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APC loss-induced intestinal tumorigenesis in *Drosophila*: Roles of Ras in Wnt signaling activation and tumor progression

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ABSTRACT

Adenomatous polyposis coli (*APC*) and *K-ras* are the two most frequently mutated genes found in human colorectal cancers. In human colorectal cancers, Wnt signaling activation after the loss of *APC* is hypothesized to be the key event for adenoma initiation, whereas additional mutations such as Ras activation are required for the progression from adenoma to carcinoma. However, accumulating data have led to conflicting views regarding the precise role of Ras in *APC* loss-induced tumorigenesis. Here, using *Drosophila* midgut as a model system, we show that in the absence of Ras, *APC* mutant epithelial cells cannot initiate hyperplasia, suggesting that Ras plays an essential role in tumor initiation. Conversely, activating Ras by expressing oncogenic Ras or Raf in *APC*-deficient cells led to a blockage of cell differentiation and to preinvasive tumor outgrowth, characteristics that are shared by advanced colorectal carcinoma in humans. Mechanistically, we find that Ras is not required for Wnt signaling activation after *APC* loss, although Ras hyperactivation is able to potentiate Wnt signaling by increasing the cytoplasmic and nuclear accumulation of Armadillo/β-catenin via mechanisms independent of JNK/Rac1 or PI3K-Akt signaling, partly owing to the downregulation of DE-cadherin. Together with the data from gene expression analyses, our results indicate that both parallel and cooperative mechanisms of Wnt and Ras signaling are responsible for the initiation and progression of intestinal tumorigenesis after *APC* loss.

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Introduction

Adenomatous polyposis coli (*APC*) is a human disease gene whose mutation is responsible for familial adenomatous polyposis (FAP) (Groden et al., 1991; Kinzler et al., 1991), a dominantly inherited disease characterized by early onset of intestinal polyps that eventually develop into carcinoma if left untreated. *APC* has also been found to be mutated in most sporadic colon cancers (Powell et al., 1992). Numerous studies have established that *APC* negatively regulates Wnt signaling by forming a protein destruction complex that targets β-catenin for degradation (Bienz and Clevers, 2000; Clevers, 2006; MacDonald et al., 2009). Loss-of-function and gain-of-function studies of *APC* and β-catenin in mice have suggested that aberrant Wnt signaling activation after *APC* loss is responsible for the initiation of intestinal adenoma. Consistent with this suggestion, some sporadic human colon carcinomas carry stabilizing mutations in β-catenin, rather than mutations in *APC* (Iwao et al., 1998; Morin et al., 1997; Sparks et al., 1998). Therefore, hyperactivation of Wnt signaling is generally viewed as the key event for the initiation of intestinal adenoma after *APC* loss, although progression to carcinoma requires additional mutations, such as K-ras, which is found to be mutated in approximately 40% of colon cancers (Bos et al., 1987; Forrester et al., 1987).

The molecular events that are thought to initiate adenoma formation after *APC* loss have been recently revisited. Using an in vivo zebrafish model and human cells, Phelps et al. found that adenoma formation after *APC* loss is mediated by the transcriptional repressor CtBP1 rather than the nuclear localization of β-catenin and subsequent Wnt pathway activation. *APC* loss alone is insufficient to cause nuclear localization of β-catenin; however, when the loss of *APC* is combined with the activation of oncogenic K-ras, they are able to promote Rac1-dependent nuclear localization of β-catenin and Wnt pathway activation (Phelps et al., 2009). A two-step model has therefore been proposed for colon cancer development after *APC* loss: first, CtBP1 contributes to adenoma initiation immediately after *APC* loss; second, oncogenic KRAS activation and β-catenin nuclear localization promote the progression from adenoma to carcinoma (Phelps et al., 2009). This model is consistent with the observations that nuclear localization of β-catenin has been observed in late, but not early, adenomas in FAP patients and was frequently observed in sporadic colon

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carcinomas (Amos-Landgraf et al., 2007; Anderson et al., 2002; Blaker et al., 2003). But the data do contradict the observations that the presence of nuclear β-catenin in adenomas is not associated with K-ras mutations (Fodde and Tomlinson, 2010; Obrador-Hevia et al., 2010). Although previous studies have revealed a cooperative relationship between *APC* loss and K-ras mutation in adenoma development (Janssen et al., 2006), the immediate consequences of *APC* loss in intestinal cells have not been thoroughly studied. Therefore, the requirements for oncogenic K-ras in Wnt signaling activation and in the development of *APC* loss-induced intestinal adenoma requires further investigation.

The *Drosophila* midgut has recently emerged as an attractive system in which to study intestinal homeostasis and tumorigenesis regulated by Wnt/Wingless (Wg) signaling (Biteau et al., 2011; Casali and Batlle, 2009; Cordero et al., 2009). The midgut epithelium is monolayered, and its renewal is dependent on the proliferation of basally-localized intestinal stem cells (ISCs) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006), whose activity is normally regulated by multiple signaling pathways, including Wg, JAK/STAT, EGFR/Ras and insulin signaling (Biteau et al., 2011; Jiang and Edgar, 2011). Notably, deletions of *Drosophila APC* genes in the midgut lead to overproliferation of ISCs and epithelial hyperplasia that is characterized by the formation of multilayered epithelium and the accumulation of aberrantly differentiated progenitor cells (Lee et al., 2009), which suggests that the *Drosophila* midgut could be used to model adenoma development following *APC* loss. Inhibition of Wg signaling can largely suppress the overproliferation phenotype caused by *APC* loss, demonstrating that intestinal hyperplasia after *APC* loss is at least partially mediated by the hyperactivation of Wg signaling (Lee et al., 2009). However, because EGFR/Ras signaling is normally activated in ISCs to maintain a baseline of ISC activity (Biteau and Jasper, 2011; Buchon et al., 2010; Jiang et al., 2011; Xu et al., 2011), the requirements of Ras signaling for Wg signaling activation and epithelial hyperplasia following *APC* loss become obscure.

In this study, we investigated the requirement for Ras in Wg signaling activation following *APC* loss in the *Drosophila* midgut and studied whether oncogenic Ras activation could promote the progression of intestinal hyperplasia following *APC* loss. We found that Ras is required for the initiation of intestinal hyperplasia after *APC* loss but it is not required for Wg signaling activation, although oncogenic Ras activation could potentiate Wg signaling by promoting cytoplasmic and nuclear Armadillo (Arm, the *Drosophila* β-catenin) accumulation. Oncogenic Ras activation is able to promote tumor progression after *APC* loss; however, this function cannot be explained by its ability to potentiate Wnt signaling. Together with the results from gene expression analyses, we propose that a cooperative function of Wg and Ras signaling is the driving force for the initiation and progression of intestinal tumorigenesis following *APC* loss.

Materials and methods

Drosophila strains

The following fly strains were used in this study:

FRT stocks: FRT82B $APC1^{Q8}$ $APC2^{g10}$ (a gift from *M. Peifer*); FRT82B ras^{e1B}; FRT82B $APC1^{Q8}$, FRT82B $APC2^{g10}$ and FRT82B $ras^{e1B}APC1^{Q8}APC2^{g10}$ were created from the above stocks. FRT82B $Axin^{S044230}$ (a gift from Y. Ahmed) (Ahmed et al., 2002);

Transgenes: UAS-RasV12 (Lee et al., 1996); UAS-$EGFR^{DN}$ (Freeman, 1996); UAS-p35; UAS-TCF^{DN} (van de Wetering et al., 1997); UAS-Raf gof (Brand and Perrimon, 1994); UAS-Rac1.V12; UAS-Rac1.L89 (Luo et al., 1994); UAS-$PI3K92E^{CAAX}$ (Leevers et al., 1996); UAS-Akt1 (Andjelkovic et al., 1995); UAS-shg-RNAi (NIG#3722R-2); UAS-hep^{ACT} (Weber et al., 2000); UAS-sgg; UAS-pygo-IR (VDRC#100724); UAS-$arm^{\triangle N}$ (a gift from A. Martinaz-Arias); Wnt signaling activation reporter TCF-lacZ contains a lacZ reporter gene driven by 6 pairs of TCF binding and helper sites, which was constructed according to previously described protocol (Chang et al., 2008).

Mosaic analysis

GFP marked clones of *Drosophila* ISCs were generated either by the flp-out technique (Neufeld et al., 1998) or the MARCM system (Lee and Luo, 2001). For clone induction, 3 to 5-day-old female flies cultured on normal food with yeast paste were subjected to a 37 °C heat shock for 30–60 min. Flies were subsequently fed with regular food supplied with yeast paste and transferred every two days.

Immunostaining and microscopy

Drosophila midguts were dissected and immunostained as described previously (Lin et al., 2008). Following antisera and dyes were used: mouse anti-Dl (Developmental Studies Hybridoma Bank (DSHB); 1:100); mouse anti-Pros antibody (DSHB, 1:300); rabbit anti-dpERK antibody (Cell Signaling, 1:200); mouse anti-Arm (DSHB, 1:10); rat anti-E-cadherin (DSHB, 1:10); rabbit anti-pAkt (Cell Signaling, 1:200); mouse anti-Dlg (DSHB, 1:200); rabbit anti-Pdm1 (a kind gift from Xiaohang Yang, 1:1000); rat anti-BrdU antibody (Abcam, 1:300); rabbit anti-phospho-Histone H3 antibody (Upstate, 1:1000); rabbit anti-aPKC (Santa Cruz, 1:200).

rabbit polyclonal anti-β-gal antibody (Cappel, 1:6000); rabbit anti-Laminin β1(Abcam, 1:300); Secondary antibodies—including goat anti-rabbit, anti-mouse, or anti-rat IgGs-conjugated to Alexa (488, 568 or Cy5) (Molecular Probes) were used at a dilution of 1:300; rhodamine-conjugated Phalloidin (Molecular Probes, 1:500); DAPI (49,69-diamidino- 2-phenylindole, Sigma; 0.1 mg/ml, 5 min incubation). Images were captured by either a Zeiss Imager Z1 equipped with an ApoTome system or a Zeiss Meta 510 confocal microscope. All images were adjusted and assembled in Adobe Photoshop and Illustrator.

BrdU labeling

Adult flies were reared on standard corn meal food with 200ul 5mg/ml BrdU(Sigma) plus 20% sucrose at 25 °C for 24 h. Midguts were dissected in Grace's insect medium and were fixed as previously described (Lin et al., 2008). Samples were incubated at 37 °C for 30 min with DNase I (Takara) following the manufacturer's instructions, and the reaction was stopped by washing samples with PBT twice. Samples were subsequently immunostained as previously described.

TUNEL labeling

In situ cell death detection kit (Roche, Cat. No. 1684795) was used to detect cell death in the midguts. Briefly, samples were fixed as described above and were incubated in TUNEL reaction mix (enzyme solution: label solution = 1:9) for 45 min at 37 °C. The reaction was stopped by washing samples with PBT three times.

Cryosectioning

The prestained midguts were immersed in 200 ul of 75% glycerol in PBS overnight at 4 °C. The bottom of a small flat

embedding mold (PELCO, Prod No.106) was covered with a layer of O.C.T (Tissue Tek) and frozen in liquid nitrogen. Midguts were horizontally placed onto the solidified O.C.T layer, covered completely with additional O.C.T, and frozen at −20 °C. 10–20 um cross-sections of midgut were generated using a Microm HM525 cryostat. Sections were placed and dried onto poly-lysine (sigma) treated glass microscope slides (Citoglas) for 30–60 min at RT, rinsed twice with PBT and mounted in 75% glycerol.

Gene expression profiling by DNA microarray

Sample preparation

For RasV12 group, 2-day-old female flies with the genotype of esg-Gal4,UAS-GFP/+; Tub-gal80^{ts}/UAS-RasV12 were subjected to heat treatment at 29 °C for 4 days. Then guts were dissected out and homogenized in TRIzol (Invitrogen). Total RNA was extracted via standard methods. Female flies with the genotype of esg-Gal4,UAS-GFP/+; Tub-gal80^{ts}/+ served as controls. For *APC* or *APC*-RasV12 group, 3-day-old female flies with the genotype of hs-flp/+; Act-gal4,UAS-GFP/+; 82B FRT, Tub-gal80/82B FRT *APC* or hs-flp/+; Act-gal4,UAS-GFP/UAS-RasV12; 82B FRT, Tub-gal80/ 82B FRT *APC* were heatshocked at 37 °C for 1–2 h and were subsequently reared at 25 °C on regular food supplied with yeast paste for 14 days. Then total RNA of guts was extracted as described above. Female flies with the genotype of hs-flp/+; Act-gal4,UAS-GFP/+; 82B FRT, Tub-gal80/ 82B FRT were employed as controls. Total RNA was prepared from three independent experiments for each group.

Microarray analysis. The independent triplicate RNA samples were subjected to analysis by high-density oligonucleotide arrays using Affymetrix GeneChip® *Drosophila* Genome 2.0 Array. All the microarray hybridization and data analysis were performed by CapitalBio corporation, Beijing, China. Differentially expressed were determined using the SAM software with more than two-fold change and a *q* value less than 0.05.

RT-PCR confirmation. A set of genes were chosen to confirm microarray data. Real time quantitative PCR was performed under the instruction of manufacturer with SYBR PrimeScript RT-PCR kit (Takara) on an ABI PRISM 7500 fast Real-time PCR System (Applied Biosystems). RT-qPCR was performed in duplicate on each of 3 independent biological replicates. GAPDH was used as an internal control for data normalization across samples. The following primers were used in this study:

Sprouty forward:5′-CACTACCAGAACGCGCTAAAC-3′
Sprouty reverse:5′-AAGCGATTGGTTGGTTGGCT-3′
Mmp1 forward:5′-ATCATGGCAGCGGCTCAATC-3′
Mmp1 reverse: 5′-TGATGTTCAGTCCCGCGAAG-3′
Mmp2 forward:5′-GGGAAAGCGGAGAATCGCAT-3′
Mmp2 reverse: 5′-AAGTGGGCAGGAAATGCCAG-3′
Timp forward: 5′-TGTGCCCTCGGGTTCAAGTGT-3′
Timp reverse: 5′- ATGACTGCGTTGGTTGGCGAG-3′
Fz3 forward:5′-TGCTCTGCTCGTCTCTGTTT-3′
Fz3 reverse:5′-ATGCACAACTCGTGCTTCTC-3′
nkd forward:5′-TCAAGATGTTGCGAAGGGCG-3′
nkd reverse:5′-CGGAATCGTTGCCCACTGAC-3′
GAPDH forward: 5′-GAAATTAAGGCCAAGGTTCAGG-3′;
GAPDH reverse: 5′-GTACCAAGAGATCAGCTTC-3′

Statistical analysis

For all experiments, the data is represented as mean ± s.e.m. All P-values were calculated using unpaired two-tailed Student's *t*-test.

Results

Drosophila APC1 and APC2 function redundantly in suppressing Wnt signaling and preventing intestinal hyperplasia

As in mammals, there are two *APC* genes in *Drosophila*, *APC*1 and *APC*2. These genes are co-expressed in many tissues and act redundantly to inhibit Wg signaling (Ahmed et al., 2002; Akong et al., 2002a, 2002b),but there are many developmental processes in which the two genes are differentially required and/or have different cellular functions, such as their roles in cytoskeleton organization, adherens junctions and spindle orientation for asymmetric cell division (Hamada and Bienz, 2002; McCartney et al., 2001; Webb et al., 2009; Yamashita et al., 2003). Previous studies have demonstrated that simultaneous deletion of *APC*1 and *APC*2 leads to epithelial hyperplasia in the *Drosophila* midgut, but whether *APC*1 and *APC*2 play redundant or separate roles in the process is unclear, especially with the observation that RNAi knockdown of *APC*1 alone is sufficient to induce ISC proliferation (Cordero et al., 2009). Using the MARCM system for induction of homologous recombination in mitotic cells, we generated several mutant clones marked by GFP – the *APC*1^{Q8} mutant (a null *APC*1 allele) (Ahmed et al., 1998), the *APC*2^{g10} mutant (a null *APC*2 allele) (McCartney et al., 2006), and the *APC*1^{Q8} *APC*2^{g10} double mutant (referred to as *APC* mutant, hereafter) – and examined their growth behavior over time, as previously described (Lin et al., 2008, 2010; Xu et al., 2011). Because ISCs are the only cells in the epithelium capable of cell division, the growth of the GFP clones carrying ISCs (or ISC clones, for simplicity) could be reflective of the ISC activity within the clones. *APC*1^{Q8} or *APC*2^{g10} mutant ISC clones behaved similarly to wild-type ISC clones, with comparable clone sizes on day 7, day 14 and day 21 after clone induction (ACI) (Figs. 1 and 2). In contrast, ISC clones lacking both *APC*1 and *APC*2 were significantly larger than either *APC*1 or *APC*2 single mutant ISC clones (Fig. 1J, K and M). On day 14 ACI, the double mutant ISC clones began to develop into multilayered epithelia (Fig. 1L and N). On day 21 ACI, many clones had fused with each other, outcompeted the inter-clonal wild-type cells (Fig. 1K), and occupied the majority of the midgut (Fig. 2H). These results demonstrate that *APC*1 and *APC*2 function redundantly to prevent intestinal hyperplasia.

To further confirm that *APC*1 and *APC*2 function redundantly in the negative regulation of Wnt signaling in the *Drosophila* midgut, we monitored the expression of a Wnt activation reporter in *APC* mutant clones. The TCF-LacZ reporter is driven by 6 pairs of TCF binding and helper sites and has been suggested to be a reliable Wnt pathway activation reporter in vivo (Chang et al., 2008). We found that the reporter had a restricted expression pattern surrounding the pouch of the wing discs where Wg is produced and within a few specific regions along the anterior–posterior axis of the adult midgut (Supplemental Fig. S1): the muscle cells surrounding the proventriculus, the muscle cells surrounding the cooper region, and the hindgut stem cells at the boundary between the midgut and hindgut. These are all places where high levels of Wg are produced (Singh et al., 2011; Strand and Micchelli, 2011; Takashima et al., 2008). These observations suggest that TCF-LacZ could be used to reflect high levels of Wg pathway activation in the midgut. Consistent with this hypothesis, we found that TCF-LacZ was upregulated in *axin* or *shaggy* mutant clones and in Arm$^{\Delta N}$ (a stable form of Arm)-expressing clones in the midgut, although it was undetectable in wild-type intestinal cells (Supplemental Fig. S1). TCF-LacZ remained undetectable in *APC*1 or *APC*2 single mutant clones (Fig. 1O) but was significantly upregulated in the double mutant clones (Fig. 1O). These observations suggest that *APC*1 and *APC*2 function redundantly in the midgut to prevent Wnt pathway hyperactivation and intestinal hyperplasia.

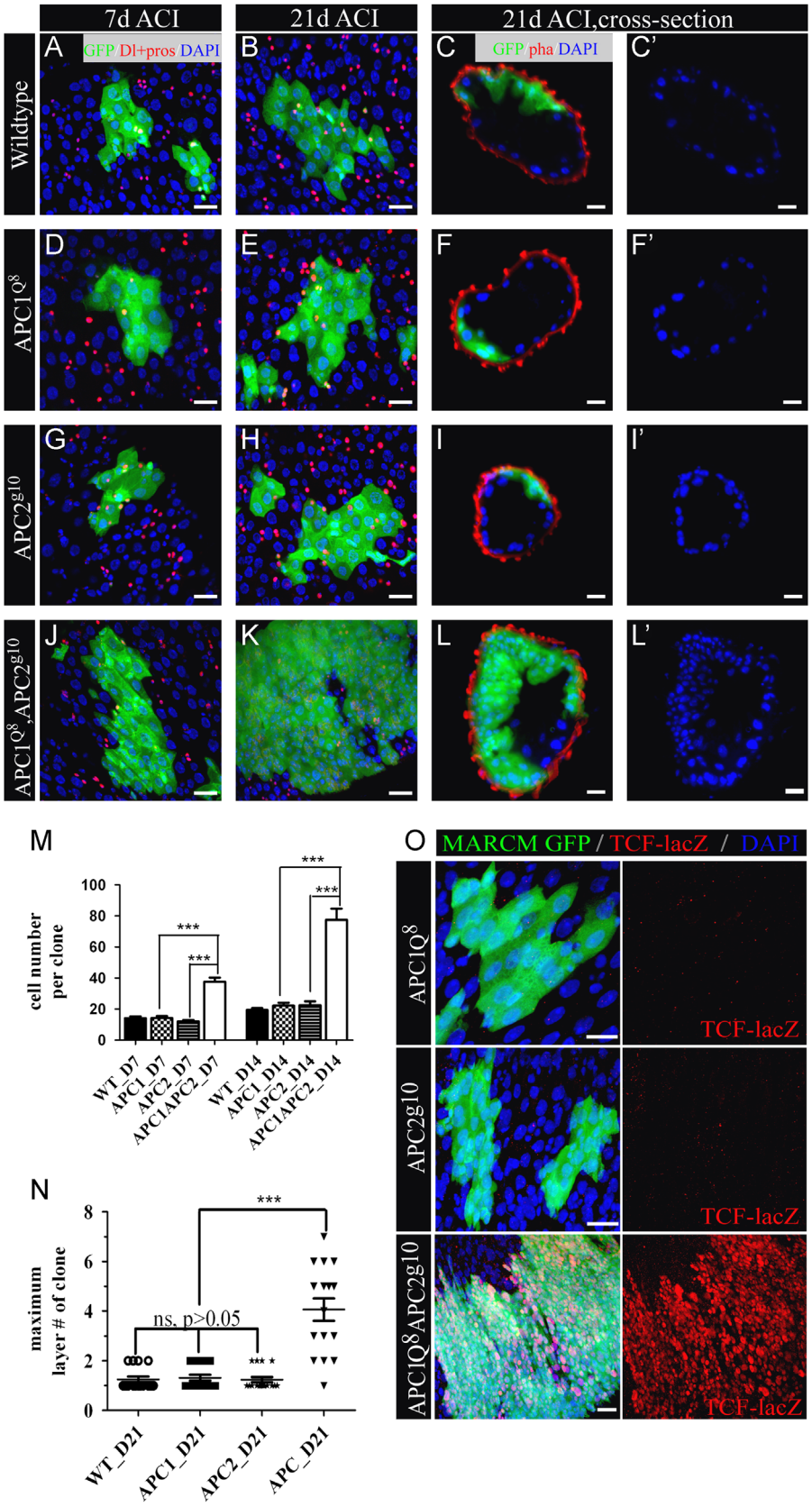

Fig. 1. *Drosophila APC*1 and *APC*2 function redundantly in preventing intestinal hyperplasia and suppressing Wnt signaling. The MARCM system was used to generate GFP labeled ISC clones of given genotypes. Superficial and cross-section view of representative clones on days 7 and 21 after clone induction (ACI) were shown and analyzed. DNA was labeled with DAPI in blue, ISCs, ee cells, and visceral muscle were marked by Dl (red in A and B, membrane), Pros (red in A and B, nucleus), and phalloidin (red in C), respectively. (A–C) Wildtype ISC clones. (D–I) Clones lacking *APC*1 (D–F) or *APC*2 (G–I) alone. (J–L′) Clones lacking both *APC*1 and *APC*2. (M) A plot showing the cell number per ISC clone of specified genotype on day 7 and 14 ACI. Values are means ± s.e.m, n=20–40 clones, ***denotes *t*-test $p < 0.001$. (N) A scatter plot shows the maximal layer of GFP-labeled intestinal epithelium on day 21 ACI. (O) The expression status of TCF-lacZ (in red) in GFP-marked *APC*1 (top), *APC*2 (middle), and *APC*1 *APC*2 double (bottom) mutant clones. Only loss of both *APC*1 and *APC*2 is sufficient to induce TCF-lacZ expression. All scale bars in this and subsequent figures represent 20 μm unless otherwise specified.

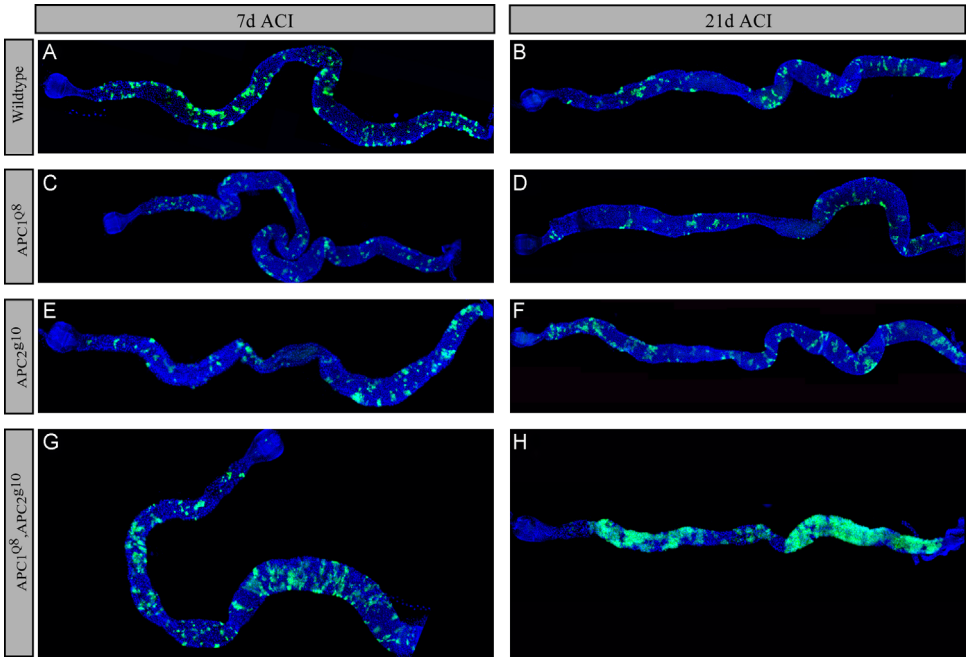

Fig. 2. *APC*1 and *APC*2 function redundantly in preventing intestinal hyperplasia. The MARCM system was used to generate GFP marked clones in 3–5 day old adult animals. Guts were dissected on day 7 and 21 ACI, respectively. (A and B) FRT82B control clones on day 7 (A) and 21 (B) ACI. Note that FRT82B clones do not undergo significant expansion from day 7 to day 21. (C–F) The MARCM clones lacking *APC*1(C and D) or *APC*2 (E and F). Note *APC*1 clones and *APC*2 clones behave similarly to FRT82B control clones. (G and H) The MARCM clones lacking both *APC*1 and *APC*2.Note significant expansion of GFP marked areas from day 7 to day 21 ACI.

The canonical Wnt signaling pathway is required for APC loss-induced intestinal hyperplasia

A large body of evidence has suggested that aberrant activation of Wnt signaling following *APC* loss is a major cause of colon adenoma formation. Similarly, in the *Drosophila* midgut, inhibiting Wg pathway activity by expressing the dominant negative form of TCF was able to at least partially prevent or delay the *APC* loss-induced hyperplasia observed on day 5 ACI (Lee et al., 2009). We observed a similar effect on day 7 ACI (Fig. 4J). Notably, large or multilayered clones were rarely observed, even on day 21 ACI (Fig. 4K and L). Consistent with the notion that the canonical Wnt signaling is downstream of APC in the development of hyperplasia, knockdown of *pygopus* (*pygo*), which encodes a nuclear protein essential for TCF-β-catenin-mediated transcription (Kramps et al., 2002), significantly suppressed *APC* loss-induced hyperplasia (Fig. 3G, H and K). In addition, forced expression of *shaggy*, which encodes the *Drosophila* glycogen synthase kinase-3 beta (GSK-3β), a kinase essential for Arm phosphorylation and subsequent degradation, was able to prevent Arm accumulation even after *APC* loss, and prevent *APC* loss-induced hyperplasia (Fig. 3J and K). This observation indicates that excessive GSK3 could make scaffold proteins dispensable for accessing β-catenin for phosphorylation and degradation. Taken together, these data strongly support that the canonical Wnt signaling acts downstream of APC in the development of midgut hyperplasia.

Normal EGFR/Ras signaling is essential for APC loss-induced intestinal hyperplasia

Studies in murine models have suggested that EGFR/Ras signaling is required for adenoma initiation following *APC* loss (Roberts et al., 2002; Torrance et al., 2000). We therefore tested whether EGFR/Ras signaling is required for the initiation of intestinal hyperplasia following *APC* loss in *Drosophila*. Two methods were used to inhibit or abolish EGFR/Ras signaling in *APC* mutant clones: expressing a dominant negative form of EGFR ($EGFR^{DN}$, 13 amino acid truncation at C-terminal of the transmembrane domain) (Freeman, 1996) or combing with a *ras* mutation, as *Apc*1, *Apc*2 and *Ras*85*D* are all located on the right arm of chromosome III, triple mutant clones can be generated by combing them all to the FRT82B chromosome. $ras85D^{e1B}$ has a missense mutation in the GTP binding domain and is a strong loss-of-function allele. Consistent with these characteristics, $ras85D^{e1B}$ mutant clones were devoid of phospho-ERK expression (Supplemental Fig. S2), a reporter for EGFR/Ras signaling activity. In both approaches, disrupting EGFR/Ras signaling significantly suppressed *APC* loss-induced overgrowth, as the sizes of GFP clones on day 7 and 14 ACI were significantly smaller than the clones without EGFR/Ras inhibition (Figs. 4 and 5). In addition, cross-section examination of the midguts revealed that disruption of Ras signaling also suppressed the multilayering phenotype commonly observed on day 21 ACI (Fig. 4C and F).

Next, we determined whether the inhibitory effect on hyperplasia caused by EGFR/Ras disruption was due to reduced cell proliferation and/or increased cell death. Disrupting Ras signaling in the *APC* mutant clones resulted in a significant decrease in the number of clones that contained mitotic cells, as indicated by the presence of phospho-histone H3 (PH3), compared with the clone mutant for *APC* alone (Fig. 4S). Similarly, using a BrdU incorporation assay, we observed a significant decrease in the number of diploid cells that were BrdU positive (Fig. 6). Therefore, Ras is important for *APC* loss-induced cell proliferation. We employed TUNEL labeling for cell death detection and found that there was not a significant increase of TUNEL+ cells when Ras was disrupted (Fig. 6). Additionally, the expression of the anti-apoptotic protein p35 in *APC* $ras85D^{e1B}$ compound clones (noted as *APC-ras* clones hereafter) did not alter the reduced clone-size phenotype (Figs. 4G, H and 5C). Taken together, these observations suggest that normal EGFR/Ras signaling is required for the proliferation, but not survival, of *APC*-deficient ISCs, and it is therefore crucial for the initiation of *APC* loss-induced intestinal hyperplasia.

Given that both Wnt and EGFR/Ras signaling mediate the initiation of *APC* loss-induced hyperplasia, we asked whether

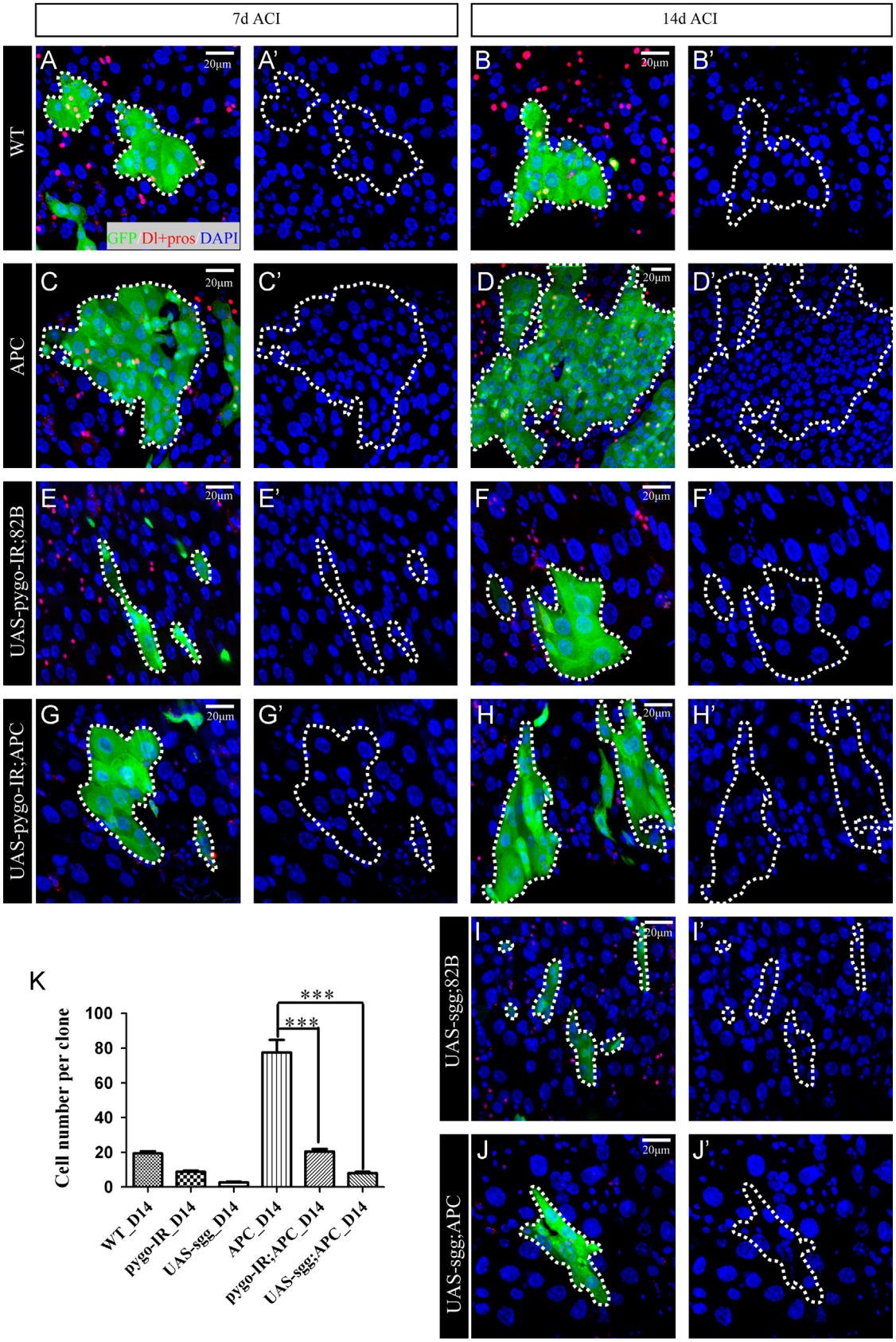

Fig.3. The canonical Wnt signaling pathway is required for *APC* loss-induced intestinal hyperplasia. (A–B) WT MARCM clones. (C–D) APC MARCM clones. (E–F) Pygo-IR expressing MARCM clones. (G–H) Pygo-IR expressing APC MARCM clones. (I) 14-day-old MARCM clones expressing sgg. (J) 14-day-old APC MARCM clones expressing sgg. (K) A plot showing the cell number per ISC clone of specified genotype on day 14 ACI. Values are means $\pm$ s.e.m, n=20–40 clones, *** denotes t-test $p < 0.001$.

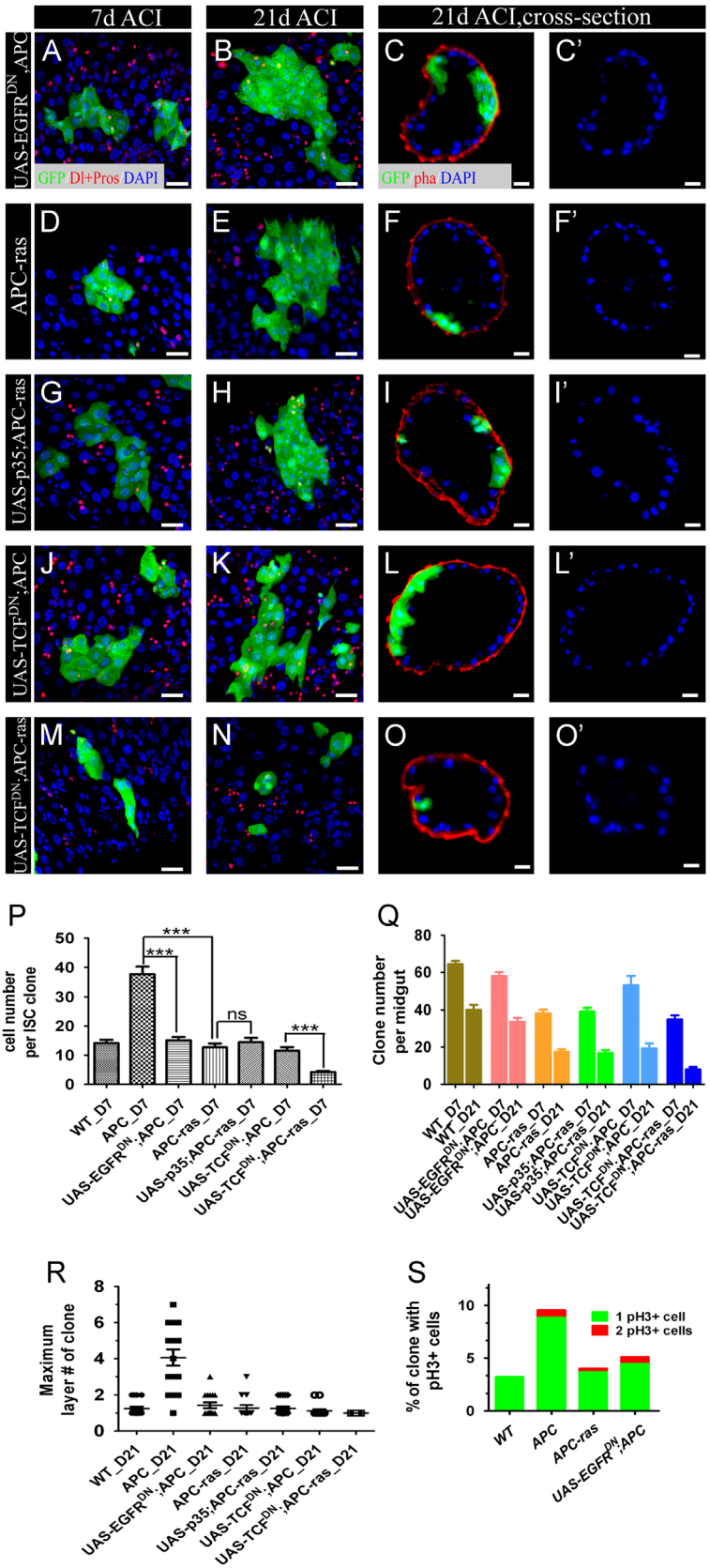

Fig. 4. Ras signaling is required for *APC* loss-induced intestinal hyperplasia. (A–C′) Overexpression of $EGFR^{DN}$ in *APC*-deficient clones significantly reduced the clone size and suppressed the formation of multilayered epithelium. (D–F′) *APC* and *ras85D* deficient clones rarely developed into hyperplasia. (G–I′) *APC-ras* clones with the expression of anti-apoptotic protein p35 behaved similarly to *APC-ras* clones. Clones rarely developed into hyperplasia. (J–L′) Expression of TCF^{DN} in *APC*-deficient clones significantly reduced the clone size and suppresses the development of hyperplasia. (M–O′) *APC-ras* clones with ectopic expression of TCF^{DN} failed to grow into large clones. (P) Quantification of clone size on day 7 ACI, measured by the total cell number within each MARCM clones of specified genotypes. $n=20$–40 clones, *** denotes $p<0.001$, ns: no significant difference. (Q) Quantification of clone number per midgut on day 7 and 21 ACI. (R) A scatter plot compares the number of epithelial layers found in each GFP-labeled clones of a given genotype on day 21 ACI. (S) The percentage of clones containing dividing cells (PH3+) on day 7 ACI. Note that disrupting of EGFR-Ras signaling significantly reduces cell proliferation.

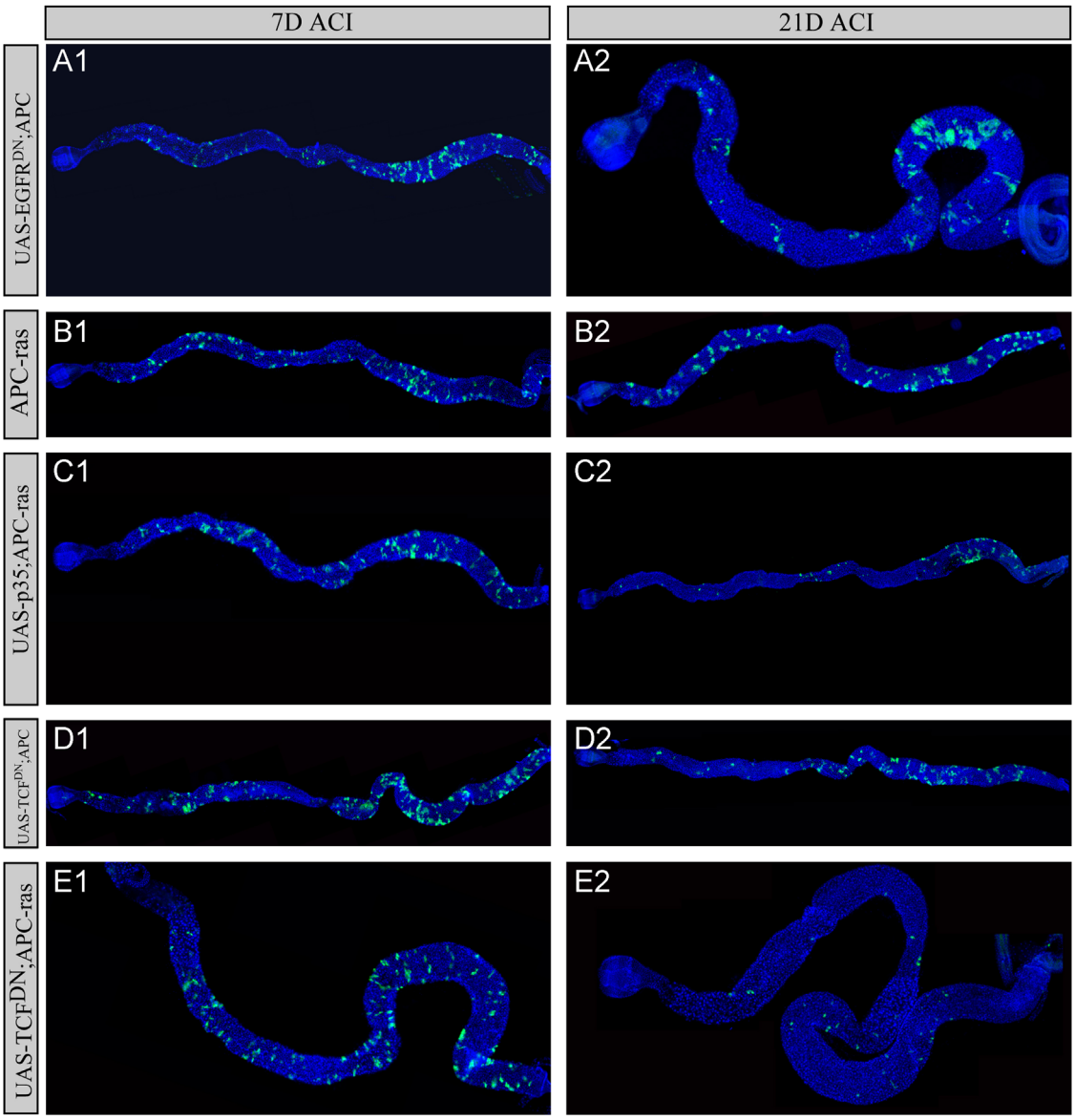

Fig. 5. Ras and Wnt signaling are both required for *APC* loss-induced hyperplasia. (A–B) *APC*-deficient clones coexpressing a dominant negative form of EGFR (A1 and A2) or lacking *Ras85D* (B1–B2) failed to overgrow examined on day 7 and day 21 ACI. (C1 and C2) Overexpression of anti-apoptotic protein p35 in *APC-ras* clones failed to rescue the reduced clone size phenotype, suggesting that loss of Ras is independent of cell death in suppressing *APC* loss- induced overgrowth. (D1 and D2) Inhibiting Wnt signaling by overexpressing a dominant negative form of TCF prevented clonal overgrowth. (E1 and E2) Simultaneous blocking Wnt signaling and Ras signaling in *APC*-deficient clones completely suppressed clonal growth. Note there is significant reduction in the size and number of clones from day 7 (E1) to 21 (E2) ACI.

simultaneous disruption of Wg and Ras signaling could further prevent *APC* loss-induced hyperplasia. Indeed, simultaneous disruption of both pathways completely abrogated the hyperplasia phenotype following *APC* loss (Figs. 4M, O and 5E), suggesting that Wg and Ras signaling act in parallel to promote the initiation of *APC* loss-induced hyperplasia. A TUNEL labeling assay revealed that there was not a significant increase in the number of TUNEL+ cells in *TCF^{DN}; APC-ras* clones (data not shown), suggesting that both pathways regulate the proliferation, but not the survival, of *APC*-deficient cells. This finding is consistent with the role of both pathways in regulating ISC proliferation during normal homeostasis.

Oncogenic Ras activation promotes the progression of APC loss-induced hyperplasia: blockage of cell differentiation and "preinvasive" outgrowth

In *APC* loss-induced tumorigenesis, oncogenic Ras activation is hypothesized to be a key transitional event for the progression from adenoma to carcinoma. To determine whether this transitional event could be modeled in *Drosophila*, we expressed oncogenic Ras (RasV12) in *APC* mutant clones (described as *APC-RasV12* clones hereafter) and examined the effects. Interestingly, during the course of clonal analyses, we observed a significant decrease in the number of GFP clones that remained in the midgut epithelium over time (Fig. 7C–E). A similar phenotype was also observed for clones overexpressing RasV12 alone (Fig. 7C–E). This decrease is likely due to epithelial delamination or sloughing induced by Ras activation (Fig. 8B and Supplemental Fig. S3) rather than cell death, as previously observed (Buchon et al., 2010). Consistent with this idea, we did not observe an obvious increase in apoptosis in *APC-RasV12* clones (data not shown). However, on day 21 ACI, approximately 35% of clones that remained in the epithelium developed into large spherical-tumor cell masses, which were termed "transformed clones" (Figs. 7F and 8C). The majority of the transformed clones resided in the anterior or posterior midgut, but not in the cooper region (middle midgut)

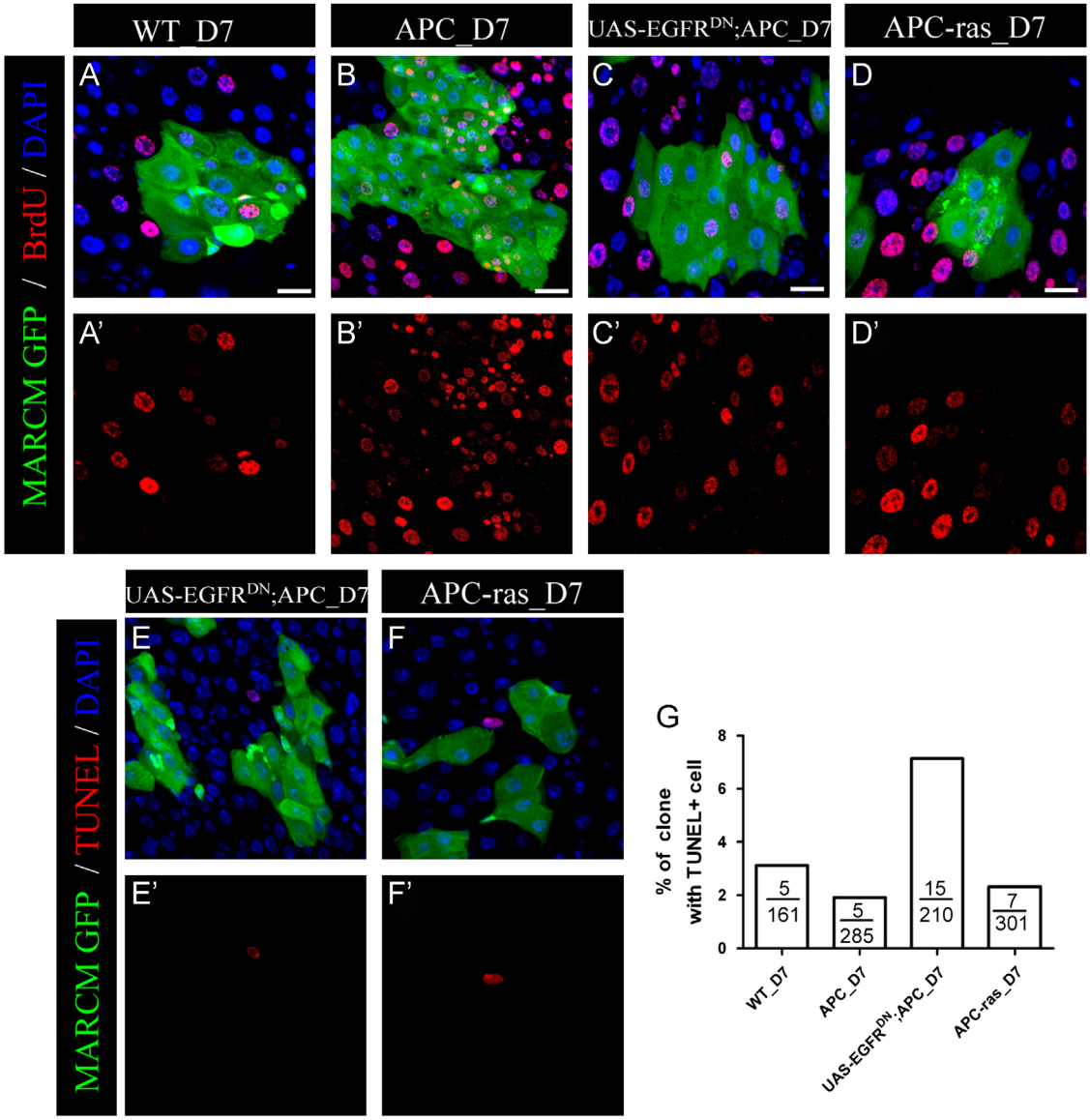

Fig. 6. Blocking Ras signaling in *APC*-deficient clones leads to reduced BrdU incorporation but does not promote cell death. (A–D, A′–D′) Detection of BrdU incorporation in the wildtype clone (A and A′), the *APC*-deficient clone (B and B′), the *APC*-deficient clone expressing $EGFR^{DN}$ (C and C′) and *APC-ras* clone (D and D′) on day 7 ACI. Note that loss of *APC* led to a significant increase in the number of BrdU+ diploid cells, and expression of $EGFR^{DN}$ or disruption of Ras could suppress this phenotype. (E and F) Detection of cell death by TUNEL labeling assay in *APC*-deficient clones expressing $EGFR^{DN}$ (E) and in *APC-ras* clones (F). Disruption of Ras signaling in *APC*-deficient clones did not lead to significant increase in cell death. (G) Quantification of percentage of clones containing TUNEL positive cell(s) on day 7 day ACI.

(Fig. 7H), suggesting that Ras hyperactivation promotes regional outgrowth of *APC*-deficient clones. The remaining 65% of clones did not develop into hyperplasia (Figs. 7F and 8D), possibly due to the counterbalance between intestinal delamination and proliferation. The size of those clones was smaller than or comparable to that of the wild-type clones; we named those clones "small clones".

We next analyzed the cellular composition of *APC-RasV12* clones by co-staining with several cellular markers, including Dl, an ISC marker; Pros, an ee cell marker; and Pdm1, an EC marker. In *APC-RasV12* small clones, the ratio of ISCs to differentiated cells was comparable to the ratio in wild-type clones, suggesting that the ISCs in the mutant clones were still able to differentiate (Fig. 8D). In *APC-RasV12* transformed clones, however, most mutant cells were small diploid cells (Fig. 8E) with weak Dl expression, and few cells expressed Pros or Pdm1 marker, suggesting that cellular differentiation was blocked in those clones. This severe blockage of cellular differentiation was rarely observed in RasV12-expressing clones or in *APC*-deficient/ Arm-activated clones (Supplemental Fig. S4).

Another striking observation was that approximately 10% (11/108) of *APC-RasV12* clones tended to extrude basally toward the surrounding muscle layer (Fig. 8F), although the underlying basement membrane remained unbroken, as indicated by the staining of Laminin B (Fig. 8H). In rare cases, the *APC-RasV12* clone was almost entirely extruded out of the surrounding muscle layers and moved to the body cavity. A similar scenario of tumor extrusion was found in the follicular epithelium in the *Drosophila* ovary, where *Psc Su(z)2* mutant follicle stem cells develop into tumor masses that basally extrude out the epithelium into body cavity without disrupting the basal lamina (Li et al., 2010). This result suggests that the *APC-RasV12* tumor cells may have a tendency to be invasive toward neighboring tissues. Because disrupted epithelial cell polarity is intricately related to malignant progression (Bilder, 2004), the observed outgrowth behavior led us to test whether cell polarity was disrupted in the mutant clones. We therefore examined several cell polarity markers in these clones. The basolateral marker Dlg was normally associated with cell membrane. This pattern of distribution remained largely normal in

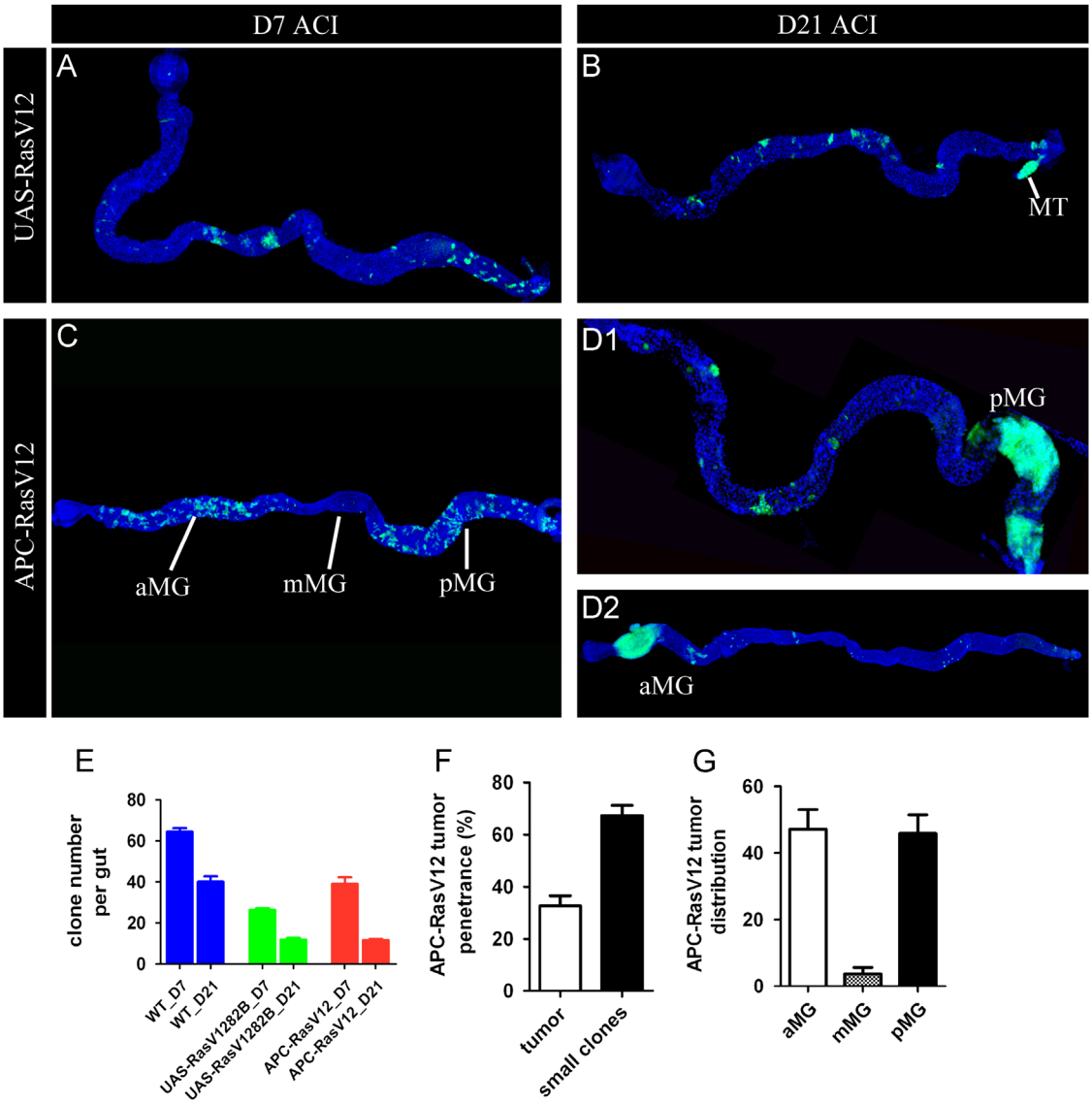

Fig. 7. Oncogenic Ras activation in *APC* clones leads to region-specific tumor outgrowth. (A–D) Overall view of midguts bearing *RasV*12 clones (A and B), or *APC-RasV*12 clones (C–D). Note that residual *APC-RasV*12 clones located at posterior midgut and anterior midgut region underwent rapid proliferation and developed into large tumor masses. (E–G) Quantification of the clone number per midgut, the ratio of tumor versus non-tumor clones within *APC-RasV*12 clones (F), and the regional distribution of *APC-RasV*12 tumors (G). aMG: anterior midgut; mMG: middle midgut; pMG: posterior midgut. MT: malphigian tubules.

*RasV*12-expressing clones and *APC*-deficient clones (Fig. 9A–C). Strikingly, a significant fraction of Dlg was dissociated from the membrane and diffused into the cytosol in *APC-RasV*12 (Fig. 9D) or *APC-Raf*^{GOF} (Fig. 9E) clones. The apical marker aPKC was normally distributed at the apical membrane of ISCs, and was undetectable in mature epithelial cells (Fig. 9F). This apical localization pattern was maintained in APC-deficient clones (Fig. 9G) or *RasV*12-expressing clones (Fig. 9H), but lost in *APC-RasV*12 clones (Fig. 9I). The appeared alternation of cell polarity is consistent with the observation that the cells in the mutant clones were severely disorganized. Taken together, these data suggest that oncogenic RasV12 is able to promote the progression of *APC* loss-induced hyperplasia, and the cells in the transformed clones show several characteristics that are distinguishable from the cells in *APC* alone mutant clones, including blockage in differentiation, appeared loss of cell polarity and a tendency to extruding out from the original tissue, a behavior that we define here as "preinvasive".

Loss of Ras results in delayed, but not failed, activation of Wg signaling following APC loss

Given that Ras and Wg signaling are both required for *APC* loss-induced ISC proliferation and intestinal hyperplasia in *Drosophila*, we asked whether Wg pathway activation following *APC* loss is also Ras-dependent in *Drosophila*. On day 7 ACI, we observed an increased accumulation of Arm in *APC*-deficient cells, but Arm protein was still associated with the cell membrane and was not detectable in the nucleus (Fig. 10M). It is probably not surprising as preferential Arm nuclear accumulation is also not observed in embryonic ectodermal cells responding to Wg signals (Peifer et al., 1994). However, as described earlier, the Wnt activation reporter TCF-LacZ was significantly upregulated in the mutant cells (Fig. 1). Therefore, following *APC* loss, Wg signaling is quickly activated despite that no detectable Arm is present in the nucleus. This indicates that nuclear Arm at an undetectable level is sufficient to activate downstream target genes. We next examined

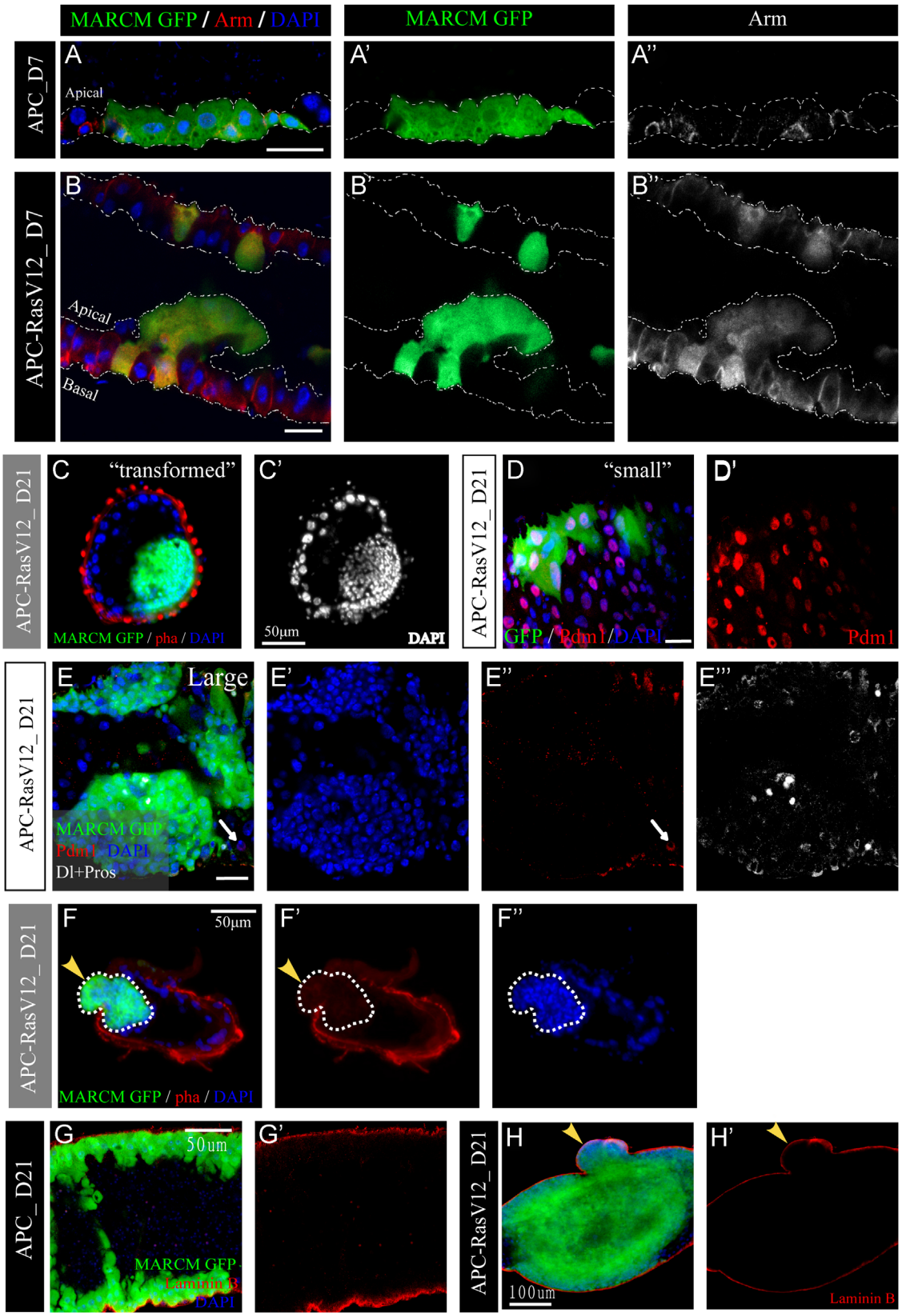

Fig. 8. Oncogenic Ras activation in *APC* clones leads to blockage of cell differentiation and preinvasive outgrowth. (A) Intestinal epithelium bearing *APC*-deficient clone remained well-organized on day 7 ACI. The lateral cell membrane was marked by Arm staining (red in A and B, white in A″ and B″). (B) Delamination of the clones was observed after *RasV*12 expression. (C–C′) A cross-section view shows a "transformed" clone. Note the spherical tumor mass was largely consist of diploid cells. (D–D′) In *APC-RasV*12 "small" clones, Pdm1 (red) labeled EC cells could be observed. (E–E‴) A sagittal-section view showed a "transformed" *APC-RasV*12 clone, which contained few differentiated ECs. Note that a fraction of cells retained Dl expression (white in E and E‴, membrane). ee cells marked by pros (white in E and E‴, nucleus) were also detected in the clone. Arrows in E and E″ indicate an adjacent EC cell marked by Pdm1 (red). (F–F″) A small fraction of *APC-RasV*12 clones (dashed line) showed aggressive outgrowth and tended to extrude out of the muscle layers, indicated by F-actin staining (Red). (G–H′) Laminin β1 staining in 21-day-old *APC*-deficient clones (G–G′) and *APC-RasV*12 clones (H–H′). Extrusion of *APC-RasV*12 clones (indicated by yellow arrowhead) did not break the basement membrane marked by laminin β1 (red).

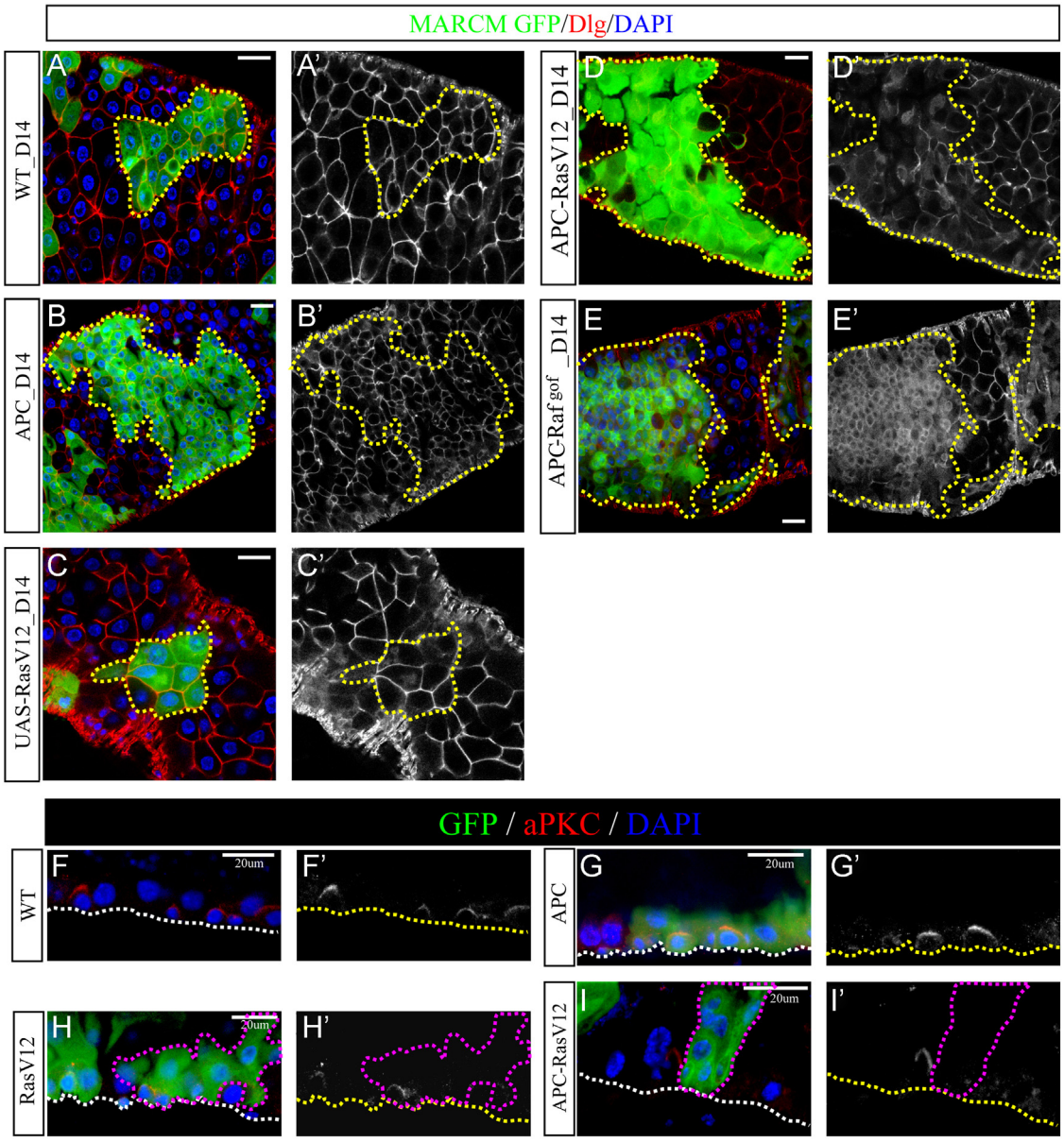

Fig. 9. Oncogenic Ras activation in *APC* clones seems to induce cell polarity changes. (A–E′) Dlg staining in MARCM clones (2 weeks ACI) of various genotypes: (A–A′) wildtype clones; (B–B′) *APC* clones; (C–C′) *RasV*12-expressing clones; (D–D′) *APC-RasV*12 clones; and (E–E′) *APC-Raf*^{gof} clones. Note that Dlg was largely localized at basolateral membrane in WT, *APC* deficient cells as well as *RasV*12-expressing cells, but a significant fraction was diffused to cytosol in *APC-RasV*12 or *APC-Raf*^{gof} clones. (F–I′) Sagittal views of aPKC staining in cells of various genotypes: (F-F′) wildtype; (G-G′) *APC* clones; (H–H′) *RasV*12-expressing clones; and (I–I′) *APC-RasV*12 clones. Note that aPKC was apically distributed in WT, *APC* deficient, as well as *RasV*12-expressing progenitor cells. However, in *APC-RasV*12 clones, the apical localization of aPKC was abolished.

the expression of Arm and TCF-LacZ in *APC-ras* clones in a time course. Similar to *APC*-deficient clones, membrane-bound Arm was also upregulated in *APC-ras* clones (Fig. 10N, Supplemental Fig. S5), and TCF-lacZ expression was still observed in the clones, suggesting that *ras* is dispensable for Wnt pathway activation following *APC* loss. However, the percentage of LacZ+ cells within each *APC-ras* clone was lower than the percentage in *APC*-deficient clones (Fig. 10A–K). On day 4 ACI, approximately 46% of *APC*-deficient clones contained cells expressing TCF-LacZ, but this number was 40% in *APC*-ras clones. Similarly, on day 14 ACI, almost all of the cells in the *APC*-deficient clones displayed TCF-LacZ expression, but only approximately 70% of cells in *APC-ras* clones expressed TCF-LacZ, although all of the cells become LacZ+ by day 21 ACI (Fig. 10L). These data suggest that Ras is not absolutely required for Wg pathway activation, but it may have a role in accelerating Wg pathway activation following *APC* loss. Because TCF-LacZ only reflect high levels of Wnt pathway activation, it remains possible that Ras could regulate low-level-Wnt response genes, whose expression changes could not be reflected by TCF-LacZ.

Oncogenic Ras or Raf activation promotes cytoplasmic and nuclear accumulation of armadillo following APC loss

Next, we evaluated whether oncogenic Ras activation is able to accelerate Wg pathway activation immediately following *APC* loss. Interestingly, we observed significant cytoplasmic and nuclear accumulation of Arm in *APC-RasV*12 clones as early as on day 4 ACI (Fig. 10O and O′). The activation of Raf in the *APC* clones had a similar effect on Arm accumulation (Fig. 10P and P′). Moreover, the activation of Raf in the *APC-ras* clones also led to cytoplasmic and nuclear accumulation of Arm (Fig. 11A) and to tumor development (Fig. 11B), which is consistent with the notion that RasV12

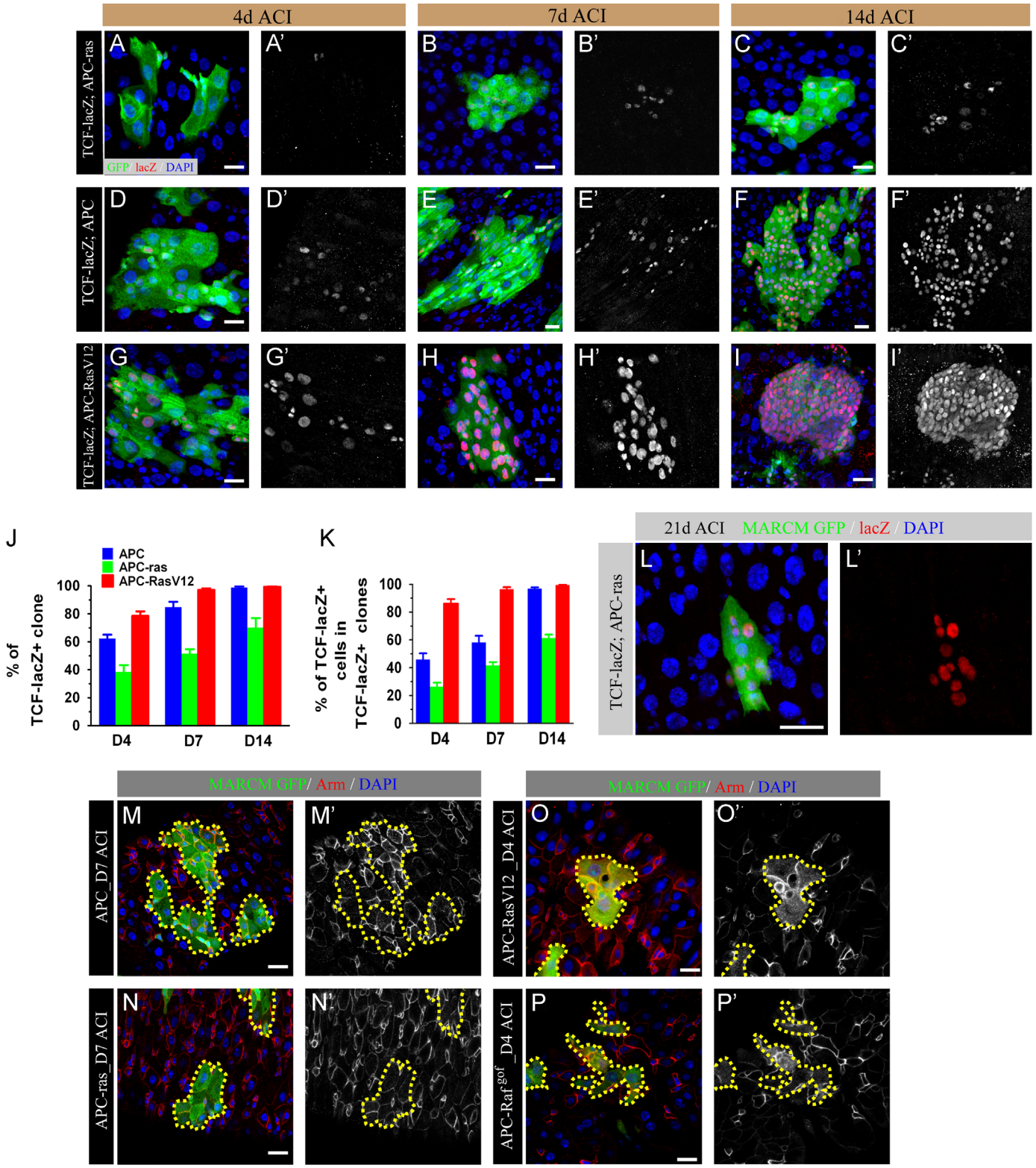

Fig. 10. The effect of Ras activity on *APC* loss-induced Wnt signaling hyperactivation. (A–I′) The expression of TCF-lacZ was examined in clones of given genotypes: *ras* (A–C′), *APC* (D–F′), *APC-RasV12* (G–I′). (J) The percentage of clones containing TCF-lacZ expressing cells. $n=15$–20 midguts. (K)The percentage of TCF-lacZ expressing cells within TCF-lacZ$^+$ clones. n=15–20 clones. (L–L′) Cells within a *APC-ras* clone on day 21 ACI were all TCF-lacZ$^+$. (M–M′) Loss of *APC* led to upregulation of membrane-bound Arm examined on day 7 ACI. (N–N′) In *APC-ras* clones, slight upregulation of membrane-bound Arm was also observed. (O–P′) Overexpression of *RasV12* (O–O′) or *Raf^{gof}* (P–P′) in *APC*-deficient clones led to cytoplasmic and nuclear accumulation of Arm.

acts through Raf to promote tumorigenesis following *APC* loss. In contrast, the overexpression of RasV12 alone had no obvious effect on the expression level of Arm or TCF-LacZ (Supplemental Fig. S5). To determine whether the accumulation of Arm in the cytoplasm and nucleus is correlated with the levels of Wg pathway activation, we examined TCF-LacZ expression in those clones. As expected, LacZ expression was significantly higher and was induced earlier in *APC-RasV12* clones compared with *APC* clones (Fig. 10G–K).

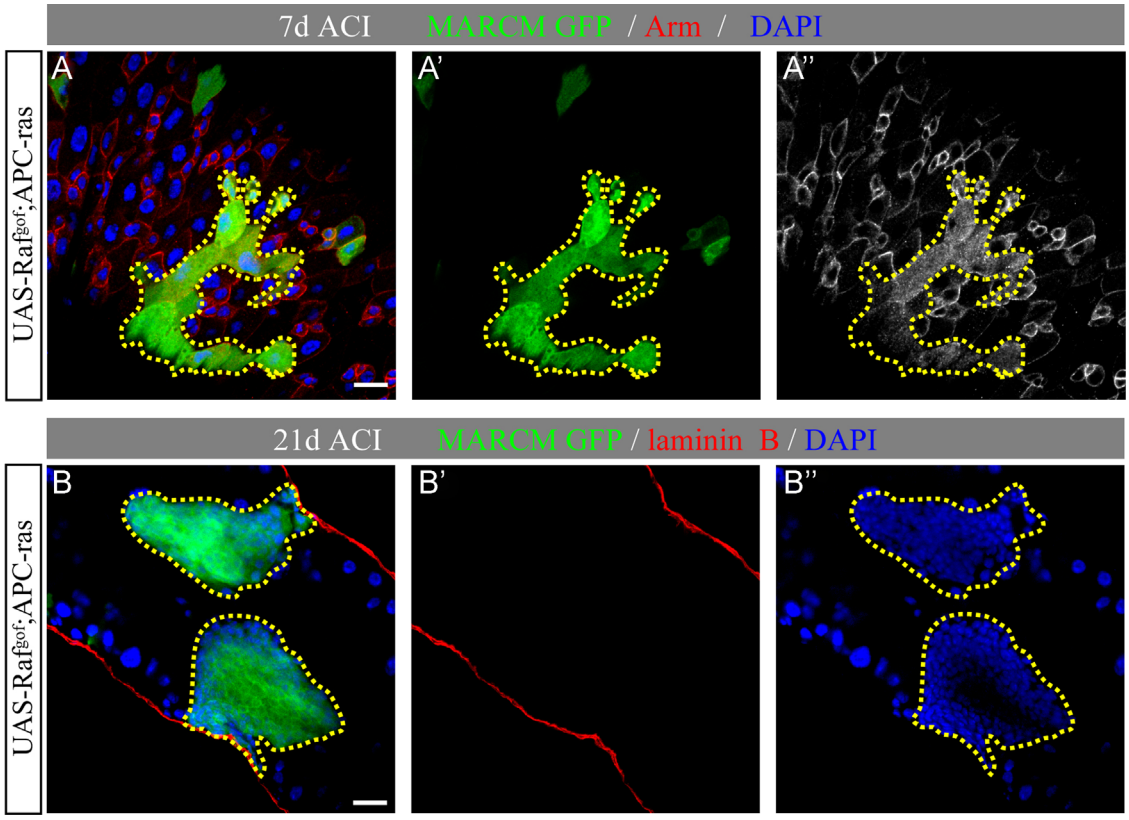

Fig. 11. RasV12 acts through Raf to promote tumor outgrowth following *APC* loss. (A–A″) Overexpression of Raf^{GOF} in *APC-ras* clones led to cytoplasmic and nuclear accumulation of Arm, suggesting that Raf acts downstream of Ras to promote mislocalization of Arm following APC loss. (B–B″) A sagittal view of midgut carrying *APC-ras* clones co-expressing *UAS-*Raf^{GOF} on day 21 ACI. Activation of Raf bypassed the requirement of Ras in *APC* loss-induced tumorigenesis. The surrounding basement membrane marked by laminin beta1 (in red) remained unbroken. Scale bars denote 20 μm.

Taken together, these data suggest that following *APC* loss, oncogenic Ras/Raf activation is able to enhance Wnt pathway activation by promoting cytoplasmic and nuclear accumulation of Arm and transcriptional activation of downstream target genes.

Ras activation-induced accumulation of arm in APC-deficient cells does not occur via the JNK or PI3K-Akt signaling pathways, but in part via the downregulation of E-cadherin

Studies in vertebrates and mammals have indicated several mechanisms that promote cytoplasmic β-catenin translocation into the nucleus. Rac1 activation has been suggested to mediate nuclear accumulation of β-catenin (Wu et al., 2008). Following the loss of *APC*, K-ras activation induces β-catenin nuclear translocation via the activation of JNK2 and Rac1 (Phelps et al., 2009). PI3K-Akt signaling that can act downstream of Ras signaling has also been proposed to have a role in phosphorylating β-catenin and promoting its nuclear localization (He et al., 2004). We therefore evaluated whether JNK or Akt1 activation after *APC* loss is sufficient to induce the nuclear accumulation of Arm in the *Drosophila* model. JNK was activated by the expression of a constitutively active form of Hep (Hep^{ACT}, the *Drosophila* JNKK), which prevented intestinal hyperplasia following *APC* loss (Fig. 12A), possibly due to JNK-mediated cell death. We did not observe obvious Arm mislocalization to the cytoplasm or nucleus in clones that remained in the epithelium on day 7 ACI (Fig. 12A). In addition, overexpression of an active form of Rac1 (Rac1.V12) in *APC* mutant clones did not cause any obvious cytoplasmic accumulation of Arm (Fig. 12B). Furthermore, Arm was still accumulated in the cytoplasm and nucleus in *APC-RasV12* clones expressing a dominant-negative form of Rac1 (Rac1.L89) (Fig. 12C), and the Wnt pathway was still hyperactivated in *APC* clones that expressed Rac1.L89, as observed by the expression of TCF-lacZ (Fig. 12D). These data suggest that JNK and Rac1 do not appear to have roles in regulating Arm nuclear localization in midgut cells. To analyze the effect of PI3K-Akt signaling on the nuclear localization of Arm, we expressed a constitutively active form of PI3K92E, $PI3K^{ACT}$, in *APC*-deficient clones. As a control for $PI3K^{ACT}$ activity, we evaluated phosphor-Akt, the active form of Akt, and found that it was undetectable in wild-type epithelial cells, but was specifically expressed in cells that expressed $PI3K^{ACT}$ (Supplemental Fig. S6). In contrast, oncogenic Ras activation in epithelial cells failed to activate Akt, which suggests that Ras activation could not activate PI3K-Akt signaling in the midgut epithelial cells and that Akt might not be involved in nuclear transportation of Arm (Supplemental Fig. S6). To further test this hypothesis, we examined the cellular distribution of Arm in *APC*-deficient clones expressing the active form of PI3K92E or Akt. Their expression did not promote the redistribution of membrane-bound Arm to the cytosol or nucleus (Fig. 12E and F). These observations suggest that Ras-induced Arm mislocalization following *APC* loss is likely not mediated by JNK or PI3K-Akt signaling in the *Drosophila* midgut.

Interestingly, we observed a significant downregulation of E-cadherin in approximately 26% (51/195) of *APC-RasV12* clones examined on day 7 ACI (Fig. 12G). In contrast, E-cadherin was slightly upregulated in *APC* clones and was not affected in clones expressing *RasV12* alone (Supplemental Fig. S7). E-Cadherin is a known binding partner of Arm/β-catenin, which functions to link cell adhesion to actin cytoskeleton in this case. Because the binding of Arm to E-cadherin can stabilize Arm on the cell membrane, the accumulation of Arm in the cytosol in *APC*-

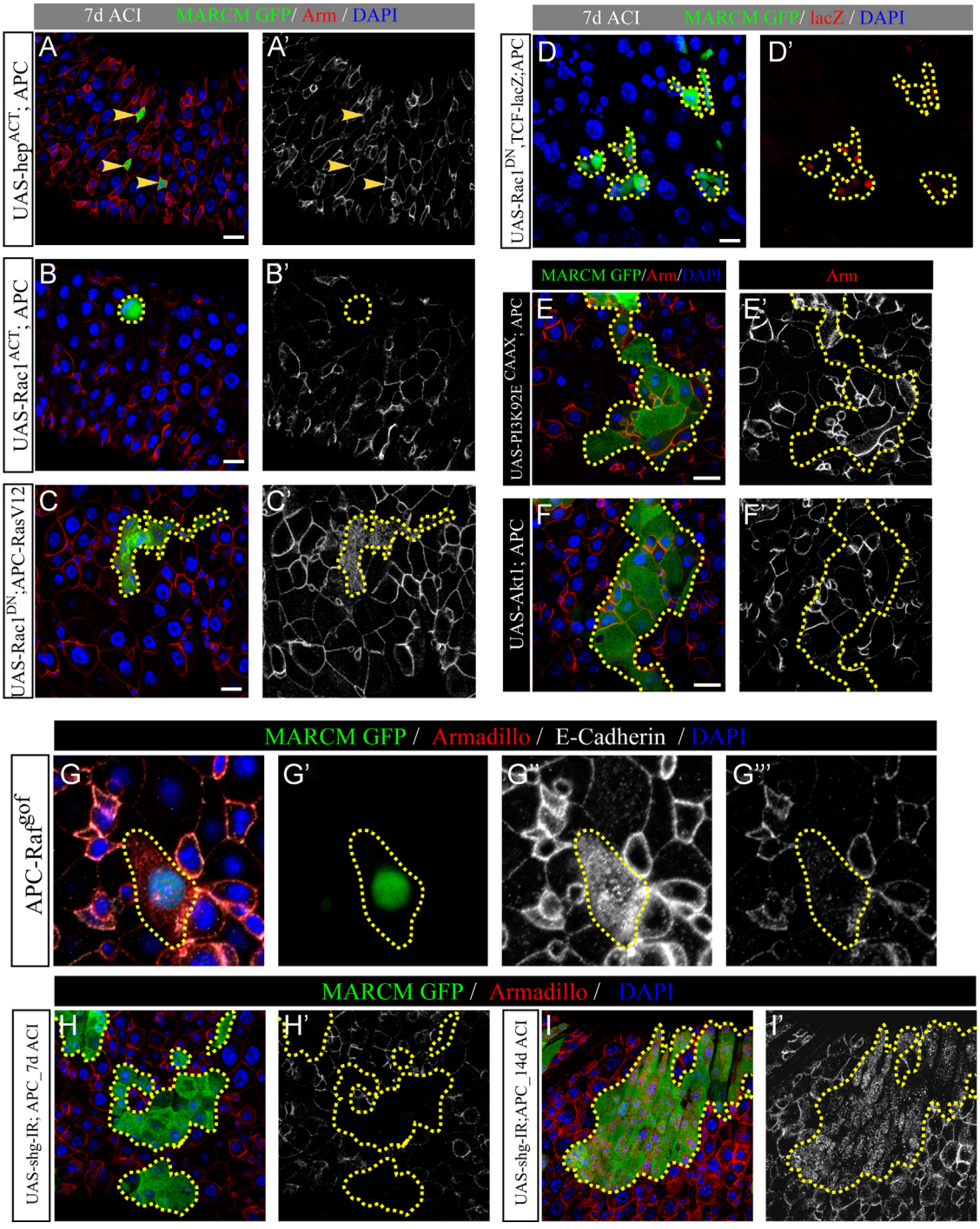

Fig. 12. Ras Activation-induced accumulation of Arm in *APC*-deficient cells does not occur via JNK or PI3K-Akt signaling pathways, but in part via downregulation of E-cadherin. (A–C) Effects of expressing hep^{ACT} (A), $Rac1^{ACT}$ (B), or $Rac1^{DN}$ (C) in *APC*-deficient clones on Arm distribution. (D) Effects of $Rac1^{DN}$ expression on TCF-lacZ expression after *APC* loss. (E–F) Effects of overexpressing PI3K92E^{CAAX} (E), or Akt1 (F) in *APC*-deficient clones on Arm distribution. (G) A *APC-*Raf^{gof} clone showed concurrence of mislocalization of Arm and down-regulation of E-cadherin. (H–I) Knockdown of E-cadherin in clones lacking *APC* led to a significant decrease in membrane-bound E-cadherin and Arm on day 7 (H), and cytoplasmic and nuclear accumulation of Arm on day 14 ACI (I).

*RasV*12 clones could be due to the downregulation of E-cadherin. We first tested whether manipulating the E-cadherin level in intestinal cells could alter Arm distribution. As expected, knocking down *shg* (the gene that encodes E-cadherin) in flp-out clones led to a reduction of Arm on the cell membrane (Supplemental Fig. S8). In contrast, forced expression of GFP-tagged E-cadherin (UAS-shg-DEFL) led to a significant accumulation of Arm in the membrane and cytoplasm, where it was largely colocalized with GFP-E-cadherin (Supplemental Fig. S8). Next, we asked whether knockdown of E-cadherin in *APC* clones is sufficient to induce Arm accumulation in the cytosol and nucleus. In early *APC*-deficient clones with shg-RNAi (day 7 ACI), we observed a significant decrease in membrane-bound E-cadherin and Arm (Fig. 12H). Strikingly, in late clones (day 14 ACI), significant cytoplasmic and nuclear accumulation of Arm became apparent (Fig. 12I). Of note, shg-RNAi also accelerated the formation of multilayered epithelium after *APC* loss. However, both ECs and ee cells were present in *APC-shg-RNAi* clones, and the ratio of differentiated cells to progenitor cells was similar to the ratio observed in *APC* clones. In addition, the cells in *APC-shg-RNAi* clones retained their

apicobasal polarity similar to *APC*-deficient cells (Supplemental Fig. S9). These observations suggest that although the down-regulation of DE-cadherin on the cell membrane may contribute to cytoplasmic and nuclear Arm accumulation, additional mechanisms must be involved in the activity through which Ras activation induces the disruption of cell polarity and blockage of cell differentiation following *APC* loss.

Ras and Wnt signaling cooperatively promote tumor progression following APC loss

Because Ras activation can further enhance Wnt pathway activation following *APC* loss, whether this function is responsible for the role of Ras in promoting tumor progression following *APC* loss is significant. As shown above, Ras is not required for Wnt pathway activation, and high levels of Wnt pathway activation could still be achieved in *APC-ras* mutant cells, albeit delayed. Moreover, forced expression of Arm^{ACT}, which presumably could further enhance Wnt pathway activation in *APC*-deficient clones, had no obvious effect on tumor progression (data not shown), suggesting that Ras signaling may function in a parallel pathway to Wnt signaling in promoting tumor progression. To further test this hypothesis, we used Affymetrix *Drosophila* genome arrays to compare the gene expression changes in the midgut with the activation of *RasV*12, loss of *APC*, or loss of *APC* combined with activation of *RasV*12. We identified and compared top 100 upregulated and top 100 downregulated genes caused by the overexpression of *RasV*12, loss of *APC*, or loss of *APC* and overexpression of *RasV*12, respectively (Fig. 13). Interestingly, there was little overlap of the genes differentially expressed in the midgut between *RasV*12 overexpression and the loss of *APC*, suggesting that Ras and *APC* regulate largely separable transcriptional programs (Fig. 13). For example, matrix metalloproteinase-1

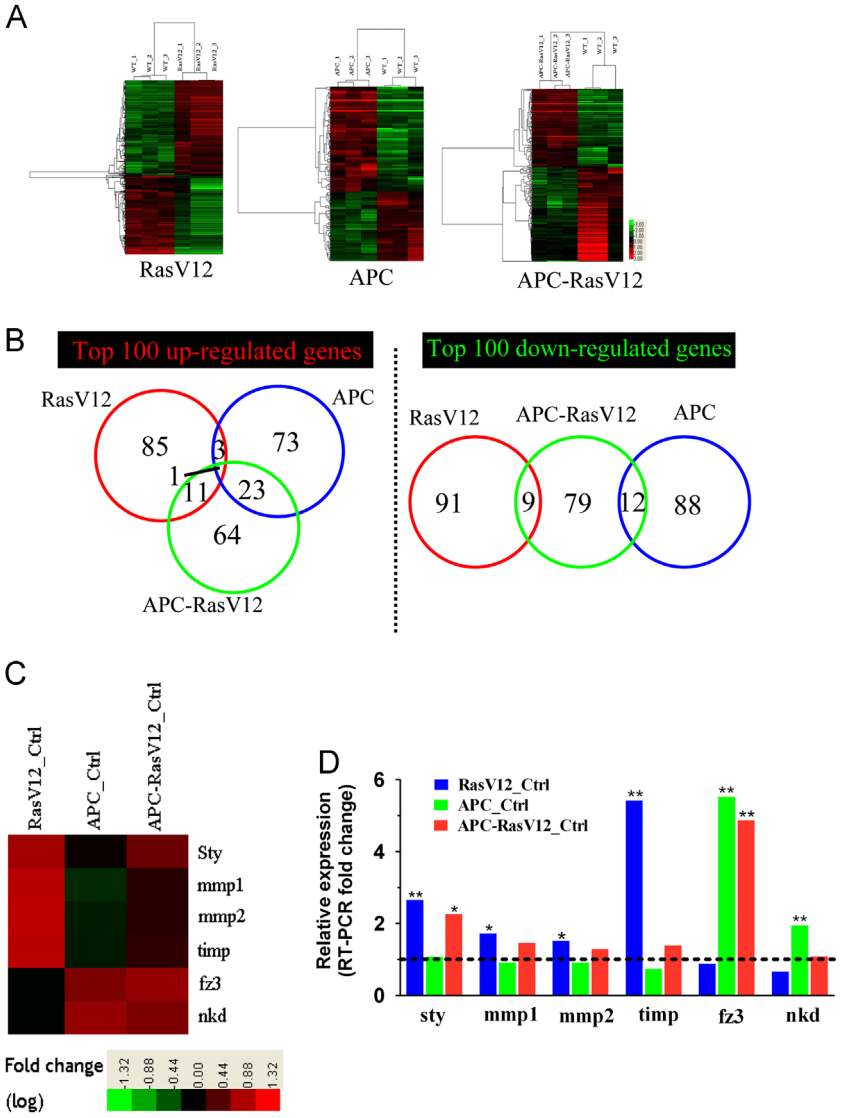

Fig. 13. Cooperative interactions between *APC* loss and oncogenic Ras/Raf activation in tumor progression. Hierarchical clustering analysis of gene expression data from *Drosophila* gene expression microarray experiments with specified genetic manipulation: *RasV*12 (overexpression of *RasV*12 driven by *esg-Gal*4), APC (*APC*-deficient clones), or *APC-RasV*12 (*APC-RasV*12 clones) compared to corresponding controls. For *RasV*12, *esg-Gal*4 > *UAS-GFP* guts were served as the control. For *APC* and *APC-RasV*12, wild-type GFP clones were served as the control. (A) Venn diagrams of top 100 up-regulated and down-regulated genes identified by microarray. There was little overlapping between the transcriptional targets of *RasV*12 and *APC loss*. (B) A list of selected genes from microarray analysis data. *Sprouty* (*sty*), *mmp*1, *mmp*2 and *timp* were significantly upregulated by *RasV*12 expression, *fz*3 and *nkd* were significantly upregulated by the loss of *APC*. (C)Validation of selected genes by quantitative RT-PCR. Samples of three independent biological replicates were used to perform qRT-PCR. Consistent with microarray data, *sty*, *mmp*1, *mmp*2 and *timp* were all significantly upregulated by RasV12. *fz*3 and *nkd* were upregulated after *APC* loss. (* denotes $p < 0.05$, ** denotes $p < 0.01$).

(mmp1) and matrix metalloproteinase-2 (mmp2), which have been implicated in metastasis, and Sprouty/Rhomboids (negative regulators of Ras signaling), are regulated by Ras, but not by APC. Conversely, fz3 and nkd, known targets of Wnt signaling, are regulated by APC, but not by Ras (Fig. 13). Notably, we identified a large set of genes exclusively responsive to the combined activation of *RasV*12 and loss of *APC*, but not regulated by *RasV*12 or loss of *APC* alone (Fig. 13). These data indicate that the cooperative activation of Ras and Wnt signaling lead to de novo activation of many genes whose expression might have significant roles in promoting tumor progression towards malignancy. Many of these genes are involved in proteolysis, nucleosome assembly, oxidation–reduction process and metabolic process, suggesting changes in these process might contribute to tumorigenesis. Taken together, our data are consistent with a model that Ras and Wnt pathway activation function in parallel and cooperatively to promote tumor progression following *APC* loss.

Discussion

Here, we studied the effect of Ras activity on the initiation and progression of the *Drosophila* model of *APC* loss-induced tumorigenesis. We found that Ras is required for the initiation of intestinal hyperplasia and that oncogenic Ras activation promotes the progression of tumors into premalignancy: mutant cells seem to have lost their cell polarity and have a tendency to invade neighboring tissues. These observations further support the *Drosophila* midgut as a useful genetic model for the understanding of the pathology of *APC* loss-induced tumorigenesis and, potentially, for drug discovery in the prevention and treatment of FAP. Importantly, we find that the proliferation of *APC* mutant clones could be further dampened by simultaneous inhibition/disruption of the Wnt and Ras pathways, which suggests that simultaneous inhibition of both Wnt and EGFR/Ras signaling could be an effective strategy for preventing adenoma development in FAP patients.

On the basis of our findings, we argue against the notion that *APC* loss alone is not sufficient for Wnt pathway activation. Using a Wg activation reporter, we found that Wg signaling is immediately activated following *APC* loss in the midgut, which is consistent with the observations in mammals that, although nuclear β-catenin is not always detected immediately following *APC* loss, Wnt target genes, such as c-Myc and Axin2, are usually upregulated (Obrador-Hevia et al., 2010; Sansom et al., 2004; Yan et al., 2001). We found that additional removal of Ras does not prevent, but delays, Wg signaling activation and that conversely, oncogenic Ras activation is able to promote Wg signaling activation following *APC* loss. These data demonstrate that Ras activation can potentiate Wnt signaling, but is not required for Wnt pathway activation. Several studies have suggested that K-ras activation can potentiate Wnt signaling in the mouse *APC* model (Janssen et al., 2006; Luo et al., 2009; Sansom et al., 2006). Interestingly, after *APC* loss, although the level of Arm protein is increased, it is still associated with the cell membrane and is not detectable in the nucleus, suggesting that transcriptional activation of Wg target genes still occurs even with an undetectable amount of Arm in the nucleus. This finding indicates that nuclear Arm/β-catenin is not a sensitive marker for Wg signaling activation. A similar scenario likely occurs in mammals as well (Fodde and Tomlinson, 2010).

Because oncogenic Ras is able to potentiate Wnt signaling following *APC* loss, an intriguing possibility is that oncogenic Ras activation might be required for a high threshold activation of Wnt signaling required for the progression from adenoma to carcinoma. Several observations in the *Drosophila* model are not in favor of a role for Ras in promoting the progression of intestinal hyperplasia by simply potentiating Wnt pathway activity. First, without *ras*, high levels of Wnt pathway activation can still be reached, although delayed, as the TCF-lacZ reporter showed full activation in *APC-ras* clones after three weeks. Second, although expression of a stable form of Arm is sufficient to promote epithelial proliferation similar to the levels caused by *APC* loss, its expression in *APC* mutant clones did not enhance the outgrowth phenotype and did not promote the preinvasive behavior observed in *APC-RasV*12 clones. A pitfall of this experiment is that Arm^{ACT} expression may not be able to further increase Arm levels in *APC* mutant clones, as APC proteins function to both sequester Arm and target Arm for destruction. Another piece of evidence is that knocking down DE-cadherin, which leads to Arm accumulation in the cytoplasm and nucleus, did not promote preinvasive outgrowth of *APC*-deficient clones. Furthermore, genetic analyses suggest that Wnt and Ras signaling function in parallel to initiate intestinal hyperplasia following *APC* loss. Finally, gene expression profiling reveals that Ras and APC control largely separable gene expression programs. Notably, many genes are de novo upregulated in *APC-RasV*12 tumors, indicating that the target genes are combinatorially regulated by TCF and the relevant Ras transcriptional activators, which might explain their synergistic effects to promoting tumor progression. Therefore, we propose that, although Ras activation can potentiate Wnt pathway activity, parallel and cooperative roles of Ras and Wnt pathway activation are likely the driving forces for promoting the progression of *APC* tumors towards malignancy.

We nevertheless explored the molecular mechanisms underlying the Wnt pathway enhancement activity of oncogenic Ras. Previous studies in vertebrates and mammals have suggested several mechanisms for K-Ras activation-induced β-catenin stabilization and nuclear localization. K-Ras could act through Akt1 to phosphorylate β-catenin at Serine 522, stabilizing β-catenin (He et al., 2007). Ras could also activate the JNK pathway and consequently Rac1, which would then promote β-catenin nuclear transport and Wnt pathway activation (Phelps et al., 2009; Wu et al., 2008). Interestingly, neither mechanism appears to be involved in Arm stabilization in *APC-RasV*12 clones in the *Drosophila* midgut. In *APC*-deficient clones, activation of either Akt1 or Rac1 is not sufficient to cause cytoplasmic or nuclear Arm accumulation. Instead, we find that a significant percentage of *APC* mutant clones display a downregulation of DE-cadherin at the cell membrane immediately after *RasV*12 is expressed. Consistently, knocking down DE-cadherin in *APC* clones is sufficient to induce Arm accumulation in the cytoplasm and nucleus. Therefore, for some *APC*-deficient clones, Ras activation could induce the downregulation of DE-cadherin and consequently Arm accumulation in the cytoplasm and nucleus. Interestingly, although knocking down DE-cadherin in *APC* clones induces Arm accumulation in the cytoplasm and nucleus and promotes epithelial proliferation, it does not cause the disruption of apicobasal polarity, suggesting that oncogenic Ras- induced cell polarity change is not via the downregulation of DE-cadherin. Changes in cell adhesion in APC mutant cells have been observed in several other *Drosophila* tissues. In the larval brain, DE-cadherin was mildly downregulated but its transcription was not altered in *APC* mutant clones. Interestingly, reduction of DE-cadherin levels could partially mimic the morphologic phenotypes observed in *APC* mutant clones, suggesting that downregulation of DE-cadherin may contribute to the phenotypes (Hayden et al., 2007). In contrast, in wing imaginal disc cells, DE-cadherin was upregulated in *APC* mutant clones, but this is not the cause of the apical constriction phenotype (Zimmerman et al., 2010), suggesting that APC could up- or down-regulate DE-cadherin levels in cell type- and/ or stage-specific manners, and this effect is also likely dependent on the activity of additional regulators, such as Ras in intestinal cells.

In cultured human breast epithelial cells, ras transformation also causes a disruption of adherens junctions (Kinch et al., 1995), suggesting that this phenomenon is not uncommon. How Ras activation following APC loss downregulates DE-cadherin is unclear and requires further study. Our array data did not reveal significant changes in levels of DE-cadherin mRNA in all three samples, indicating that down-regulation of DE-cadherin in *APC-RasV12* tumors most likely occurs at the post-transcriptional level.

One significant implication of this study is that both parallel and cooperative roles of Wnt and oncogenic Ras are likely the driving forces in promoting *APC* tumorigenesis in *Drosophila*. For a parallel function, Wnt and Ras likely promote cell proliferation through independent mechanisms, as suggested by our genetic studies. Ras activation has a unique effect on cell delamination, which leads to the loss of many *RasV12*-expressing clones from the epithelium. This function of Ras may explain why only a few *APC-RasV12* clones eventually remain in the midgut and develop into tumors. How Ras activation induces intestinal cell delamination remains uncertain; however, because Mmp1 expression can be regulated by Ras activity, Ras might induce cell delamination through remodeling of the extracellular matrix. For a cooperative function, Ras activation after *APC* loss produces several dramatic changes in cellular behavior that are not observed when either *APC* is disrupted or *RasV12* is expressed alone: blockage of differentiation, appeared loss of cell polarity and preinvasive outgrowth. Subcellular changes of DE-cadherin and Arm expression are also consequences of the cooperative effect of *APC* loss and Ras activation. Consistent with this cooperative effect, many genes, including genes involved in cell metabolism, are de novo upregulated when Ras is activated after *APC* loss (data not shown). Interestingly, an intriguing connection between cell metabolism and cancer development has been drawing much attention (DeBerardinis and Thompson, 2012). An important next step is to further elucidate the molecular mechanisms underlying the cooperative functions of Wnt and Ras signaling, which may lead to the identification of novel therapeutic targets for the prevention and treatment of gastrointestinal cancer.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2013.03.020.

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