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

Hassina Benchabane*, Edward G. Hughes*, Carter M. Takacs, Jason R. Baird and Yashi Ahmed†

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 phosphorylation and destruction complex activity is crucial to trigger Arm signaling. In the prevailing model for Wg transduction, only Axin levels limit destruction complex activity, whereas Arm 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).
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 Apc1Q8 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). Asvp-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. (C-F) In Apc1Q8 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. Apc279 has a deletion of the entire Apc2 coding region, and also disrupts the neighboring gene mRps24 (Takacs et al., 2008). In homozygous Apc279 mutants, loss of mRps24 activity induces lethality. Therefore, to obtain viable Apc279 mutant adults, a P[mRps24] transgene (Takacs et al., 2008) was recombined onto the Apc279 mutant chromosome. Apc279 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 Apc108 (Ahmed et al., 1998), P[Apc2133] (Ahmed et al., 2002), svpP[Z] (Heberlein et al. (Heberlein et al., 1991); Bloomington Drosophila Stock Center (BDSC)), UAS-arm.S10 (encoding full-length (Fortini and Rubin, 1990) (BDSC), UAS-arm.Exel; lacZ1997), rabbit anti-Apc2 (primary antibodies used for immunostaining were guinea pig anti-Apc2 (Newsome et al., 2000); provided by J. Treisman, Skirball Institute, New York), eyeless-FLP (Newcombe et al. (Newcombe 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), pM75C FRT80B (Xu and Rubin, 1993), pygo10 (Parker et al., 2002), pan1 and panERI (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 (Newcombe 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:

pygo10 mutant clones: eyeless-FLP/+; FRT82B pygo10/FRT82B arm-lacZ;

Apc2133 mutant clones: eyeless-FLP/+; FRT82B Apc2133/FRT82B arm-lacZ;


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 Immunotech). 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, 100% 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.

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 Apc108 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,
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 Apc1Q8 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 Apc1Q8 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 Apc1Q8 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 is found throughout the entire retina when Ara is expressed under the control of a GMR promoter, induces the formation of an ectopic ventral rim area with one to three rows of ommatidia expressing Ara, under the control of a GMR promoter. (E-H) Expression of full-length Arm (E,F) and constitutively active ArmS10 (G,H) under the control of a GMR promoter, respectively, leads to the expansion 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 in 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 ArmS10 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 ArmS10 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 Apc1Q8 mutant flies that are also heterozygous for the pan13 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,
as is present in flies homozygous for the hypomorphic allele pan<sup>ER1</sup> (Brunner et al., 1997), partially suppresses not only apoptosis, but also the shortened PR length in the Apc<sup>1</sup> 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 Apc<sup>1</sup> 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 Apc2 mutants that contain a strong hypomorphic allele, Apc<sup>23</sup> (see Fig. S4 in the supplementary material). In Apc<sup>23</sup> 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 Apc<sup>23</sup> 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 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 Apc<sup>23</sup> 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<sup>−15</sup>; Fig. 7E). We find similar results in pupae
that are transheterozygous for the Apc2<sup>33</sup> 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<sup>33</sup> 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<sup>33</sup> 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<sup>33</sup> 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<sup>79</sup> (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; Towlinski 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; Towlinski 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. Salaberg, 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. Lavaway 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-S68081).

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

References
Apc ensures accurate responses to Wg

Development 2565-2576.

Cell 91, 1017-1026.

Nature 407, 530-535.


Dev. Biol. 164, 328-331.

Cell 5, 577-584.

Cell 164, 367-373.

Cell 124, 3747-37485.

Nature 407, 527-530.

Development 126, 4165-4173.

Development 124, 2155-2266.

Development 124, 1655-1673.

Cell 5, 523-532.

Cell 113, 1309-1321.

Cell 72, 527-540.

Cell 71, 149-156.

Cell Biol. 1, 1-10.

Cell 71, 1037-1048.

Cell 146, 1303-1318.

Cell 146, 577-586.

Nature 32, 594-605.

Cell 126, 577-586.


Cell 146, 1303-1318.

Genes Dev. 13, 851-877.

Development 123, 2409-2418.

Development 122, 3477-3485.

Development 122, 3477-3485.

Development 124, 871-880.

Development 124, 871-880.

Development 124, 871-880.

Development 124, 871-880.

Development 124, 871-880.

Development 124, 871-880.

Development 124, 871-880.

Development 124, 871-880.