Essential requirement for β -arrestin2 in mouse intestinal tumors with elevated Wnt signaling

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β -Arrestins (Arrb) participate in the regulation of multiple signaling pathways, including Wnt/ β -catenin, the major actor in human colorectal cancer initiation. To better understand the roles of Arrb in intestinal tumorigenesis, a reverse genetic approach (*Arrb*^{-/-}) and in vivo siRNA treatment were used in *Apc* ^{Δ 14/+} mice. Mice with Arrb2 depletion (knockout and siRNA) developed only 33% of the tumors detected in their *Arrb2*-WT littermates, whereas Arrb1 depletion remained without significant effect. These remaining tumors grow normally and are essentially Arrb2-independent. Unsupervised hierarchical clustering analysis showed that they clustered with 25% of *Apc* ^{Δ 14/+}; *Arrb2*^{+/+} tumors. Genes overexpressed in this subset reflect a high interaction with the immune system, whereas those overexpressed in Arrb2-dependent tumors are predominantly involved in Wnt signaling, cell adhesion, migration, and extracellular matrix remodeling. The involvement of Arrb2 in intestinal tumor development via the regulation of the Wnt pathway is supported by ex vivo and in vitro experiments using either tumors from *Apc* ^{Δ 14/+} mice or murine *Apc*^{Min/+} cells. Indeed, Arrb2 siRNAs decreased the expression of Wnt target genes in cells isolated from 12 of 18 tumors from *Apc* ^{Δ 14/+} mice. In *Apc*^{Min/+} cells, Arrb2 siRNAs completely reversed the increased Wnt activity and colony formation in soft agar induced by Apc siRNA treatment, whereas they did not affect these parameters in basal conditions or in cells expressing constitutively active β -catenin. We demonstrate that Arrb2 is essential for the initiation and growth of intestinal tumors displaying elevated Wnt pathway activity and identify a previously unsuspected molecular heterogeneity among tumors induced by truncating *Apc* mutations.

tumor initiation | carcinogenesis

Biallelic disruption of tumor-suppressor genes (1) and the acquisition of dominant-activating mutations of oncogenes (2) are considered as founding events of tumorigenesis. Tumor diversity, the soil of tumor evolution, is thought to emerge with time from these genetic alterations. In the case of colorectal tumorigenesis, the earliest genetic event is the activation of Wnt signaling through the genetic disruption of the tumor-suppressor gene adenomatous polyposis coli (*APC*) (3). It is estimated that 80% of colorectal tumors carry somatic mutations of *APC* and, although its loss of function is associated with microadenoma formation (4), the mechanisms underlying macroscopic tumor development remain elusive.

Nonvisual β -arrestins, β -arrestin1 (Arrb1) and β -arrestin2 (Arrb2), are ubiquitous proteins initially described for their role in G protein-coupled receptors desensitization, sequestration, and internalization (5). More recently, many studies have uncovered unexpected functions for Arrb as scaffold proteins for many signaling molecules in the cytoplasm and nucleus, thus regulating gene expression and cellular responses (6). As an example, Arrb modulate ERK, c-Jun NH2-terminal kinase, and phospho-inosi-

tide 3-kinase, kinases that are strongly activated in malignant cells (6). Arrb are overexpressed in late-stage cancers, such as human glioblastomas (7) and breast cancer (8), and have been assigned several functions in tumor cells, including proliferation (9), apoptosis (10), migration, and invasion (11). Arrb were also reported to regulate Wnt signaling in nonintestinal models (12). Indeed, Arrb1 interacts with phosphorylated dishevelled (Dsh) 1 and Dsh2 and enhances transcription factor-4 (Tcf-4)-mediated transcriptional activity in HEK293 cells (13). Arrb2 interacts with Dsh2 to mediate Wnt5A-stimulated endocytosis of Frizzled 4 (14) and interacts with Axin and Dsh after Wnt3A stimulation of mouse embryo fibroblasts, resulting in inactivation of glycogen synthase kinase 3 β and stabilization of β -catenin (15). Thus, we hypothesized that Arrb could play a role in intestinal tumorigenesis initiated by the activation of Wnt signaling, and we studied the effects of Arrb gene deletion on intestinal tumorigenesis using *Apc* ^{Δ 14/+} mice, a relevant mouse model of human intestinal carcinogenesis (16). Our results establish a critical role for Arrb2 at an early stage of adenoma formation and unravel a previously unsuspected diversity among *Apc* ^{Δ 14/+} mouse tumors.

Results

Depletion of Arrb2, but Not Arrb1, Decreases Intestinal Tumor Number in *Apc* ^{Δ 14/+} Mice. To unravel the role of Arrb in intestinal tumorigenesis, *Apc* ^{Δ 14/+} mice, which spontaneously develop intestinal tumors (16) that express heterogeneous levels of Arrb1 and Arrb2 mRNA (Fig. S1A), were crossed with *Arrb1* or *Arrb2*-null mice (17, 18). Arrb1 or Arrb2 protein expression was not detected in mucosa from *Apc* ^{Δ 14/+}; *Arrb1*^{-/-} (Fig. 1A) or *Apc* ^{Δ 14/+}; *Arrb2*^{-/-} (Fig. 1B), respectively. Although Arrb1 depletion had no significant effect, 3.5-mo-old *Apc* ^{Δ 14/+}; *Arrb2*^{-/-} mice displayed a significantly lower number of tumors (33%) than control littermates (Fig. 1C). This was indeed due to β -arrestin2 deletion and not to a compensatory change in Arrb1 mRNA levels, because the latter were not modified in *Arrb2*^{-/-} mice (Fig. S1B and

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Fig. 1. Depletion of Arrb2, but not Arrb1, decreases tumor number in $Apc^{\Delta14/+}$ mice. Absence of Arrb1 (A) or Arrb2 (B) in intestinal mucosa from $Apc^{\Delta14/+}$; $Arrb1^{-/-}$ and $Apc^{\Delta14/+}$; $Arrb2^{-/-}$, respectively, was verified by Western blot. (C) Tukey box plots show number of adenomas per mouse in 3.5-mo-old $Apc^{\Delta14/+}$; $Arrb1^{+/+}$ ($n = 11$), $Apc^{\Delta14/+}$; $Arrb1^{-/-}$ ($n = 15$), and $Apc^{\Delta14/+}$; $Arrb2^{-/-}$ ($n = 19$) mice. $*P = 0.004$ $Apc^{\Delta14/+}$; $Arrb2^{-/-}$ vs. $Apc^{\Delta14/+}$; $Arrb1^{+/+}$. Effect of Arrb1 and Arrb2 siRNA treatment on Arrb1 (D) and Arrb2 (E) mRNA expression in adenomas and adjacent healthy mucosa. $n = 4$ tumors or healthy tissues from different mice; $*P < 0.05$ vs. corresponding control siRNA. (F) Tukey box plots show the number of adenomas per $Apc^{\Delta14/+}$ mouse after treatment with siRNA targeting either the Arrb1 ($n = 9$) or Arrb2 mRNA ($n = 9$), compared with control siRNA. $n = 7$; $*P < 0.05$, Arrb2 vs. control siRNA.

C). Analysis of $Arrb1^{-/-}$ and $Arrb2^{-/-}$ mice revealed no alterations in the architecture or in the proliferation (Ki67 immunostaining) of the small intestine or the colon (Fig. S2).

In additional experiments, specific siRNAs against Arrbs were administered during 4 wk to 4.5-mo-old $Apc^{\Delta14/+}$ to determine the impact of Arrb1 and Arrb2 deletion on the fate of preexisting tumors. The efficacy of Arrb1- and Arrb2-targeting siRNAs was validated on CMT-93 mouse colorectal cancer cells in vitro (Fig. S3 A and B). Efficient in vivo delivery of Arrb2 siRNAs to intestinal crypts and tumors was verified (Fig. S3 C and D), and these siRNAs selectively decreased the expression level of Arrb1 (by $\approx 50\%$) and Arrb2 mRNA (by $\approx 60\%$) in healthy and tumor tissue (Fig. 1 D and E). Treatment of $Apc^{\Delta14/+}$ mice with Arrb2 siRNA significantly decreased (by 65%; SI Discussion) the number of tumors, whereas Arrb1 siRNA treatment had no significant effect (Fig. 1F). All these data suggest that Arrb2 plays a crucial role in intestinal tumorigenesis in $Apc^{\Delta14/+}$ mice.

Arrb2 Is Essential for the Initiation and Growth of Some, but Not All Intestinal Tumors in Apc-Mutated Mice. To assess the effect of Arrb2 deficiency on tumor growth, we measured the size of

tumors remaining in both models of Arrb2-depleted $Apc^{\Delta14/+}$ mice. Surprisingly, the decreased tumor number was not accompanied by a decrease in tumor size in both $Apc^{\Delta14/+}$; $Arrb2^{-/-}$ (Fig. 2A) and Arrb2 siRNA-treated mice (Fig. 2B). Furthermore, there was no difference in Ki67 and cyclin-D1 staining or in BrdU incorporation in residual tumors of $Apc^{\Delta14/+}$; $Arrb2^{-/-}$ mice and Arrb2 siRNA-treated $Apc^{\Delta14/+}$ mice compared with randomly selected tumors in their respective controls (Fig. 2C), indicating that the proliferative status does not discriminate tumors that manage to grow in $Apc^{\Delta14/+}$; $Arrb2^{-/-}$ mice from overall $Apc^{\Delta14/+}$ tumors. Taken together, these results suggest that Arrb2 is essential for the initial development and growth of 67% of tumors in $Apc^{\Delta14/+}$ mice, whereas remaining tumors are essentially independent from Arrb2. Thus, because the decreased number of tumors in $Apc^{\Delta14/+}$; $Arrb2^{-/-}$ mice is not accompanied by a decrease in tumor size and cannot be explained by a change in proliferation or migration rate (Fig. S4), we analyzed whether Arrb2-dependent and -independent tumors displayed different molecular profiles.

Molecular Analysis of Arrb2-Dependent and -Independent Tumors in $Apc^{\Delta14/+}$ Tumors. Although the same Apc mutation is present in every cell of $Apc^{\Delta14/+}$ mice, the identification of Arrb2-dependent and -independent tumors was suggestive of an early tumor heterogeneity in this mouse model. Thus, we hypothesized that molecular differences may allow us to discriminate two subset of tumors in $Apc^{\Delta14/+}$; $Arrb2^{+/+}$ mice and that remaining tumors in $Apc^{\Delta14/+}$; $Arrb2^{-/-}$ animals may bear a closer molecular resemblance to one of these subsets. To test this hypothesis, we analyzed the gene expression profiles of tumors of 16 $Apc^{\Delta14/+}$; $Arrb2^{+/+}$ and 13 $Apc^{\Delta14/+}$; $Arrb2^{-/-}$ tumors.

Unsupervised hierarchical clustering identified two major clusters: one (T1) containing 75% of the $Apc^{\Delta14/+}$; $Arrb2^{+/+}$ tumors, and the other (T2) containing the remaining tumors from $Apc^{\Delta14/+}$; $Arrb2^{+/+}$ (T2a) along with all tumors from $Apc^{\Delta14/+}$;

Fig. 2. Depletion of Arrb2 has no impact on residual tumor size distribution and proliferation in $Apc^{\Delta14/+}$ mice. (A) Graph shows the mean number of tumors of increasing sizes per mouse in 3.5-mo-old $Apc^{\Delta14/+}$; $Arrb2^{+/+}$ ($n = 11$) and $Apc^{\Delta14/+}$; $Arrb2^{-/-}$ ($n = 19$) mice ($P = 0.2$). (B) Graph shows the number of tumors of increasing sizes per mouse, in mice that received either control ($n = 7$) or Arrb2 ($n = 9$) siRNA ($P = 0.16$). (C) Depletion of Arrb2 did not affect the proliferation status of residual adenomas: expression of Ki67, BrdU, and cyclin D1. (Scale bars, 50 μ m.)

The tumor heterogeneity described above at the gene expression level was confirmed at the protein expression level by immunofluorescence. Indeed, a large majority of tumors in *Apc*^{Δ14/+} mice expressed high levels of Prox1 and Cldn2 and low levels of Cd45, whereas the remaining tumors expressed low levels of Prox1 and Cldn2 proteins and high levels of Cd45. These data suggest that the former are the Arrb2-dependent tumors and that the latter are infiltrated by immune cells and represent the Arrb2-independent subgroup (Fig. 3E). All these results reflect the presence of a significant molecular heterogeneity between Arrb2-dependent and -independent tumors downstream from the *Apc* mutation and suggest that Arrb2-dependent tumors have a higher level of Wnt target activation than their Arrb2-independent counterparts.

Ex Vivo Arrb2 Down-Regulation Differentially Decreases Wnt Target Gene Expression in Cells Isolated from *Apc*^{Δ14/+} Mouse Tumors. To further highlight the causal link between Arrb2 and the Wnt pathway in Arrb2-dependent tumors from *Apc*^{Δ14/+} mice, tumor cells were isolated from 18 adenomas stemming from three different *Apc*^{Δ14/+} mice, and the impact of Arrb2 siRNA was analyzed on the expression of three Wnt target genes (*Myc*, *Lgr5*, and *Cd44*). Although siRNA treatment efficiently down-regulated Arrb2 in all samples (Fig. 4A), cells from 12 of 18 adenomas only displayed reduced expression of *Myc*, *Lgr5*, and *Cd44* mRNA (Fig. 4B–D), whereas their expression was unaffected by Arrb2 down-regulation in 2 out of every 6 tumors per mouse. In addition, cells from the 12 responsive tumors displayed higher levels of *cldn2*, a representative gene of the T1 subgroup (Fig. 4E). Moreover, Arrb2 down-regulation induced a decrease of Wnt/β-catenin activity in SW480 human colorectal cancer cells, which display elevated Wnt signaling due to a genetic inactivation of *APC*, but not in RKO cells, a colorectal cancer cell line displaying WT *APC* and normal Wnt signaling (Fig. S6). These results confirm that Arrb2 is essential in cells with elevated Wnt activity.

Arrb2 Knockdown Prevents the Increase of β-catenin/Tcf-4 activity and Colony Formation Induced by *Apc* Depletion in Vitro. To ascertain whether Arrb2 could be essential for the initial step of tumor development in tumors with elevated Wnt activity, Arrb2 or control siRNA was used in murine *Apc*^{Min/+} cells in which a loss of the second *Apc* allele was mimicked in vitro using *Apc* siRNA. Efficacy of these siRNA is demonstrated in Fig. S4. As expected, β-catenin/Tcf-4 transcriptional activity and the number of colonies increased when *Apc* expression was diminished (Fig. 5B and C). Arrb2 depletion reversed this phenotype, whereas it was ineffective in control cells, which carry a heterozygous *Apc* mutation (Fig. 5B and C). In the same cells, we found that the effect of Arrb2 siRNA on *Apc* siRNA-induced Wnt activity (Fig. 5B) and colony formation (Fig. 5C) was reversed by expression of a constitutively active form of β-catenin (ΔN87b-cat). In conclusion, these results strongly support the hypothesis that Arrb2 is only essential for the initiation of tumors in an elevated Wnt signaling context and is acting upstream from or on the *Apc*/Gsk3β/axin complex.

Discussion

In this study, we identify the involvement of Arrb2 in intestinal tumorigenesis induced by truncating *Apc* mutations. We demonstrate that 67% of tumors were absent upon Arrb2 depletion in *Apc*-mutated mice. The remaining 33% grew normally and clustered with 25% of tumors present in *Apc*^{Δ14/+}; *Arrb2*^{+/-} mice in an unsupervised clustering analysis of global gene expression profiles. This observation unravels a previously unsuspected molecular heterogeneity among these tumors. One of these tumor subgroups only displays a higher level of Wnt activity, and we provide evidence in favor of a selective role for

Fig. 4. Ex vivo Arrb2 down-regulation decreases Wnt target gene expression in cells isolated from 67% of *Apc*^{Δ14/+} mouse tumors. (A–D) Impact of Arrb2 siRNA on Arrb2 (A), *Myc* (B), *Lgr5* (C), and *Cd44* (D) mRNA expression in tumor cells isolated from 18 adenomas (tumors 1–18) from three different *Apc*^{Δ14/+} mice (six adenomas per mouse), compared with control siRNA. (E) Expression of *Cldn2*, a typical gene overexpressed in T1 tumors, was quantified in cells isolated from tumors that responded to Arrb2 siRNA for Wnt target genes (adenomas 1, 2, 3, 4, 7, 8, 9, 10, 13, 14, 15, and 16) compared with the ones that did not respond (adenomas 5, 6, 11, 12, 17, and 18). ΔCt are represented (*Cldn2* Ct–*Gapdh* Ct), thus high ΔCt values reflect low *Cldn2* expression, and vice versa. Consequently, tumor samples 5, 6, 11, 12, 17, and 18 show a higher ΔCt and therefore express lower levels of *Cldn2*, in line with their low response to the Arrb2 depletion.

Fig. 5. Arrb2 depletion reverses tumorigenicity induced by *Apc* loss of function but not by constitutively active β -catenin. (A) Impact of *Apc* and Arrb2 siRNA treatment on Arrb2 ($n = 5$; $*P = 0.003$ and $**P = 0.007$) and *Apc* ($n = 5$; $*P = 0.0003$ and $**P < 0.0001$) mRNA expression in *Apc*^{Min/+} cells compared with control siRNA. (B) Analysis of the impact of siRNA-mediated *Apc* and/or Arrb2 down-regulation on transcriptional activity of the β -catenin–Tcf-4 complex using a luciferase reporter system, modulated or not by transfecting cells with a mutated β -catenin $\Delta N87\beta$ -cat ($n = 3$; $*P < 0.001$ compared with control cells and $**P < 0.001$ compared with *Apc* siRNA). (C) *Apc*^{Min/+} colony formation in soft agar after depletion of *Apc* or Arrb2 or both compared with control siRNA modulated or not by transfecting cells with a mutated β -catenin $\Delta N87\beta$ -cat. Number of colonies obtained from 30 random fields per condition ($n = 4$; $*P < 0.001$ compared with control cells and $**P < 0.001$ compared with *Apc* siRNA).

Arrb2 in this subgroup via a modulation of Wnt target gene expression.

The complete absence of 67% of tumors in both Arrb2 siRNA and *Arrb2*^{−/−} *Apc*-mutated mice, without any detectable effect on the size of remaining tumors, suggests that Arrb2 plays an essential role in tumor initiation. The interplay between Arrb2 and the Wnt pathway detected in our study is likely to play a role in this effect. In *Apc*-mutated mice, partial or complete inactivation of the second *Apc* allele is the earliest event of tumor initiation (4), and threshold levels of Tcf-4 activity must be attained to initiate intestinal tumors (21). Our results indicate that the reduction of Arrb2 is only effective to decrease β -catenin/Tcf-4 activity and colony formation in soft agar when Wnt activity is experimentally increased in *Apc*^{Min/+} cells. In line with these data, we found that multiple Wnt target genes are overexpressed in the 75% of Arrb2-dependent tumors identified in *Apc*^{Δ14/+} mice, reflecting a higher β -catenin activity in these tumors compared with the Arrb2-independent subgroup. This is in line with what has been observed in patients with familial adenomatous polyposis, who also carry germinal *APC* mutations. In these patients, different adenomas do not seem to always undergo similar alterations of the second allele (22). Such

differences in *Apc* modulation likely lead to differences in β -catenin activity (23). We therefore hypothesize that Arrb2 is essential for intestinal tumor initiation in a subset of *Apc*^{Δ14/+} adenomas displaying higher Tcf-4 activity, whereas it is dispensable for tumors originating in a lower Tcf-4 activity context. Our ex vivo and in vitro data support this hypothesis and indicate that the regulation of the Wnt pathway by Arrb2 can occur directly at the level of epithelial tumor cells. These data are also in agreement with the observation showing that the renewal of healthy intestinal crypts is similar in *Apc*^{Δ14/+}; *Arrb2*^{−/−} and in *Apc*^{Δ14/+}; *Arrb2*^{+/+} mice, corroborating the lack of effect of Arrb2 depletion on the growth or survival of cells with subthreshold Tcf-4 activity. Finally, this dependency clearly does not require overexpression of Arrb2, because Arrb2-dependent tumors actually express slightly lower levels of Arrb2 than the subgroup of independent tumors.

What could be the molecular mechanism involved in the regulation by Arrb2 of *Apc* mutation-induced tumorigenesis? Because the inhibitory effects of Arrb2 depletion is only detected in models in which the β -catenin degradation complex (*Apc*/Gsk3 β /Axin) is at best poorly effective (*Apc*^{Δ14/+} mice or *Apc*^{Min/+} cells treated with *Apc* siRNA), and because the expression of a constitutively activated β -catenin mutant reversed Arrb2 siRNA inhibition on Wnt activity and colony formation induced by *Apc* depletion in *Apc*^{Min/+} cells, it is tempting to speculate that Arrb2 regulates Wnt signaling upstream from that complex. Several proteins known to interact with Arrb2 and involved in the Wnt signaling pathway could thus be potential candidates, such as Dvl2 (14) and Src (11). Depletion of Dvl2 decreases tumor number in *Apc*^{Min/+} mice (24), which is in favor of the fact that Dvl2 can affect β -catenin/Tcf-4 activity even in a context whereby the β -catenin degradation complex is lost. Furthermore, the interaction of Dvl2 with Src has been shown to be required for the tyrosine phosphorylation of β -catenin that promotes β -catenin/Tcf-4 activity involved in ovarian cancer cell invasion and metastasis (11). Thus, in our model, Dvl2 and Src might be potential partners by which Arrb2 regulates *Apc* mutation-induced tumorigenesis.

Unexpectedly, the identification of two subsets of *Apc*^{Δ14/+} tumors with very different sensitivity toward Arrb2 also helped us unravel a previously unsuspected early heterogeneity among these tumors at the gene expression level. The gene set enriched in Arrb2-dependent *Apc*^{Δ14/+} mouse tumors includes numerous genes that promote the evolution toward malignant and invasive tumors. Indeed, in vitro and in vivo studies have linked the expression of Itga6, Foxc1, and Mmp7, -10, and -12 to increased tumor cell invasion, migration, and metastasis (25–27). Target genes of the Wnt signaling pathway such as Prox1, Sox4, Sox9, Lgr5, and Cldn2 are also involved in tumorigenesis and cancer progression (20, 28–31). By contrast, genes highly represented in Arrb2-independent tumors encode molecules mainly involved in T- and B-cell differentiation and activation, such as Cd40, Cd74, Cd86, and Il21r. In agreement with the present work, a gene profile related to the immune system correlated to underexpressed genes involved in tumor invasion and metastasis in carcinogen-induced intestinal tumors from *Bcl9/Bcl9l*^{−/−} mice (32). Our results and those of that study thus provide strong arguments to suggest that a dynamic interaction between cancer cells and cells from the immune system may control tumor progression toward malignancy.

In conclusion, our data provide evidence that Arrb2 is essential for the initiation and growth of intestinal tumors displaying elevated Wnt pathway activity and identify a previously unsuspected molecular heterogeneity among tumors induced by truncating *Apc* mutations. This molecular diversity could have a clinical impact because it may allow the early detection of adenomas with a potential for progression toward the invasive colorectal carcinoma stage.

Materials and Methods

Detailed protocols are given in *SI Materials and Methods*.

Animals. All animal procedures were carried out in accordance with French government regulations (*services vétérinaires de la Santé et de la Production Animale, Ministère de l'Agriculture*). *Arrb1* and *Arrb2*-deficient mice (*Arrb1*^{−/−} and *Arrb2*^{−/−}, C57BL/6 background) were kindly provided by Dr. R. J. Lefkowitz (Duke University Medical Center, Durham, NC) (17, 18). Genotypes of *Arrb1*^{−/−} mice were determined by quantitative PCR, as described in *SI Materials and Methods*, and *Arrb2*^{−/−} mice as previously described (33). *Apc*^{Δ14/+} mice were a generous gift of Christine Perret (Institut Cochin, Paris) (16).

In Vivo siRNA Experiments. *Apc*^{Δ14/+} mice (4.5 mo old) received *Arrb1* siRNA (nine mice) or *Arrb2* siRNA (nine mice) or nonsilencing control siRNA (seven mice). siRNA duplexes were complexed with cationic liposomes prepared from RPR209120 [2-(3-[Bis-(3-amino-propyl)-amino]-propylamino)-N-dite-tradecylcarbamoyle-methyl-acetamide] and DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; Avanti Polar Lipids) (1/1 M/M) (34). Equal volumes of cationic liposome (30 nmol) and nucleic acid (5 μg siRNA) in 0.9% NaCl were mixed and incubated for 30 min at room temperature. siRNA (sequences in *SI Materials and Methods*) were injected i.p. twice a week for 4 wk.

Adenoma Scoring and Immunohistochemistry. Mice received an i.p. injection of 2 mg BrdU (Sigma-Aldrich) 2 h or 48 h before being killed. Postmortem, the whole intestinal tract was removed and reversed on a skewer to count the polyps. When possible, 1 to 2 tumors (≈2–3 mm) were removed from the jejunum and kept in RNAlater at −80 °C for further RNA extraction. Tissue

fixation and immunohistochemistry on thin sections of paraffin-embedded tissue were performed essentially as described previously (35).

Gene Expression Profiling by Oligonucleotide Microarray. Methods for RNA labeling, microarray processing, and analysis of microarray data are available in *SI Materials and Methods*. Microarray data are accessible at the Gene Expression Omnibus database under accession number GSE24577.

Statistical Analysis. All data were represented as mean ± SEM, except for the Tukey boxplots that express the first and the third quartiles by the upper and lower horizontal lines in a rectangular box, inside which the horizontal line represents the median. When distribution was normal (assessed with a Skewness and Kurtosis test), an unpaired *t* test was used for two-group comparisons, and a one-way ANOVA followed by a Bonferroni post hoc test was used for comparisons of three or more groups. In other cases, a Mann-Whitney test was used for two-group comparison and a Kruskal-Wallis test followed by Dunn's post hoc test was used for three-group comparisons. For all tests, a *P* value of <0.05 was considered statistically significant.

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