. (E) Arm-CAAX and (F) wild-type Arm in stage 9 embryos. Arm-CAAX is heavily enriched at the plasma membrane, and accumulates at lower levels in a punctate fashion in the cytoplasm. Wild-type Arm accumulation is lower at the membrane and heavier in the cytoplasm. Arm-CAAX levels are elevated in segmentally reiterated cells, presumably those receiving Wg. (G-J) Arm-TM localization. G-H are stained with anti-myc (anti-myc is red in H; the green channel shows phalloidin for detection of cortical actin); I is stained with anti-Arm, recognizing both Arm-TM and endogenous wild-type Arm. (G) Stage 11 embryo. Arm-TM accumulates in rings in the perinuclear region of each cell. (H) Simultaneous localization of Arm-TM (red) and cortical actin (green). The rings of Arm-TM are inside cells and not at the plasma membrane. (I) Arm-TM accumulates to levels higher than wild-type endogenous Arm. A stage 11 Arm-TM expressing embryo (top) is paired with a non-transgenic sibling at stage 9 (bottom), the peak accumulation stage of wild-type Arm. (J) Arm-TM expressed in *Xenopus* embryos localizes to a perinuclear region and is excluded from nuclei.

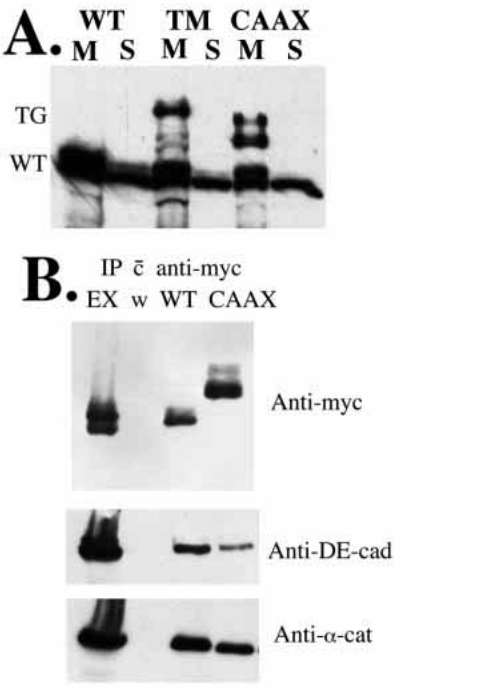

Fig. 3. Arm-CAAX is membrane-tethered and binds DE-cadherin and α-catenin. (A) Cell extracts from embryos expressing no transgene, Arm-TM or Arm-CAAX were fractionated into soluble (S=S100) and membrane (M=P100) fractions. The soluble fraction was concentrated ten-fold. Wild-type (WT) is found in both soluble and membrane fractions, while Arm-TM and Arm-CAAX (TG=transgene) are completely absent from the soluble fraction. (B) DE-cadherin and α-catenin co-IP with both wild-type Arm and Arm-CAAX. Proteins were immunoprecipitated (IPed) with anti-myc antibody from extracts of non-transgenic embryos (w) or from embryos expressing wild-type myc-tagged Arm^{S2} (WT) or Arm-CAAX (CAAX). Total extract of Arm^{S2} (EX) or IPs as indicated were fractionated by SDS-PAGE and sequentially immunoblotted with anti-myc, anti-DE-cad and anti-α-catenin antibodies.

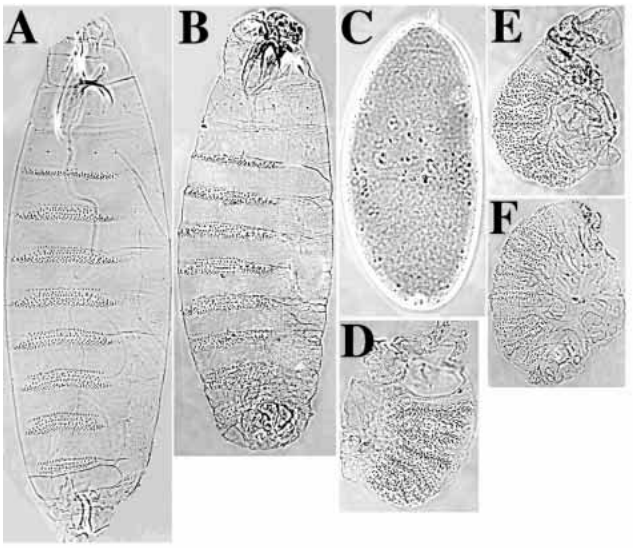

Fig. 4. Membrane-tethered Arm cannot signal on its own. (A) Wild-type embryo; anterior cells of each segment secrete denticles and posterior cells secrete naked cuticle. Embryos expressing Arm-CAAX were also wild-type. (B) Wild-type embryos expressing Arm-TM die with failure of head involution. (C) Embryo maternally and zygotically mutant for arm^{XP33} (still in vitelline membrane). Epithelial tissues are disrupted; embryos only secrete scraps of cuticle. (D) arm^{XP33} embryo expressing Arm-CAAX. Note complete rescue of cuticular integrity, but total failure to rescue Wg signaling defects. (E) Embryo maternally and zygotically mutant for arm^{XM19}, with defects in Wg signaling. (F) Embryo maternally and zygotically mutant for arm^{XM19} and also expressing Arm-TM. Arm-TM has little rescuing ability.

is substantially impaired in signaling (Peifer et al., 1994a). Arm-TM did not significantly promote signaling by Arm^{XM19} (Fig. 4F), leading to only subtle rescue of signaling, while similar expression of Arm-WT fully rescued signaling. This further suggests that tethered Arm cannot signal on its own, and suggests that the activation of Wnt signaling caused by over-expression of either βcat-TM (Miller and Moon, 1997) or Arm-TM (Fig. 1B) in *Xenopus* occurs at least in part by activation of the endogenous βcat.

Added nuclear localization or export signals do not alter Arm localization

While our current model for Wg signaling suggests that the key regulatory step is control of Arm stability, another potential point of regulation is nuclear import. In fact, Arm localization in the ectoderm supports this possibility (Fig. 5L). We knew that cells that do not receive Wg accumulate Arm at the plasma membrane, while cells that receive Wg also accumulate Arm in the cytoplasm and nucleus at relatively equal levels (Peifer et al., 1994a). However, upon more detailed examination we found that at the borders of the stripes of Arm accumulation one sees cells with higher levels of Arm in the cytoplasm than in the nucleus (Fig. 5L, arrows), suggesting that nuclear entry may also be regulated.

To further examine this issue, we attempted to alter Arm nuclear import by adding heterologous NLS or NES signals. Very similar βcat constructs were previously examined in *Xenopus* (Miller and Moon, 1997), where the added signals dramatically redirected βcat. We first tested these Arm constructs in *Xenopus*, where the added signals substantially altered the localization of Arm as expected. Arm-WT accumulates at high levels in nuclei and at lower levels at the cortex (Fig. 5A), while Arm-NLS accumulates almost exclusively in nuclei (Fig. 5B) and Arm-NES levels in nuclei are substantially reduced relative to the cortex (Fig. 5C).

We then examined these constructs in *Drosophila*. Arm-WT accumulated at the plasma membrane of all cells (Fig. 5D) and at much higher levels in a subset of cells whose position and number varied from embryo to embryo (Fig. 5G). This uneven accumulation likely reflects the mosaic expression of *e22c*-GAL4 (Fig. 2A,B). In cells where Arm-WT accumulated to the highest levels, it accumulated relatively uniformly in the cytoplasm and the nucleus, though occasionally the level in the nucleus seemed a bit lower (Fig. 5G, arrow).

We then compared the localization of Arm-NLS and Arm-NES in *Drosophila*, using both anti-myc antibody, which specifically recognizes mutant protein, and antibodies against Arm which recognizes both wild-type endogenous and mutant

protein. Both Arm-NLS (Fig. 5E,H,K) and Arm-NES (Fig. 5F,I) expressing embryos had higher levels of overall Arm (endogenous plus transgenic Arm) in all cells relative to non-transgenic siblings, as well as having substantially elevated Arm accumulation in a subset of cells. In *Drosophila*, unlike in *Xenopus*, the added NLS or NES signals have no noticeable effect on subcellular localization; the localizations of Arm-WT (Fig. 5G,J), Arm-NLS (Fig. 5H,K) and Arm-NES (Fig. 5I) were essentially indistinguishable. These data support a model in which Arm localization is primarily determined by the availability and affinity of its binding partners (see below).

We then analyzed the biological function of these mutant Arm proteins, first in a wild-type background. We tested six Arm-NLS lines, five Arm-NES lines, and, as a control, five Arm-WT lines. Three of the six Arm-NLS lines tested had dominant phenotypes suggesting constitutive activity in Wg signaling (Fig. 6B,C); two had a strongly activated phenotype similar to arm^{S10} (Fig. 6B), and the other was weaker (Fig. 6C). The other three Arm-NLS lines had no dominant effects. The dominant Arm-NLS lines had elevated levels of Arm protein accumulation (see below). In contrast, five of the six Arm-NES lines and all five Arm-WT lines had no dominant effects. The sixth Arm-NES line had a weak dominant effect, resulting in a novel phenotype with aspects of both constitutive activation and dominant negative activity; denticles were ablated at the midline, but additional denticles were found in the lateral naked cuticle region (Fig. 6D). We also examined the ability of Arm-NLS and Arm-NES to rescue the arm^{XM19} maternal and zygotic mutants. Arm-NES behaved indistinguishably from Arm-WT in this assay (Fig. 6F vs. 6I). Arm-NLS lines also rescued Wg signaling defects. Arm-NLS lines without dominant effects behaved like Arm-WT (Fig. 6G), while an Arm-NLS line with constitutive activity also showed this activity in the arm^{XM19} background (Fig. 6H). These data suggest that enhancing nuclear import subtly promotes signaling.

Levels of expression and phosphorylation

To determine whether observed differences in function seen were due to differences in expression levels, we examined each mutant for protein accumulation. Arm-CAAX, under the control of the *arm* promoter, accumulated to levels similar to or slightly lower than those of wild-type Arm (Fig. 7D). We expressed the other constructs under the control of *e22c*- GAL4. *e22c*-GAL4-driven Arm-WT is nearly undetectable before 6 hours of embryonic development, reaches approx. 10% of endogenous Arm levels by 6-12 hours, matches endogenous Arm by 12-18 hours, and substantially exceeds endogenous Arm at 18-24 hours (Fig. 7A). Arm-NES and Arm-TM accumulated to levels similar to or somewhat lower than those of Arm-WT. Arm-NLS lines varied more substantially, with some lines accumulating more protein than Arm-WT while others accumulated less (Fig. 7A,B); lines with higher levels of accumulation had dominant phenotypes. Ser/Thr

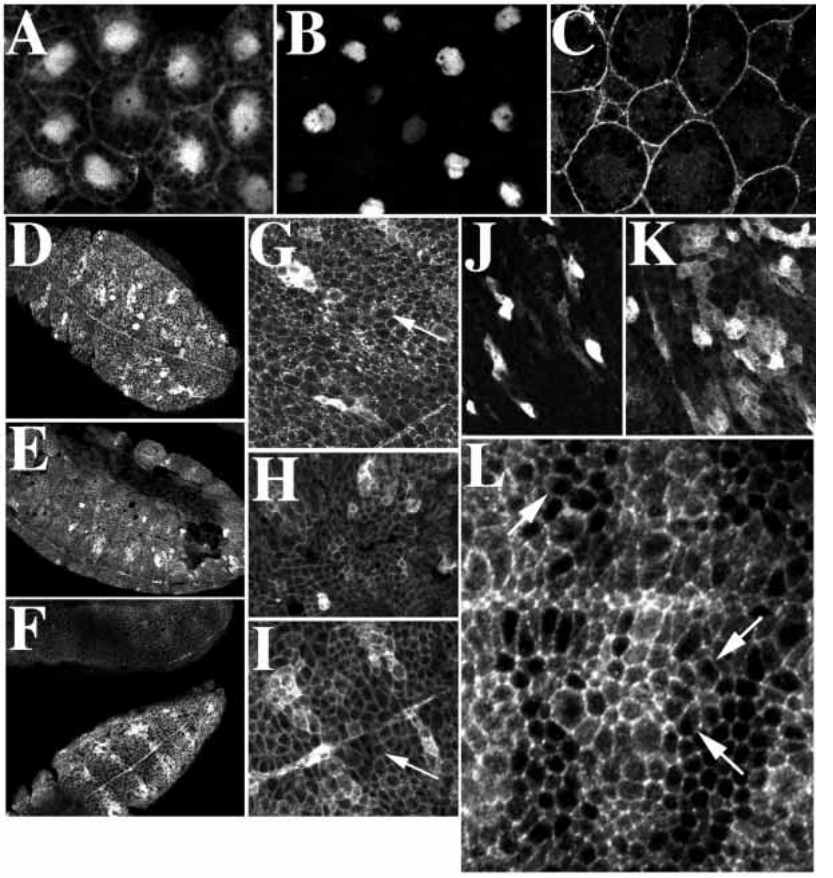

Fig. 5. Added NLS or NES alter Arm's intracellular localization in *Xenopus* but not *Drosophila*. (A-C) Localization of Arm mutants in wild-type *Xenopus* embryos. Arm-WT (A) localizes primarily to nuclei, with lower levels at the cortex. Arm-NLS (B) localizes almost exclusively to nuclei. Arm-NES (C) localizes primarily to the cortex, though some remains in nuclei. D-K. Arm-WT, Arm-NLS, or Arm-NES in wild-type *Drosophila* embryos, visualized using either anti-myc antibody, recognizing only transgene-encoded protein or anti-Arm which also recognizes endogenous Arm. All were driven by *e22c*-GAL4. (D,G) Stage 11 embryos expressing Arm-WT stained with anti-Arm. Arm-WT accumulates in all cells, primarily at the plasma membrane. Certain cells accumulate Arm-WT at substantially higher levels, both in the cytoplasm and nuclei. The number and position of such cells varies from embryo to embryo. Slight nuclear exclusion is sometimes seen in Arm-WT (arrow in G), Arm-NLS and Arm-NES (arrow in I). (E,H) Arm-NLS accumulation at stage 11 (visualized with anti-myc) is very similar to that of Arm-WT. All cells accumulate Arm-NLS at the plasma membrane while a few cells accumulate higher levels of Arm-NLS in the cytoplasm and nucleus (H). (F,I) Arm-NES accumulation at stage 11 (F, anti Arm; I, anti-myc). Arm-NES accumulates at the plasma membrane of all cells, and at higher levels in stripes of cells in each segment, presumably cells receiving Wg signal. (F) Arm-NES expressing embryos (bottom) accumulate higher levels of total Arm (wild-type endogenous plus Arm-NES) than sibling non-transgenic embryos (top). (J) Arm-WT accumulation at stage 14 visualized with anti-Arm. (K) Arm-NLS at stage 13, visualized with anti-myc. No nuclear enrichment is evident. (L) Arm nuclear import may be regulated in some cells. Wild-type embryo stained with anti-Arm. At the borders of the Arm stripes, certain cells have higher levels of Arm in the cytoplasm than in nuclei (arrows).

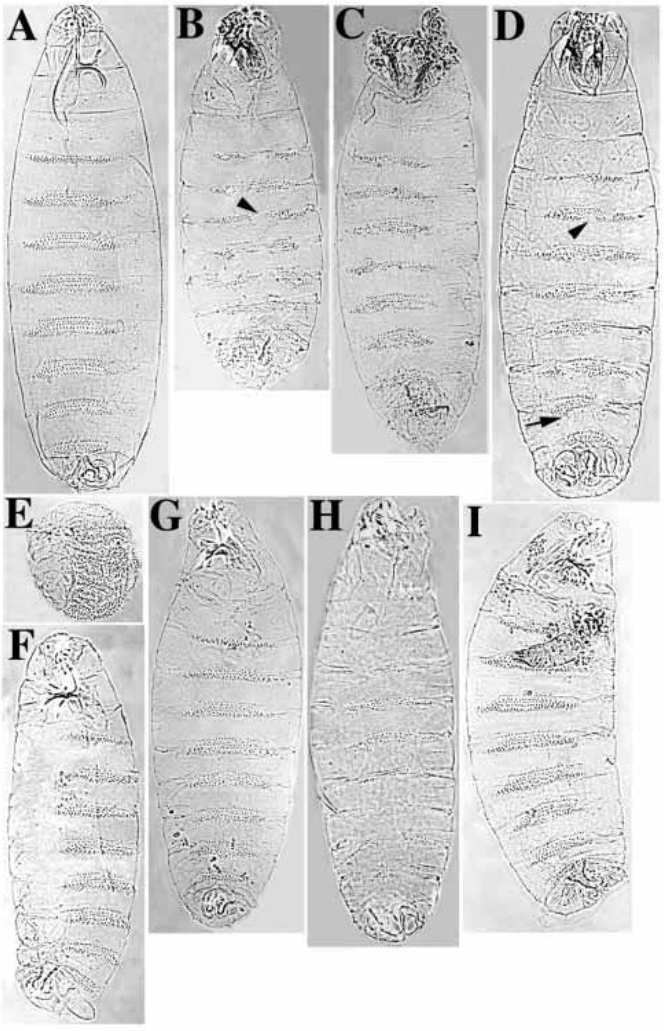

Fig. 6. Arm-NLS lines often have a dominant activated phenotype. (A) Wild-type embryo. (B,C) Two lines expressing Arm-NLS in a wild-type background. Note dominant activated phenotype, resulting in partial loss of denticle belts and their replacement by naked cuticle (arrowhead). (D) The sole Arm-NES line with a dominant phenotype. Note partial loss of denticles along the ventral midline (arrowhead), and occasional ectopic denticles laterally (arrow). (E) Embryo maternally and zygotically mutant for arm^{XM19}. (F) Embryo maternally and zygotically mutant for arm^{XM19} expressing Arm-NES. The segment polarity phenotype is substantially rescued. (G,H) Embryos maternally and zygotically mutant for arm^{XM19} expressing Arm-NLS. The segment polarity phenotype is substantially rescued, and in dominant lines the dominant phenotype remains. (I) Embryo maternally and zygotically mutant for arm^{XM19} expressing Arm-WT.

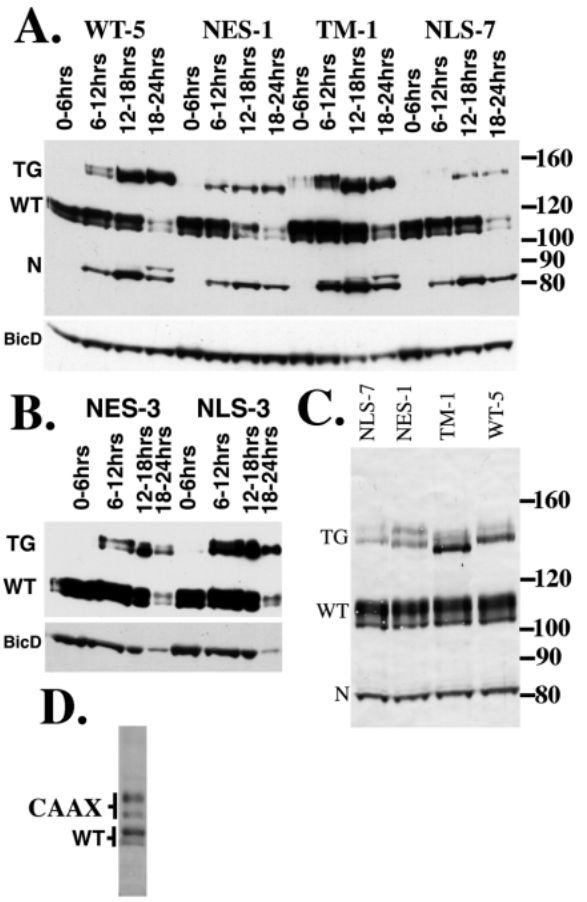

Fig. 7. Accumulation levels of Arm mutant proteins. Extracts from embryos expressing UAS-Arm constructs driven by *e22c*- GAL4 were immunoblotted with anti-Arm. A and B were reprobed with anti-BicD to control for loading. In A and C MW markers are indicated. (A-B) Time courses, after fertilization, of expression of Arm mutants. The species of Arm is indicated at the left. Identity of the transgene lines is indicated above; number suffix indicates insertion line. (C) Phosphorylation isoforms of Arm mutant proteins. Wild-type Arm (WT), neural Arm (N), transgene-encoded Arm (TG). (D) Level of expression and phosphorylation of Arm-CAAX. WT, wild-type Arm; CAAX, Arm-CAAX.

phosphorylation of Arm alters its mobility on SDS-PAGE (Fig. 7C; Peifer et al., 1994b). We can thus roughly estimate whether mutant proteins are normally phosphorylated. Arm in adherens junctions is more highly phosphorylated than soluble Arm; thus mutants which accumulate in the cytoplasm have lower apparent phosphorylation (Pai et al., 1997). Arm-CAAX phosphorylation was similar to that of endogenous Arm (Fig. 7D), consistent with its adherens junction localization. Arm-WT accumulated the same phosphoisoforms as wild-type Arm, but the relative levels of the most highly phosphorylated isoforms were lower, perhaps due to its accumulation in the cytoplasm and nucleus of certain cells (Fig. 5G). Arm-NLS, Arm-NES and Arm-TM behave like Arm-WT.

DISCUSSION

Recent work in vertebrates and *Drosophila* (reviewed by Cox and Peifer, 1998) suggested that Arm/β-cat acts together with nuclear partners in the TCF/LEF family, prompting a model in which Arm/βcat and TCF's form bipartite transcription factors which activate Wg/Wnt-responsive genes. However, data from the *Xenopus* system called into question the requirement for nuclear localization of Arm/βcat in signaling. We thus carried out a series of experiments to distinguish between these two hypotheses in *Drosophila*.

In *Drosophila*, membrane-tethered Arm cannot signal on its own

Membrane-tethered versions of βcat or of its paralog plakoglobin are incapable of entering the nucleus, and thus by the current model should be unable to signal. When expressed in *Xenopus* embryos, however, both activate Wnt signaling (Merriam et al., 1997; Miller and Moon, 1997). These data were explained in different ways by the two groups. One called the current model into question, suggesting that the real role of Arm/βcat is to bind and thus inactivate TCF/LEF family members, which otherwise would repress Wnt-responsive genes (Merriam et al., 1997). The other group presented evidence suggesting that membrane-tethered βcat sequesters APC, thus allowing endogenous wild-type βcat to accumulate and transduce signal rather than be destroyed (Miller and Moon, 1997).

To distinguish among these possibilities we removed endogenous Arm/βcat. We created two tethered versions of Arm, and demonstrated by cell fractionation that both are effectively membrane-tethered. When we expressed Arm-CAAX in embryos nearly null for endogenous Arm, it rescued the embryo's adherens junction defects but totally failed to function in Wg signaling; in contrast, wild-type Arm expressed at the same level fully rescues the pattern. Likewise, Arm-TM has very little or no signaling function in *Drosophila*, though it can activate Wnt responsive genes when over-expressed in *Xenopus*. Thus membrane-tethered Arm cannot signal on its own in *Drosophila*, suggesting that the signaling activity seen in *Xenopus* is at least in part due to endogenous βcat. In addition, neither Arm-CAAX nor Arm-TM activate Wg signaling in wild-type embryos, unlike what occurs in *Xenopus*. In flies, the level of misexpression was relatively low (≤2× normal Arm); this excess Arm can be accommodated without disrupting normal signaling. The levels of misexpression in frogs may be much more substantial, overloading the normal machinery for Arm/βcat destruction. While our data demonstrate that membrane-tethered Arm is not sufficient to trigger Wg signaling in *Drosophila*, we should note that our data do not refute the idea that Arm/βcat may also antagonize the repressive activity of TCF/LEF proteins (Riese et al., 1997; Brannon et al., 1997; Cavallo et al., 1998; Roose et al., 1998). This mechanism, championed by Klymkowsky and colleagues (Merriam et al., 1997), is likely to play an important part in Wg/Wnt signaling, and in some contexts, such as in early *C. elegans* embryos, may play the critical role in this process.

Arm intracellular localization is regulated by the availability and affinity of its binding partners

Many proteins contain signals directing their intracellular localization to specific subcellular compartments. Arm plays essential roles in a variety of cellular locations: at the plasma membrane, within the nucleus and likely in the cytoplasm. In each location it has distinct protein partners. Thus Arm localization must be regulated in a more complex fashion.

We favor a model in which Arm localization is regulated primarily by its binding partners. The capacity of each partner to bind Arm is determined both by the partner's accumulation level and its affinity for Arm. Data in vivo suggest that among Arm's known partners, cadherins have the highest affinity, with APC and dTCF having lower and lowest affinities, respectively. Thus, in embryos with reduced levels of Arm, the remaining Arm is exclusively associated with cadherins, as assayed by immunolocalization and by function (Cox et al., 1996). ≥70% of cellular Arm is cadherin-associated (Peifer, 1993). When cadherin binding sites are saturated, excess Arm binds to APC/Axin, leading to its destruction and thus preventing accumulation of free Arm. While APC levels, at least in mammalian cells, are relatively low relative to the total pool of βcat, Arm bound to APC is rapidly targeted for destruction, thus opening the way for the binding of additional Arm. Normally the destruction machinery can not only dispose of all non-junctional Arm, but is likely not near saturation, as Arm synthesis can be increased several-fold without biological consequences. When the destruction machinery is inactivated either by Wg signal or mutation, however, Arm is synthesized but not destroyed, and thus levels of Arm rise. APC can bind Arm but is likely rapidly saturated, allowing accumulation of sufficient Arm to allow dTCF to effectively compete for binding. DE-cadherin, dAPC, dTCF and other possible unknown partners together account for virtually all the Arm in a normal embryo; little if any free Arm is present (Peifer, 1993).

This model helps explain the differences in localization of the Arm-NLS and Arm-NES in flies and frogs. In *Xenopus*, added NLS or NES signals dramatically altered Arm's intracellular distribution as expected, while in *Drosophila* the distribution of Arm-WT, Arm-NLS and Arm-NES are indistinguishable. We propose that this reflects differences in the level of expression. In flies, mutant Arm accumulates at near wild-type levels, so its binding partners can accommodate the additional protein. Arm bound to cadherin at the plasma membrane is unavailable for nuclear import; likewise Arm in a complex with dTCF is not available for export. Thus Arm-NLS and Arm-NES localization is primarily determined by their binding partners, resulting in a near normal localization. In contrast, Arm-NLS and Arm-NES expression levels in *Xenopus* likely exceed that of both endogenous βcat or its binding partners. Free Arm is thus accessible to the nuclear import and export machinery, allowing its localization to be altered.

Given this, is nuclear localization of Arm a regulated step in Wg signaling in normal cells? The fact that a subset of cells accumulate cytoplasmic but not nuclear Arm suggests that nuclear import may be regulated. In the simplest situation, addition of an NLS ought to promote Arm nuclear accumulation and trigger signaling, while addition of a NES should antagonize signaling. However, heterologous targeting signals had only subtle effects on signaling. Arm-NES signaled like Arm-WT, while only a subset of the Arm-NLS lines were activated for signaling. We thus envision a different scenario. In cells in which the destruction machinery is on, no free Arm is available for nuclear import or export. In cells with intermediate levels of Wg signaling, the destruction machinery may be slowed, allowing accumulation of cytoplasmic Arm in complex with APC, but not to sufficient levels to saturate APC and allow nuclear import. Only when signaling is fully activated would sufficient free Arm accumulate for nuclear import. Addition of an NLS would thus only alter the balance in cells near the signaling threshold. Further, if nuclear Arm is bound to dTCF, it may be inaccessible to the nuclear export machinery. The mechanisms by which Arm/βcat enters nuclei remain unclear; dTCF-dependent and independent pathways

may exist (Huber et al., 1996; Orsulic and Peifer, 1996). The recent observation that βcat may mediate its own nuclear transport independent of importins further complicates the issue (Fagotto et al., 1998). We must thus now direct our efforts to establishing whether additional levels of regulation occur, beyond the simple regulation of Arm/βcat stability.

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