

Adenomatous polyposis coli is present near the minimal level required for accurate graded responses to the Wingless morphogen

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The mechanisms by which the Wingless (Wg) morphogen modulates the activity of the transcriptional activator Armadillo (Arm) to elicit precise, concentration-dependent cellular responses remain uncertain. Arm is targeted for proteolysis by the Axin/Adenomatous polyposis coli (Apc1 and Apc2)/Zeste-white 3 destruction complex, and Wg-dependent inactivation of destruction complex activity is crucial to trigger Arm signaling. In the prevailing model for Wg transduction, only Axin levels limit destruction complex activity, whereas Apc is present in vast excess. To test this model, we reduced Apc activity to different degrees, and analyzed the effects on three concentration-dependent responses to Arm signaling that specify distinct retinal photoreceptor fates. We find that both Apc1 and Apc2 negatively regulate Arm activity in photoreceptors, but that the relative contribution of Apc1 is much greater than that of Apc2. Unexpectedly, a less than twofold reduction in total Apc activity, achieved by loss of Apc2, decreases the effective threshold at which Wg elicits a cellular response, thereby resulting in ectopic responses that are spatially restricted to regions with low Wg concentration. We conclude that Apc activity is not present in vast excess, but instead is near the minimal level required for accurate graded responses to the Wg morphogen.

KEY WORDS: Adenomatous polyposis coli, Armadillo, Wingless, *Drosophila*

INTRODUCTION

In *Drosophila*, the secreted glycoprotein Wingless (Wg) activates a signal transduction pathway that is important for cell proliferation, cell fate specification and apoptosis during development. Wg functions as a morphogen, emanating from a localized source to elicit long-range, concentration-dependent cellular responses (Neumann and Cohen, 1997; Zecca et al., 1996). Wg elicits its biological effects by binding to the Frizzled and Arrow co-receptors (Bhanot et al., 1996; Bhanot et al., 1999; Chen and Struhl, 1999; Kennerdell and Carthew, 1998; Muller et al., 1999; Tamai et al., 2000; Wehrli et al., 2000). Activation of these receptors results in the stabilization and nuclear accumulation of Armadillo (Arm), a key transcriptional activator in the pathway (Riggleman et al., 1990). In the absence of Wg, Arm is targeted for phosphorylation and subsequent proteolysis by a destruction complex composed of the scaffolding protein Axin, the two Adenomatous polyposis coli proteins (Apc1 and Apc2), and two kinases, the glycogen synthase kinase 3 homologue Zeste-white 3 (Zw3), and casein kinase 1 (Ahmed et al., 1998; Hamada et al., 1999; Hayashi et al., 1997; Liu et al., 2002; McCartney et al., 1999; Siegfried et al., 1992; Willert et al., 1999; Yanagawa et al., 2002; Yu et al., 1999). Upon Wg stimulation, this destruction complex is inactivated, thereby resulting in increased Arm levels and transcriptional activity (Peifer et al., 1994; Siegfried et al., 1994; Tolwinski et al., 2003).

Previous work indicated that a spatial gradient of Wg activity is crucial for proper patterning of the wing and leg primordia (Lecuit and Cohen, 1997; Neumann and Cohen, 1996; Struhl and Basler,

1993; Zecca et al., 1996). A more recent study reveals that proper eye development also requires precise, concentration-dependent cellular responses to a gradient of Wg activity (Tomlinson, 2003). The retina is composed of approximately 800 ommatidia, each of which contains six outer (R1-R6) and two inner (R7 and R8) photoreceptors (PRs) (reviewed by Cook and Desplan, 2001; Mollereau and Domingos, 2005; Wolff and Ready, 1993). Ommatidia can be grouped into three functional categories, pale, yellow, and dorsal rim area (DRA), which are distinguished by opsin expression and light sensitivity. The outer PRs in each category express the same opsin, *Rhodopsin 1* (*Rh1*), whereas the inner PRs express one of four distinct opsins (Fortini and Rubin, 1990). Inner PRs in pale and yellow ommatidia express *Rh3* or *Rh4* in R7, and *Rh5* or *Rh6* in R8, respectively, allowing for color discrimination. By contrast, all inner PRs within DRA ommatidia are characterized by expression of *Rh3* and the transcription factor *homothorax* (*hth*), and by an increased diameter of their light-sensing organelles, or rhabdomeres (Fortini and Rubin, 1990; Tomlinson, 2003; Wernet et al., 2003). The DRA ommatidia function as polarized light sensors, and are spatially restricted to the two outermost rows at the dorsal margin of the retina, extending up to the dorsoventral equator.

Wg is required for proper patterning of the peripheral retina, and is expressed within a ring of cells in the presumptive head capsule that surrounds the retina. The spread of Wg from the head capsule to the retina results in a gradient of Wg morphogen activity that specifies three distinct fates in the peripheral ommatidia (Tomlinson, 2003) (Fig. 1A). The highest levels of Wg, found at the very perimeter of the eye, induce apoptosis of all photoreceptors at the retinal edge, leaving behind a peripheral rim of pigment cells (Lin et al., 2004; Tomlinson, 2003). Intermediate Wg levels, found just inside the pigment rim, specify the DRA ommatidia (Tomlinson, 2003; Wernet et al., 2003). Even lower Wg levels are sufficient to induce the formation of ommatidia that lack bristles, which are restricted to the three outermost rows of the retina (Cadigan et al., 2002; Tomlinson, 2003).

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How is a gradient of Wg activity translated into quantitatively distinct levels of Arm signaling that induce qualitatively distinct cellular responses? Specifically, how do the different components in the destruction complex contribute to the level of Arm signaling? Biochemical studies have indicated that Axin levels are approximately 5000-fold lower than the level of other members of the destruction complex, and have led to the model that Axin is the only limiting

component, whereas Apc is present in vast excess (Lee et al., 2003; Salic et al., 2000). To address this model, we examined how the reduction of Apc activity to different degrees affects Arm signaling, both in the absence of Wg, and within the Wg gradient. We assayed three concentration-dependent readouts of Arm signaling in PRs: DRA fate specification, shortening of PR length, and apoptosis, which are induced by progressively higher levels of Arm signaling. We find that both Apc1 and Apc2 negatively regulate Arm signaling in photoreceptors, but that the relative contribution of Apc1 is much greater than that of Apc2. Unexpectedly, we also find that a less than twofold reduction in total Apc activity, achieved by loss of Apc2, decreases the effective threshold at which Wg elicits a cellular response, thereby resulting in ectopic responses that are spatially restricted to regions with low Wg concentration. These results indicate that within the range of the Wg gradient, Apc activity is not present in vast excess, but instead is near the minimal level required for accurate patterning.

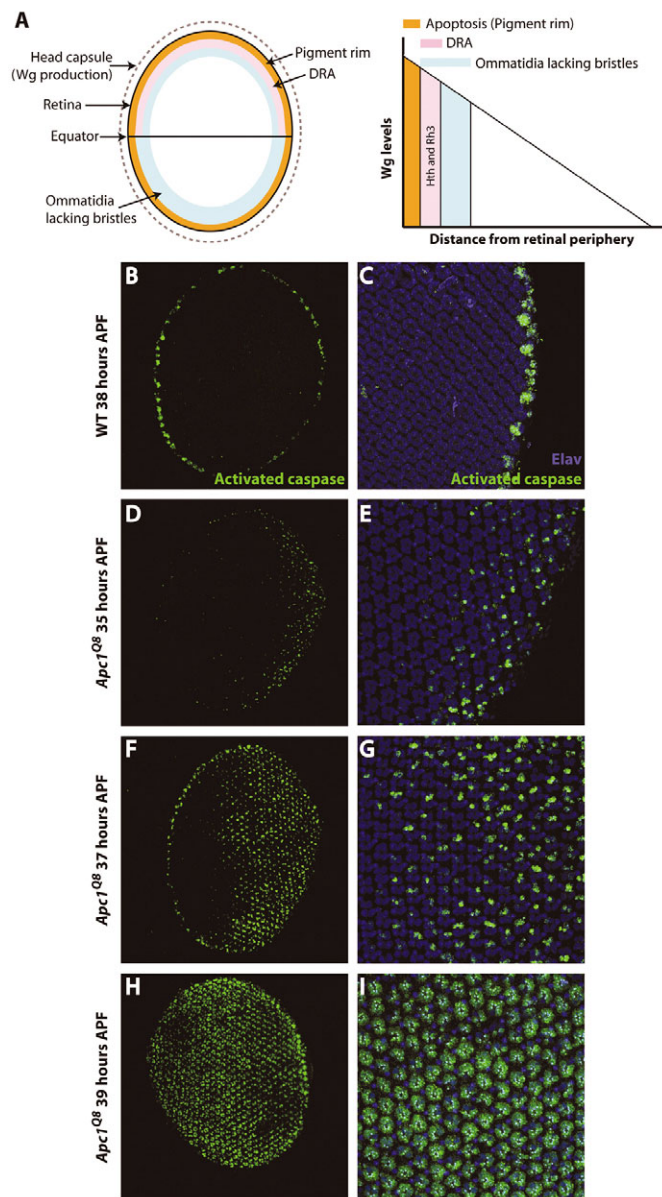


Fig. 1. *Apc1* loss results in apoptosis of photoreceptors. (A) Schematic of the retina. High levels of Wg signaling induce apoptosis of photoreceptors (orange), intermediate levels induce photoreceptors to adopt a DRA fate (pink), and low levels result in ommatidia that lack bristles (blue). (B-I) Pupal retinas were stained with antibodies against activated caspase (green) and Elav (blue). Elav is expressed in all photoreceptors. (B,C) In wild-type pupal retinas at 38 hours after puparium formation (APF), activated caspases are present only at the periphery. (D-I) In *Apc1^{Q8}* mutants, activated caspases are observed throughout the pupal retina. Caspase activation in photoreceptors is initially observed in the posterior retina at 35 hours APF (D,E), and, over the next four hours, extends to cover the entire retina (F-I).

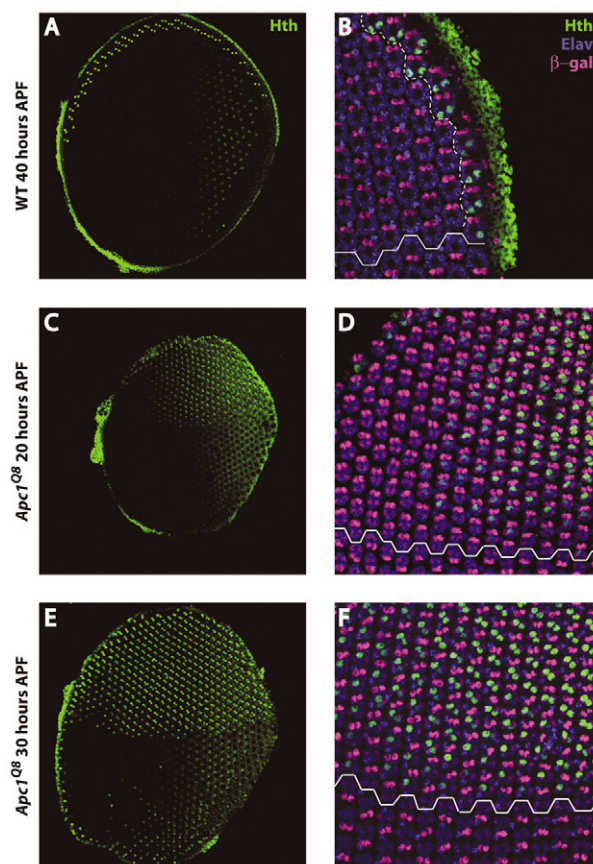


Fig. 2. *Apc1* inactivation induces an ectopic dorsal rim area (DRA) fate. Pupal retinas were stained with antibodies against Homothorax (Hth; green), Elav (blue; marking all photoreceptors) and β -gal (magenta). A *svp-lacZ* insertion allows demarcation of the dorsoventral equator (indicated by a solid white line in B, D and F). In all panels, dorsal is oriented to the top. (A,B) In wild-type pupal retinas, *hth* is expressed in all inner photoreceptors in the DRA, as well as in some pigment cells and the head capsule surrounding the retina. *Hth* expression (dashed white line in B) is restricted to one or two rows of ommatidia at the dorsal edge of the retina, and extends up to, or one ommatidial cluster above, the equator (B). (C-F) In *Apc1^{Q8}* mutant retinas, *hth* is expressed throughout the dorsal half of the retina, extending up to the equator or to one row above the equator (C,D: 20 hours APF; E,F: 30 hours APF).

MATERIALS AND METHODS

Fly stocks and genetics

All crosses were performed at 25°C. *Apc2*⁷⁹ has a deletion of the entire *Apc2* coding region, and also disrupts the neighboring gene *mRps24* (Takacs et al., 2008). In homozygous *Apc2*⁷⁹ mutants, loss of *mRps24* activity induces lethality. Therefore, to obtain viable *Apc2*⁷⁹ mutant adults, a *P[mRps24]* transgene (Takacs et al., 2008) was recombined onto the *Apc2*⁷⁹ mutant chromosome. *Apc2*³³ has a deletion of 1738 nucleotides that includes sequences encoding the first 349 amino acids, extending to the fifth Armadillo repeat (Takacs et al., 2008).

Other stocks used were *Apc1*^{Q8} (Ahmed et al., 1998), *P[Apc2*^{13.5}] (Ahmed et al., 2002), *svp*⁰⁷⁴⁸²-*PZ* [Heberlein et al. (Heberlein et al., 1991); Bloomington Drosophila Stock Center (BDSC)], *Rh3-lacZ* (Fortini and Rubin, 1990) (BDSC), *UAS-arm.Exel* (encoding full-length Arm, Exelixis, BDSC), *UAS-arm*^{S10} (Pai et al., 1997), *elav-GAL4* (Lin and Goodman, 1994) (BDSC), *GMR-Gal4 12*, line MF815 (Freeman, 1996), *long GMR-Gal4* and *short GMR-Gal4* (Wernet et al., 2003), *FRT82B arm-lacZ* [Vincent et al. (Vincent et al., 1994); provided by J. Treisman, Skirball Institute, New York], *eyeless-FLP* [Newsome et al. (Newsome et al., 2000); provided by J. Treisman], *Df(3L)H99 FRT80B* [White et al. (White et al., 1994); provided by F. Davidson, National Cancer Institute, Bethesda, MD], *Df(3R)w6* (provided by M. Bienz, MRC Laboratory of Molecular Biology, Cambridge, UK), *UAS-ara* (Gomez-Skarmeta et al., 1996), *piM75C FRT80B* (Xu and Rubin, 1993), *pygo*¹⁰ (Parker et al., 2002), *pan*¹³ and *pan*^{ER1} (Brunner et al., 1997), and *P[mRps24]*. Canton S flies were used as wild-type controls.

Generation of mitotic eye clones

Clones of mutant retinal cells were generated by FLP-mediated recombination (Xu and Rubin, 1993), using *eyeless-FLP* (Newsome et al., 2000). Clones were detected by loss of expression either of an *arm-lacZ* transgene in pupal retinas, or of a *P[w+]* transgene in adult eyes.

Genotypes for generating mutant eye clones were as follows:

*pygo*¹⁰ mutant clones: *eyeless-FLP/+; FRT82B pygo*¹⁰/*FRT82B arm-lacZ*;

*Apc2*³³ mutant clones: *eyeless-FLP/+; FRT82B Apc2*³³/*FRT82B arm-lacZ*;

Df(3L)H99 mutant clones in the homozygous *Apc1*^{Q8} mutant: *eyeless-FLP/+; Df(3L)H99 FRT80B Apc1*^{Q8}/*piM75C FRT80B Apc1*^{Q8}.

Immunohistochemistry

Primary antibodies used for immunostaining were guinea pig anti-Apc2 (GP10) 1:12,000 (Takacs et al., 2008), rabbit anti-Apc1 1:400 (Hayashi et al., 1997), rabbit anti-β-Gal 1:5000 (Cappel), mouse anti-β-Gal 1:500 (Promega),

guinea pig anti-Hth 1:500 (Abu-Shaar et al., 1999), rabbit anti-Hth 1:1000 (Kurant et al., 1998), mouse (9F8A9) or rat (7E8A10) anti-Elav 1:10 (Developmental Studies Hybridoma Bank, DSHB), rabbit anti-cleaved caspase-3 1:100 (Cell Signaling Technology), mouse anti-Arm 1:10 (N2 7A1, DSHB). Secondary antibodies were goat or donkey Alexa Fluor 488 or 568 conjugates 1:200 (Molecular Probes), and goat or donkey Cy3 or Cy5 conjugates 1:200 (Jackson Immunochemicals). Fluorescent images were obtained on a Leica TCS SP UV confocal microscope.

Pupal retinas were dissected in PBS, then fixed in 4% paraformaldehyde, 10 mM NaH₂PO₄ (pH 7.2) for 20 minutes, washed with PBS, 0.1% Triton X-100, and incubated in PBS, 0.1% Triton X-100, 10% BSA for 30 minutes at room temperature. For analysis with Apc1 and Arm antibodies, pupal retinas were fixed using heat/methanol (Ahmed et al., 1998). Incubation with primary antibodies was performed at 4°C overnight in BNT (PBS, 250 mM NaCl, 1% BSA, 1% Tween 20) or, if anti-cleaved caspase 3 was used, TBS [50 mM Tris-HCl (pH 7.4), 150 mM NaCl]. Incubations with secondary antibodies were for 2 hours at room temperature, or 30 minutes at room temperature if retinas were stained with the anti-cleaved caspase-3 antibody.

Histology

Adult eyes were dissected and fixed for whole mount X-Gal staining (Tomlinson, 2003), or were embedded in plastic resin (Durcupan, Fluka), sectioned to 1 μm and stained with Toluidine Blue (Cagan and Ready, 1989; Wolff, 2000).

RESULTS

Loss of Apc1 results in cellular responses characteristic of both high-level and intermediate-level Arm signaling activity in retinal photoreceptors

Inactivation of *Apc1* results in ectopic Arm signaling, which induces the apoptotic death of all retinal PRs (Ahmed et al., 1998). PR apoptosis, as visualized by the presence of an activated caspase, begins at the posterior edge of the *Apc1*^{Q8} null mutant retina by 35 hours after puparium formation (APF), and expands to encompass the entire retina by 39 hours APF (Fig. 1D-I). An identical onset and duration of caspase expression is observed in this ectopic apoptosis of all PRs resulting from Apc1 loss and in the developmentally regulated PR apoptosis induced by high-level Arm signaling restricted to the retinal periphery (Lin et al., 2004; Tomlinson, 2003) (Fig. 1B,C; see also Fig. S1 in the supplementary material). Indeed,

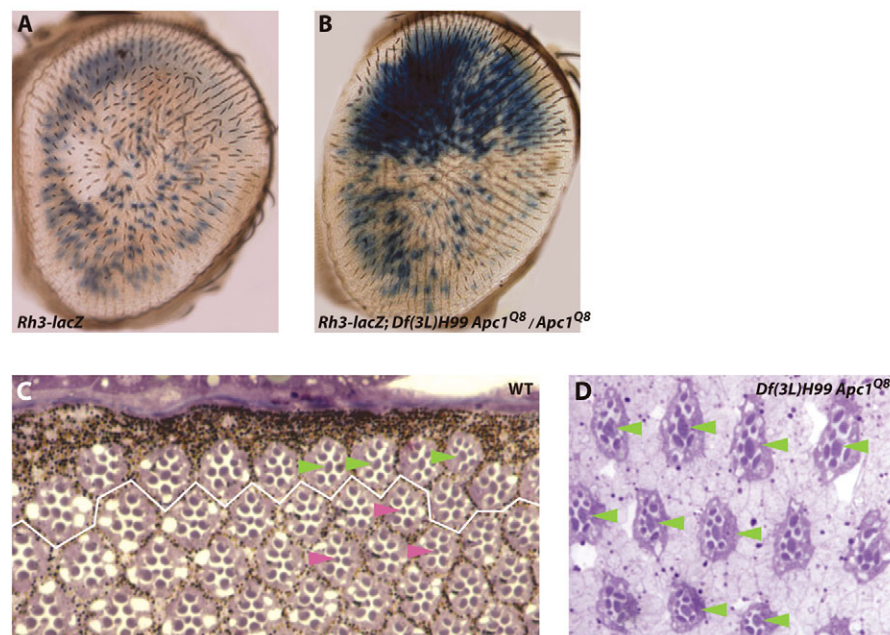


Fig. 3. Ectopic DRA ommatidia in the *Apc1* mutant retina. (A) Whole-mount adult retina of *Rh3>lacZ* flies stained with X-gal. Rh3 is expressed in 30% of all R7 cells, as well as in all R7 and R8 cells of the DRA ommatidia. (B) In *Apc1*^{Q8} flies heterozygous for *grim*, *reaper* and *hid* (*Df(3L)H99*), Rh3 is present in an increased number of PRs throughout the dorsal half of the retina. (C) Adult eye section from the dorsal-most regions of a wild-type retina. Ommatidia with a DRA fate are found above the solid white line, and are characterized by inner photoreceptors with enlarged rhabdomeres (green arrowheads). (D) A *Df(3L)H99* mutant clone located away from the retinal periphery in the *Apc1*^{Q8} mutant. Inner PRs with enlarged rhabdomeres are observed in many ommatidia that are not restricted to the dorsal rim (green arrowheads).

both of these retinal apoptotic responses can be observed simultaneously (Fig. 1D,F). These data suggest that ectopic apoptosis of all PRs upon *Apc1* loss is induced by high-level Arm signaling.

A gradient model for Wg signaling predicts that if *Apc1* loss results in high-level Arm signaling in all PRs, responses that require lower levels of Arm signaling would also be induced in these cells, but may be obscured by their apoptotic death. To address this hypothesis, we examined a response to intermediate-level Arm signaling, DRA fate specification (Wernet et al., 2003; Tomlinson, 2003). If either Wg or Arm is ectopically expressed at intermediate levels, many PRs inappropriately express *hth* and adopt a DRA fate (Tomlinson, 2003; Wernet et al., 2003). Conversely, inactivation of any one of several transducers of Wg signaling, including the cytoplasmic effector Disheveled (Dsh), the co-receptors Arrow (Arr) and Frizzled (Fz and Fz2; previously known as DFz2), or the transcriptional co-activator Pygopus (Pygo) (Kramps et al., 2002; Parker et al., 2002; Thompson et al., 2002), partially disrupts *hth* expression and DRA fate specification (Wernet et al., 2003; Tomlinson, 2003) (see Fig. S2 in the supplementary material).

We examined *hth* expression in the *Apc1^{Q8}* null mutant at 20 and 30 hours APF, prior to the time at which high-level Arm signaling induces PR apoptosis. We found a marked expansion in the range of *hth* expression throughout the dorsal half of the *Apc1* mutant retina (Fig. 2A-F). The expanded *hth* expression is apparent in the posterior retina by 20 hours APF (Fig. 2C,D). By 30 hours APF, ectopic *hth* expression is present in nearly all inner PRs in the dorsal half of the retina, and extends either up to or one row above the dorsoventral equator (Fig. 2E,F).

This ectopic *hth* expression suggests that *Apc1* mutant PRs would have adopted a DRA fate had they survived. To test this hypothesis, we prevented apoptosis in the *Apc1^{Q8}* mutant by using a deficiency that eliminates three cell-death effector genes *reaper*, *head involution defective (hid)* and *grim* (Lin et al., 2004; White et al., 1994). Inhibition of PR apoptosis reveals that many dorsal PRs adopt a DRA fate in the *Apc1* mutant adult, as indicated by both ectopic *Rh3* expression and increased rhabdomere diameter (Fig. 3). In wild-type flies, *Rh3* is present not only in both R7 and R8 of all DRA ommatidia, but also in 30% of R7 cells in pale ommatidia that are randomly distributed throughout the retina (Chou et al., 1996; Papatsenko et al., 1997) (Fig. 3A). By contrast, in the *Apc1* mutant retina, *Rh3* is found in many more inner PRs throughout the entire dorsal half of the retina (Fig. 3B), and a corresponding increase in the rhabdomere diameter of these inner PRs, indicative of DRA fate, is also observed (Fig. 3C,D). Taken together, our data support the previously proposed model for a retinal Wg gradient (Tomlinson, 2003), and also indicate that *Apc1* loss induces both PR apoptosis in response to high-level Arm signaling, and DRA fate specification, which is triggered by intermediate-level Arm signaling.

Intermediate-level Arm signaling is sufficient to induce ectopic *homothorax* expression in a fraction of ventral ommatidia

In the wild-type pupal retina, *Hth* is restricted primarily to the dorsal eye (Fig. 2A,B) by the dorsal selector genes *araucan (ara)*, *caupolican* and *mirror*, which encode homologous homeodomain transcription factors that form the Iroquois complex (IRO-C) (Gomez-Skarmeta et al., 1996; Wernet et al., 2003). Therefore, our expectation was that, in the *Apc1* mutant, ectopic *hth* expression would also be confined to the dorsal retina. However, we find that

ectopic *hth* expression is not only induced throughout the dorsal half of the retina, but is also present in a fraction of ventral PRs in the *Apc1^{Q8}* mutant (Fig. 4A,B).

Thus we sought to determine whether in the *Apc1* mutant, ectopic Arm signaling partially overrides the dorsal restriction of *hth* expression by IRO-C. Ectopic expression of any one member

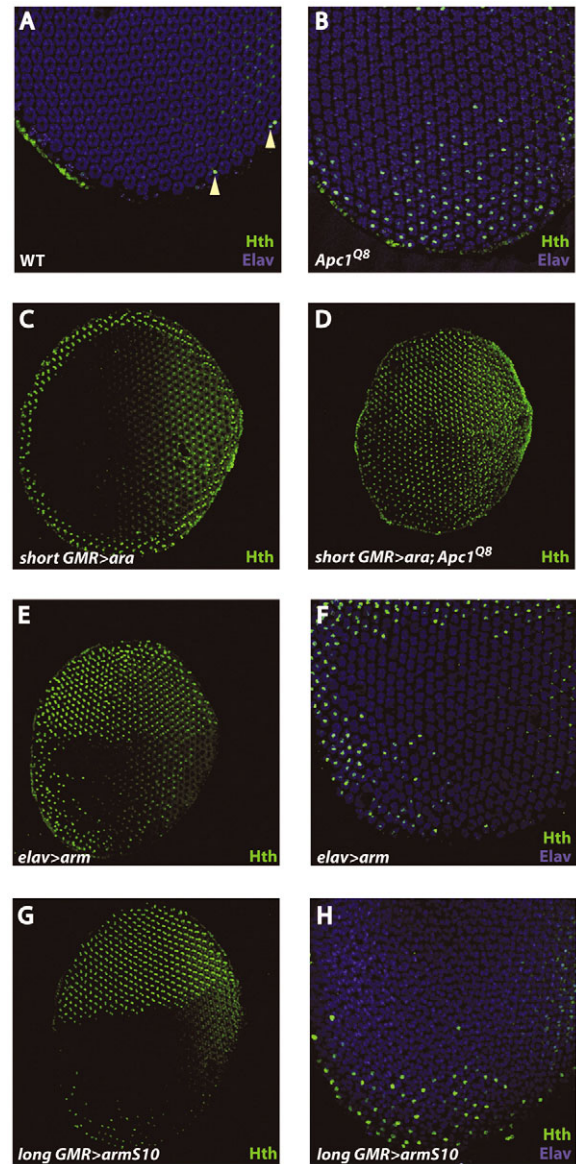


Fig. 4. Ectopic Wg signaling induces DRA fates in a fraction of ventral ommatidia. (A-D) *Apc1* loss and ectopic Arm signaling induces expression of *Hth* (green) in some ventral ommatidia. Photoreceptors are marked with anti-*Elav* (blue). (A) In wild-type retinas, *hth* expression is observed in rare photoreceptors at the ventral rim (arrowheads). (B) In *Apc1^{Q8}* mutant retinas, 30 hours APF, many photoreceptors near the ventral pole express *hth*. (C) In otherwise wild-type retinas, ectopic expression of the IRO-C protein *Ara*, under the control of a *GMR* promoter, induces the formation of an ectopic ventral rim area with one to three rows of ommatidia expressing *hth*. (D) In *Apc1^{Q8}* flies, *Hth* is found throughout the entire retina when *Ara* is expressed under the control of a *GMR* promoter. (E-H) Expression of full-length *Arm* (E,F) and constitutively active *Arm^{S10}* (G,H) under the control of *elav* and *long GMR* promoters, respectively, leads to the expansion of *Hth* expression (green) within the entire dorsal half of the retina, as well as in a fraction of ventral ommatidia. Photoreceptors are marked with anti-*Elav* (blue).

of the IRO-C complex in the ventral ommatidia of wild-type flies induces a large increase the number of PRs at the ventral periphery that express *hth*, resulting in the formation of an ectopic 'ventral rim area', containing one to three rows of *hth*-expressing ommatidia (Tomlinson, 2003; Wernet et al., 2003) (Fig. 4A,C). We expressed *ara* ectopically in all *Apc1* mutant PRs using an eye-specific *GMR* promoter, and found that, in the presence of *Ara*, all ommatidia in the ventral *Apc1* mutant retina express *hth*, although not always in both R7 and R8 (Fig. 4D). Thus, although *Apc1* loss induces *hth* expression in both the dorsal and ventral retina, this expression is restricted primarily to the dorsal half by the IRO-C proteins.

To determine whether Arm signaling is sufficient to induce *hth* expression not only in all dorsal ommatidia, but also in a fraction of ventral ommatidia, we expressed the constitutively activated Arm^{S10} protein (Pai et al., 1997) under control of a *GMR* promoter. In this genotype, no PR apoptosis is observed, indicating that high-level Arm signaling is not induced; however, intermediate-level Arm signaling in these *GMR>armS10* retinas is sufficient to induce *hth* expression not only in nearly all dorsal ommatidia, but also in a fraction of ventral ommatidia (Fig. 4G,H). To determine whether higher levels of Arm signaling would induce *hth* expression in all ventral ommatidia, we expressed full-length Arm in all photoreceptors under the control of an *elav* promoter, which drives strong expression in all neurons. In this genotype, nearly all PRs undergo apoptosis, indicating that high-level Arm signaling is induced throughout the retina (data not shown). We find that in *elav>arm* flies, *hth* expression not only expands to encompass the entire dorsal half of the retina, but also is present in an increased number of ventral ommatidia (Fig. 4E,F). However, despite the presence of high-level Arm signaling in all PRs, *hth* expression is found only in a fraction of ventral ommatidia, as is also observed in the *Apc1* mutant (Fig. 4B). We conclude that in a subset of ventral ommatidia, intermediate-level Arm signaling in PRs is sufficient to override the requirement for IRO-C in inducing *hth* expression. However, neither intermediate nor high-level Arm signaling can completely override this IRO-C requirement in all ventral ommatidia.

Intermediate-level Arm signaling induces a shortened photoreceptor length

In the wild-type retina, photoreceptors extend the entire length of the ommatidium, tapering slightly in diameter from the lens to the base (Wolff and Ready, 1993) (Fig. 5A-C). Inhibition of apoptosis in *Apc1* mutants, either by expression of the caspase inhibitor p35, or by elimination of the cell death effectors *reaper*, *grim* and *hid*, reveals that the majority of PRs are present, but that these surviving PRs are markedly shorter (Ahmed et al., 1998) (Fig. 5D,E). By comparison to wild-type PRs, the surviving *Apc1* mutant PRs have slightly reduced diameters at the very apical surface of the retina; at more basal levels, PRs are either not detectable or the PR diameter is markedly diminished. Reducing the gene dosage of *arm* by only one-half suppresses this shortening of PR length, indicating that elevated Arm levels induce the shortened PR morphology (Ahmed et al., 1998).

To determine whether Arm-mediated signaling results in shortened PRs, we used a *GMR* promoter to express the activated Arm^{S10} protein ubiquitously in otherwise wild-type retinas. As noted above, no PR apoptosis is observed in this genotype, indicating a lack of high-level Arm signaling; however, we find that intermediate-level Arm signaling in *GMR>armS10* retinas induces a shortening of PR length, resulting in a morphological appearance that is identical to that induced by *Apc1* loss (Ahmed et al., 1998) (Fig. 5F-H). These data suggest that although high-level Arm signaling induces apoptosis, intermediate-level Arm signaling induces both a DRA fate and a shortening of PR length.

Arm functions not only as a transcriptional activator, but also as an essential component in the formation and maintenance of cadherin-based adherens junctions (Cox et al., 1996; Muller and Wieschaus, 1996). To determine which of these functions results in shortened PR length, we analyzed the requirement for Arm's co-transcriptional activator dTCF/Pangolin (Pan) (Brunner et al., 1997; van de Wetering et al., 1997). In *Apc1*^{Q8} mutant flies that are also heterozygous for the *pan*¹³ null allele (Brunner et al., 1997), cell death is partially suppressed, but a severe defect in PR length persists; shortened PRs are observed in all ommatidia (Fig. 6A,B) (Ahmed et al., 1998). However, a further reduction in dTCF activity,

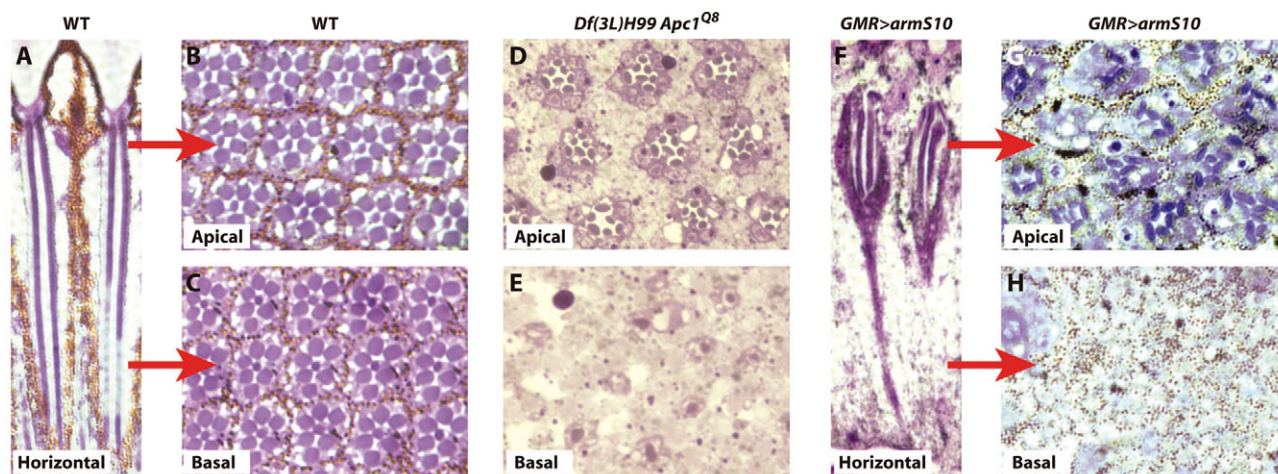


Fig. 5. Activation of Arm signaling leads to a morphological defect in photoreceptor length. (A) Horizontal section from a wild-type adult eye. Photoreceptors extend from the base to the apical surface of the retina. (B,C) Cross-section of wild-type adult retina. Most photoreceptors are present at the apical surface (B) and basal surface (C) of the retina. (D,E) Apical (D) and basal (E) cross-sections of an *Apc1*^{Q8} retina containing a large clone of cells homozygous mutant for *grim*, *reaper* and *hid* (*Df(3L)H99*). (F-H) In eyes expressing constitutively active Arm^{S10} under the control of a *GMR*-GAL4 driver, photoreceptor length is shortened (horizontal section in F, apical cross-section in G and basal cross-section in H).

as is present in flies homozygous for the hypomorphic allele *pan^{ER1}* (Brunner et al., 1997), partially suppresses not only apoptosis, but also the shortened PR length in the *Apc1* mutant (Fig. 6C,D). These data indicate that the shortened PR length requires both Arm and dTCF activity, and therefore is likely to involve Arm/dTCF-mediated transcription.

We note, unexpectedly, that even in wild-type adults, markedly shortened PRs are found in the outer rows of the retina, where Wg activity is present (Fig. 6E,F). By contrast, we do not find any shortened PRs in ommatidia located in the center of the eye, where Wg is absent (data not shown). These results raise the possibility that the shortened PR morphology, which is aberrantly induced in all PRs by ectopic Arm signaling upon *Apc1* loss, recapitulates another physiological response to normal Wg/Arm signaling at the retinal periphery.

We sought to determine whether we could distinguish the levels of Arm signaling that induce DRA fate specification from those that induce shortening of PR length. We found that although reducing the *arm* gene dosage by only one-half in the *Apc1* mutant was sufficient to partially suppress both the cell death and the shortened PR length (Ahmed et al., 1998), there was no suppression of ectopic *hth* expression (data not shown). Together, these results indicate that three distinct responses to Arm signaling are specified by three distinct levels of Arm activity: PR apoptosis is induced by the highest levels of Arm signaling, shortened PR length is induced by lower levels, and even lower levels are sufficient to induce *hth* expression.

Reduction in Apc activity by less than twofold decreases the effective threshold at which Wg elicits a cellular response

To test the model that Apc is present in vast excess, we examined the effects of relatively modest reductions in Apc activity on Arm signaling. The two *Drosophila* Apc proteins have largely redundant functions, such that, in most cell types, the complete loss of either Apc protein singly has no functional consequence on Wg-dependent patterning (Ahmed et al., 2002; Akong et al., 2002a). One exception is in retinal photoreceptors, in which both Apc proteins are required to negatively regulate Arm, but the relative contribution of Apc1 towards total Apc activity is much greater than that of Apc2. Although both Apc proteins are expressed in PRs (see Fig. S3 in the supplementary material), Apc2 levels are low enough that inactivation of Apc1 is sufficient to induce ectopic, high-level Arm signaling (Ahmed et al., 1998; Ahmed et al., 2002) (Fig. 1). Overexpression of Apc2 can compensate for Apc1 loss, revealing that even in PRs, the two Apc proteins are functionally equivalent (Ahmed et al., 2002). Thus, the reduction of Apc2 levels in photoreceptors provides an opportunity to determine the effects of a less than twofold reduction in total Apc activity on Arm signaling.

We examined the three concentration-dependent PR responses to Arm signaling in homozygous *Apc2³³* mutants that contain a strong hypomorphic allele, *Apc2³³* (see Fig. S4 in the supplementary material). In *Apc2³³* mutants, we find no increase in the number of PRs that either undergo apoptosis or have a shortened length when compared with wild type, the two cellular responses that require higher levels of Arm signaling (data not shown). By contrast, we find that more photoreceptors express *hth* in *Apc2³³* mutants (Fig. 7). Strikingly, in the *Apc2* mutant, ectopic *hth* expression occurs only in PRs that are immediately adjacent to those that normally express *hth*, resulting in a wider zone of *hth* expression. Specifically, in wild-type flies, *hth* expression is restricted to the most peripheral ommatidia in the

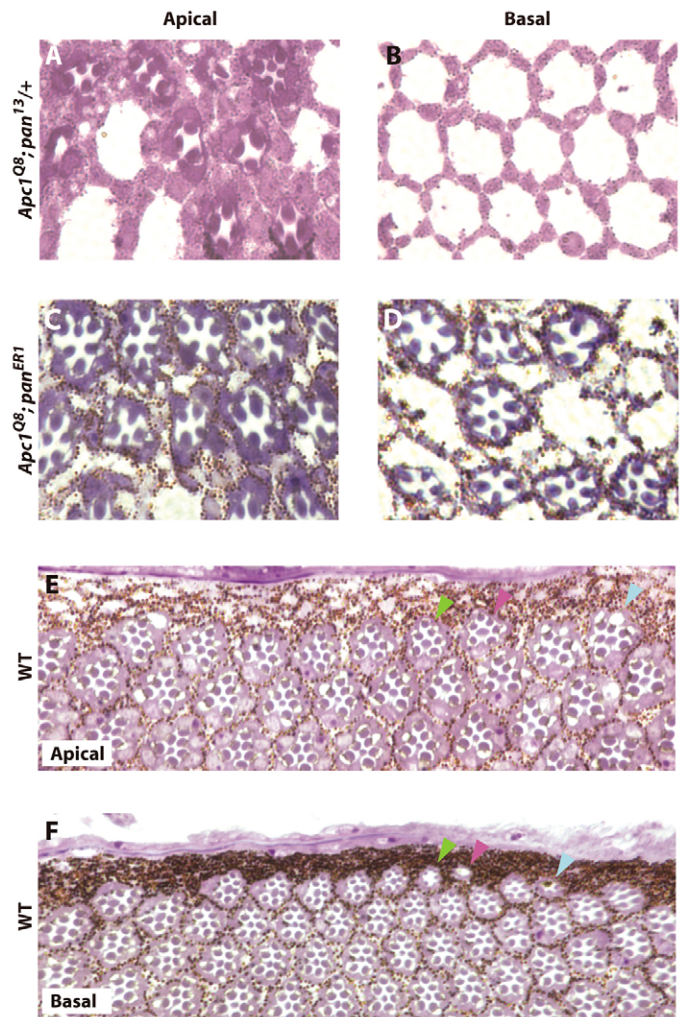


Fig. 6. Shortened PR length in the *Apc1* mutant results from ectopic activation of Arm/dTCF (Pan) signaling. (A,B) Apoptosis of photoreceptors is partially suppressed in *Apc1^{Q8}* mutants by reducing the gene dosage of *pan* by one-half, using the *pan¹³* null allele. However, the shortened PR morphology is not suppressed. (A) Apical section; (B) basal section. (C,D) Upon further reduction of *pan* activity, in flies homozygous for the hypomorphic *pan^{ER1}* allele, both apoptosis and the shortening of PR length are partially suppressed in the *Apc1^{Q8}* mutant. (C) Apical section; (D) basal section. (E,F) Cross-section of the dorsal-most part of a wild-type adult retina at an apical (E) and basal (F) level. In the outermost rows of the retina, where Wingless signaling is at an intermediate level, some photoreceptors do not extend the entire length of the retina (color-coded arrowhead indicates the same ommatidium at an apical and basal level).

dorsal half of the retina, and also to a small fraction of ommatidia in the 'second row' and 'third row', which are immediately adjacent to the outermost row (Wernet et al., 2003) (Fig. 7A, arrowheads). By contrast, in homozygous *Apc2³³* mutants, approximately twice as many ommatidia in the second row express *hth* (Fig. 7B,E). In addition, 82% of *Apc2* mutant retinas ($n=51$), but only 28% of wild-type retinas ($n=37$), contain at least one ommatidium in the third row that expresses *hth*. The increased number of ommatidia in the second and third row of the *Apc2* mutant retina that ectopically express *hth* is highly significant ($P<10^{-15}$; Fig. 7E). We find similar results in pupae

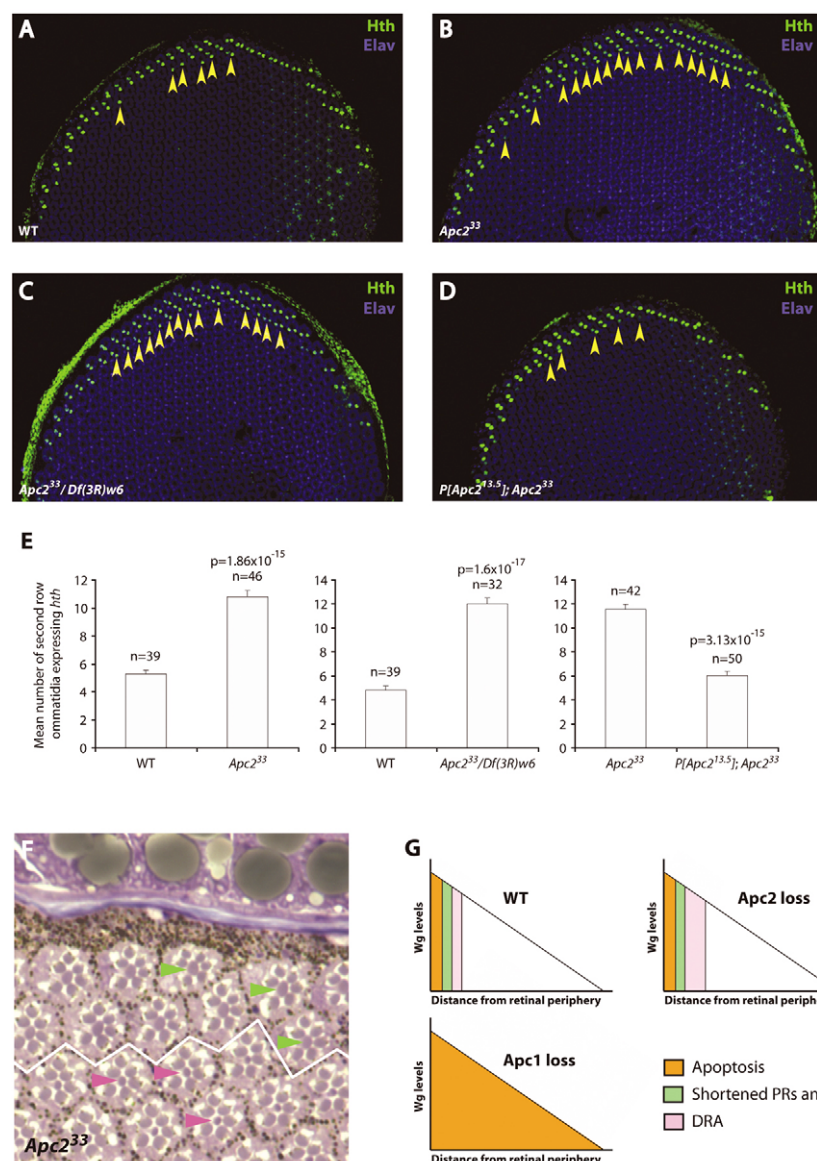


Fig. 7. Loss of *Apc2* increases the width of the dorsal rim area. Second and third row ommatidia expressing *Hth* (green) are marked with yellow arrowheads. Photoreceptors are marked with anti-Elav (blue). The number of second and third row ommatidia expressing *hth* increases when *Apc2* activity is severely reduced. (A) Wild-type, (B) *Apc2*³³/*Apc2*³³ and (C) *Apc2*³³/*Df(3R)w6*. (D) The number of second row ommatidia expressing *Hth* is reduced to normal levels in *Apc2*³³ mutants with two copies of an *Apc2* transgene, *P[Apc2*^{13.5}]. (E) Average number of second row ommatidia expressing *Hth* for each genotype. Standard errors are shown. (F) Adult eye section from the dorsal-most retina of a homozygous *Apc2*³³ mutant. Ommatidia with a DRA fate are found above the solid white line and are characterized by inner photoreceptors with enlarged rhabdomeres (green arrowheads). There is an increased number of ommatidia with a DRA fate when compared to wild-type retinas (see Fig. 3C). (G) Schematic of the retina in wild-type, *Apc1* mutant and *Apc2* mutant flies. Loss of *Apc1* induces apoptosis of all photoreceptors (orange). Loss of *Apc2* causes an expansion of the DRA fate (pink).

that are transheterozygous for the *Apc2*³³ allele and a chromosomal deficiency that eliminates the entire *Apc2* gene, *Df(3R)w6* (Fig. 7C,E). In addition, the introduction of two copies of an *Apc2* transgene (Ahmed et al., 2002) into homozygous *Apc2*³³ mutants reduces the number of second and third row ommatidia expressing *hth* to wild-type levels, while having no effect on *hth* expression in wild-type flies (Fig. 7D,E; see Fig. S5 in the supplementary material). Together, these data rule out the possibility that the ectopic *hth* results from background mutations on the *Apc2*³³ chromosome. These results indicate that reducing total Apc activity by less than twofold can shift the threshold for response to Wg.

To determine whether ectopic *hth* expression in the *Apc2* mutant is sufficient to induce an ectopic DRA fate, we analyzed sections from the retinal dorsal rim. By comparison with wild-type retinas, *Apc2*³³ mutant retinas have many more inner PRs with enlarged rhabdomeres, indicative of DRA ommatidia, in the second row of the dorsal rim (compare Fig. 3C with Fig. 7F). We also observed an increased number of DRA ommatidia in the second row in *Apc2* mutants homozygous for a deletion that eliminates the entire coding

region of the *Apc2* gene, *Apc2*⁷⁹ (see Fig. S4 in the supplementary material; data not shown). In summary, these data indicate that loss of *Apc2* results in ectopic Arm signaling, and thereby results in an increased number of PRs that aberrantly adopt a DRA fate. Furthermore, the PRs adopting an ectopic DRA fate are immediately adjacent to PRs that normally adopt a DRA fate. Thus, our results indicate that Apc activity is not present in vast excess, but instead is present near the minimal level required for accurate patterning in response to the Wg morphogen.

DISCUSSION

Previous genetic studies have provided conclusive evidence that the two *Drosophila* Apc proteins are crucial negative regulators of Arm signaling (Ahmed et al., 2002; Akong et al., 2002a). Simultaneous inactivation of both Apc proteins results in ectopic Arm signaling in nearly all, if not all, cells, indicating that Apc is required to prevent Arm signaling in the absence of Wg stimulation. In contrast with the prevailing model for Wg transduction, which proposes that Apc is present in vast excess, the work presented here reveals that a less than twofold reduction in Apc activity can shift the threshold for the

response to Wg. We conclude that by negatively regulating Arm, Apc prevents ectopic Arm activity not only where Wg is absent, but also within the range of the Wg gradient.

Translation of a gradient of Wg morphogen activity to quantitatively distinct levels of Arm signaling is required to induce concentration-dependent cellular responses, although the mechanisms by which this occurs remain uncertain. Our results reveal that in regions of low Wg concentration, reducing total Apc activity by less than twofold results in aberrant cell fate specification (Fig. 7G). A morphogen model predicts that the low Wg concentration present in this region of the gradient is below the threshold necessary to trigger a detectable cellular response. We find that this is the only region within the Wg gradient where a relatively small reduction in total Apc activity elicits an ectopic cellular response, and this response is characteristic of intermediate-level Arm signaling. Thus, our results reveal that Apc activity is in excess in regions where Wg is absent, but is not in vast excess within the range of the Wg gradient. Together, our data indicate that Apc activity is present near the minimal level required to prevent ectopic Arm signaling and thereby ensure accurate graded responses.

In *Xenopus* egg extracts, the levels of Axin are several magnitudes lower than the levels of other proteins in the destruction complex, suggesting that only Axin is a limiting component in Arm proteolysis, whereas Apc is present in vast excess (Lee et al., 2003; Salic et al., 2000). How can these biochemical data be reconciled with our in vivo data, which indicate that Apc is not present in excess within the range of the Wg gradient? One possibility is that the levels of Apc in *Xenopus* eggs are much greater than those present in *Drosophila* photoreceptors. Alternatively, total Apc levels could be present in excess regardless of cell type or organism, but the relevant pool contributing to destruction complex activity, distinguished by either post-translational modification and/or intracellular localization, might be present near threshold levels. A correlation between the degree of reduction in the activity of the fly and mammalian Apc proteins with the level of β -catenin/Arm signaling has been demonstrated in several other developmental contexts and in tumorigenesis (Ahmed et al., 2002; Akong et al., 2002a; Akong et al., 2002b; Benhamouche et al., 2006; Hayden et al., 2007; Kielman et al., 2002; McCartney et al., 2006; Smits et al., 1999). Thus data from diverse experimental models indicate that the level of Apc contributes to the level of β -catenin/Arm signaling.

How is a gradient of Wg concentration translated into quantitatively distinct levels of Arm activity? Upon Wg stimulation, inactivation of the Axin/Zw3/Apc destruction complex is the primary event that triggers Arm signaling (Peifer et al., 1994; Siegfried et al., 1994; Tolwinski et al., 2003). Inactivation of Axin is important for downstream signal transduction in response to Wg stimulation, and is likely to be mediated by the translocation of Axin to the plasma membrane, and/or the degradation of Axin (Cliffe et al., 2003; Tamai et al., 2004; Tolwinski et al., 2003). Thus the local Axin concentration is likely to have a significant role in determining whether the destruction complex is assembled, and consequently is important in regulating Arm stability. Our findings provide in vivo evidence that the level of destruction complex activity is crucial for accurate patterning in response to Wg, and is dependent not only on Axin, but also on the maintenance of Apc activity above a minimal level. We conclude that within the range of the Wg gradient, both Axin and Apc are present near threshold levels, and that, together, they achieve the precise levels of destruction complex activity required for accurate graded responses.

We thank C. Pikielny, S. Ogden and A. Mehra for valuable discussion; A. Salzberg, K. Basler, A. Tomlinson, C. Desplan, K. Cadigan, M. Bienz, M. Peifer, J. Treisman, S. Campuzano, R. Mann, F. Davidson, the Bloomington *Drosophila* Stock Center, the Berkeley *Drosophila* Genome Project, and the Developmental Studies Hybridoma Bank for flies and antisera; V. Marlar for technical support; and A. Lavanway and A. Tomlinson for technical advice. This work was supported by the Norris Cotton Cancer Center, the Emerald Foundation, the Scholars Program of the General Motors Cancer Research Foundation, the American Cancer Society (IRG-82-003-21), the National Cancer Institute (RO1CA105038), and the Howard Hughes Medical Institute, through an award from the Biomedical Research Support Program for Medical Schools to Dartmouth Medical School (76200-560801).

Supplementary material

Supplementary material for this article is available at
<http://dev.biologists.org/cgi/content/full/135/5/963/DC1>

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