Development 127, 2629-2642 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
DEV4333



# Rac1 mutations produce aberrant epithelial differentiation in the developing and adult mouse small intestine

**Thaddeus S. Stappenbeck[1,2] and Jeffrey I. Gordon[1,*]**

Departments of [1]Molecular Biology and Pharmacology and [2]Pathology, Washington University School of Medicine, St Louis, MO 63110, USA
*Author for correspondence (e-mail: jgordon@molecool.wustl.edu)

*Accepted 28 March; published on WWW 23 May 2000*

## SUMMARY

**The mouse small intestinal epithelium undergoes continuous renewal throughout life. Previous studies suggest that differentiation of this epithelium is regulated by instructions that are received as cells migrate along crypt-villus units. The nature of the instructions and their intracellular processing remain largely undefined. In this report, we have used genetic mosaic analysis to examine the role of Rac1 GTPase-mediated signaling in controlling differentiation. A constitutively active mutation (Rac1Leu61) or a dominant negative mutation (Rac1Asn17) was expressed in the 129/Sv embryonic stem cell-derived component of the small intestine of C57Bl/6-ROSA26↔129/Sv mice. Rac1Leu61 induces precocious differentiation of members of the Paneth cell and enterocytic lineages in the proliferative compartment of the fetal gut, without suppressing cell division. Forced expression of the dominant negative mutation inhibits epithelial differentiation, without affecting cell division, and slows enterocytic migration along crypt-villus units. The effects produced by Rac1Leu61 or Rac1Asn17 in the 129/Sv epithelium do not spread to adjacent normal C57Bl/6 epithelial cells. These results provide in vivo evidence that Rac1 is involved in the import and intracellular processing of signals that control differentiation of a mammalian epithelium.**

Key words: Rac1, Epithelial differentiation, Cell migration, Intestine, Genetic mosaic analysis, Mouse

## INTRODUCTION

The adult mouse intestinal epithelium undergoes rapid and continuous renewal throughout life. Proliferation, differentiation and death are coordinated to maintain a proper cellular census. These programs are executed in anatomically well-defined units consisting of invaginated flask-shaped crypts of Lieberkühn and evaginated finger-like villi. Crypts represent the proliferative units of the intestine. Each crypt contains a population of 4-5 long-lived multipotent stem cells anchored at or near its base (Cheng and Leblond, 1974c; Bjerknes and Cheng, 1981a,b, 1999). The daughters of these stem cells undergo 4-6 rounds of division in the middle and upper portions of crypts and give rise to four distinct epithelial lineages. Three of these, enterocytes, goblet and enteroendocrine cells complete their differentiation as they migrate upwards, out of the crypt, onto adjacent villi (Cheng, 1974a; Cheng and Leblond, 1974a-c). These cells move up the villus in coherent well-ordered columns (Schmidt et al., 1985) over a 3-5 day period. Upon reaching the villus tip, they undergo apoptosis and/or extrusion (Hall et al., 1994). The fourth lineage (Paneth cell) executes its terminal differentiation program during a downward migration to the crypt base (Cheng, 1974b). Each crypt contains 30-50 mature Paneth cells with an average lifespan of 18-23 days (Cheng et al., 1969; Troughton and Trier, 1969; Cheng, 1974b). Paneth cells secrete a variety of factors involved in regulation of cell proliferation (e.g. epidermal growth factor), modification of the extracellular matrix (e.g. matrilysin; Wilson et al., 1997) and host defense against microbial pathogens (e.g. cryptdins; Ouellette, 1997; Wilson et al., 1999).

Villi first appear at embryonic day (E) 15, as a proximal-to-distal wave of cytodifferentiation converts the pseudostratified intestinal epithelium to a simple monolayer. Crypt morphogenesis is not completed until the end of the second postnatal week (Calvert and Pothier, 1990). Studies of chimeric mice, composed of cells from two distinct genetic backgrounds, indicate that as crypts form from the intervillus epithelium, their stem cell population undergoes ‘purification’ from polyclonality (i.e. a mixture of both genotypes) to monoclonality so that all active stem cells in a fully formed crypt are ultimately descended from a progenitor of a single genotype (Schmidt et al., 1988). Absorptive enterocytes, mucus-producing goblet cells, and enteroendocrine cells are apparent in late fetal life, while mature Paneth cells do not appear until postnatal day (P) 7 to P14 (Bry et al., 1994).

A central question concerning this renewing epithelium is how decisions about proliferation, differentiation and death are coordinated. Recent in vivo studies suggest that these decisions are not exclusively cell autonomous. Forced expression of E-cadherin in epithelial cells distributed throughout the length of crypt-villus units slows their rate of migration (Hermiston et

al., 1996). If regulation of intestinal epithelial differentiation were entirely cell autonomous, expression of terminal differentiation markers should be affected by the time that has elapsed since migrating cells exit the cell cycle, and not by the position they occupy along the crypt-villus axis. However, the location of expression of terminal differentiation markers in the intestinal epithelium of these E-cadherin overproducing mice is unchanged, even though migration is slowed appreciably (Hermiston et al., 1996). This finding suggests that as epithelial cells arrive at specific locations along crypt-villus units, they receive instructions that allow them to proceed through their differentiation programs. The results invite an obvious question: what is the nature of these instructions and what molecules are involved in their transmission and interpretation? In the present study, we have examined the role of Rac1, a member of the Rho family of GTP-binding proteins.

The Rho GTPase family includes at least eight distinct groups of proteins. Thus far, the best characterized members are Rho (A, B and C subtypes), Cdc42 (Cdc42Hs and G25K), and Rac (1, 2 and 3; Mackay and Hall, 1998; Aspenström, 1999). Studies in cultured cells have demonstrated that members of this family integrate information from a variety of signaling pathways and act as molecular switches to mediate effects on proliferation, differentiation, or migration (Mackay and Hall, 1998). For example, Rac1 stimulates quiescent 3T3 cells to progress through $G_1$/S (Olson et al., 1995). It also affects transcription of muscle-specific genes in C2C12 cells (Takano et al., 1998) and serum response factor-dependent genes in 3T3 cells (Hill et al., 1995). In addition, Rac1 increases the activities of NFκB (Sulciner et al., 1996) and MAP kinases in 3T3, HeLa, and/or COS-7 cells (Coso et al., 1995; Minden et al., 1995). Activation of Rho GTPase family members in quiescent 3T3 fibroblasts stimulates rapid formation of specialized actin structures: RhoA creates stress fibers and associated focal adhesions, Cdc42 forms filopodia, while Rac1 is involved in formation of lamellipodia and associated small focal complexes (Ridley and Hall, 1992; Ridley et al., 1992; Nobes and Hall, 1995; Hall, 1998). Experiments performed with cultured epithelial cells have shown that Rac GTPases can modulate cell polarity (Takaishi et al., 1997; Jou and Nelson, 1998), cell-cell adhesion (Braga et al., 1997; Hordijk et al., 1997), tight junction function (Jou et al., 1998), as well as cell spreading and migration (Ridley et al., 1995; Keely et al., 1997).

Cell culture-based studies indicate that the outcome of Rac1 signaling varies among cell types and likely depends upon the availability of different downstream effectors, and the extracellular signal 'context' (Sander et al., 1998; Aspenström, 1999). There have been a few in vivo tests of Rac function. Forced expression of a dominant negative Rac1 in the *Drosophila* wing disc epithelium disrupts adherens junction formation and produces cell death (Eaton et al., 1995). Perturbing Rac activity affects axonal growth/guidance in *C. elegans* and *Drosophila* (Kaufmann et al., 1998; Steven et al., 1998). *Rac1*$^{-/-}$ mice die by E9.5 with severe developmental abnormalities. The mesoderm appears to be most severely affected, with numerous cells undergoing apoptosis (Sugihara et al., 1998). Mouse *Rac2* is expressed primarily in hematopoietic lineages. *Rac2*$^{-/-}$ mice are viable but exhibit defective neutrophil migration and phagocytosis (Roberts et. al., 1999). Forced expression of wild-type Rac2, or a constitutively activated form of the protein, in transgenic mice using thymus-specific transcriptional regulatory elements from the *lck* gene, produces apoptosis in developing thymocytes (Lores et al., 1997). A constitutively active Rac1 expressed in the cerebellar Purkinjie neurons of transgenic mice has differential effects on axons, dendritic trunks and spines (Luo et al., 1996).

There are no reports of the results of in vivo tests of Rac function in mammalian epithelia. In the present study, we have examined the effects of forced expression of constitutively active and dominant negative Rac1 mutations in the developing and adult small intestinal epithelium of chimeric mice. Our results indicate that this protein plays an important role in regulating cellular differentiation.

## MATERIALS AND METHODS

### Generation of chimeric mice

pL596hGHpNeoΔB$_2$ (Hermiston et al., 1996) contains nucleotides −596 to +21 of the rat fatty acid binding protein gene (*Fabpl*) linked to nucleotides +3 to +2150 of the human growth hormone gene (*hGH*). The neomycin resistance gene, under the control *pgk* regulatory elements, is positioned downstream of *hGH*. Plasmids containing cDNAs encoding previously characterized, constitutively active and dominant negative human Rac1 mutations, each with an N-terminal c-myc epitope tag, were generously provided by Alan Hall (University College, London). The constitutively active mutation contains a Gln61→Leu substitution (abbreviated Rac1Leu61) (Lamarche et al., 1996). The dominant negative mutation contains a Thr17→Asn substitution (Rac1Asn17). 600 bp *Cla*I/*Eco*RI fragments, containing Rac1Leu61 or Rac1Asn17, were blunt-end ligated to *Bam*HI-digested (and Klenow treated) pL596hGHpNeoΔB$_2$ so that the Rac1 sequences were placed between the *Fabpl* and *hGH* sequences. The 5.2 kb *Fabpl*-Rac1Leu61-hGH-*pgk*neo and *Fabpl*-Rac1Asn17-hGH-*pgk*neo inserts in the resulting plasmids (pLFRac1Leu61 and pLFRac1Asn17, respectively) were excised with *Xba*I, separated from vector sequences by electrophoresis in low melting point agarose, extracted from the gel, and electroporated into D3 129/Sv ES cells (Hermiston and Gordon, 1995a). Stably transfected ES cells were selected using G418, cloned, and the presence of *Fabpl*-Rac1Leu61-hGH-*pgk*neo or *Fabpl*-Rac1Asn17-hGH-*pgk*neo in various clones was verified by PCR using primers that recognize Rac1 sequences and sequences in exon 2 of hGH (5′-AAATACCTGGAGTGCTCGGC-3′; 5′-GGCAGAGCAGGCCAAA-AGCC-3′).

Cloned ES cell lines, or nontransfected D3 ES cells (controls) were injected into C57Bl/6-ROSA26 blastocysts (Wong et al., 1998) to produce B6-ROSA26↔129/Sv chimeric mice. Chimeras were produced from 6 different ES cell clones containing *Fabpl*-Rac1Leu61, and from 6 different ES cell clones containing *Fabpl*-Rac1Asn17. A 'line' of chimeric-transgenic mice refers to animals generated using a given ES cell clone. 'Normal chimeras' refer to mice produced from non-transfected D3 ES cells. The 129/Sv contribution in adult chimeras was determined by coat color, and in the embryos and neonates by an electrophoretic glucose 6-phosphate isomerase (GPI) isoenzyme assay of limb tissue (Nagy and Rossant, 1992).

### Maintenance of mice

All mice used in this study were housed in microisolator cages, in a barrier facility, under a strict 12 hour light cycle. Mice were fed a standard irradiated chow diet (PicoLab Rodent Chow 20, Purina Mills Inc.) ad libitum. Mice were maintained in a specified pathogen-free state (i.e. free of Hepatitis, Minute, Lymphocytic Choriomeningitis,

Ectromelia, Polyoma, Sendai, Pneumonia, and mouse adeno viruses, enteric bacterial pathogens, and parasites).

### Assays for transgene expression

#### Reverse transcriptase-PCR

Total cellular RNA was isolated from the entire small intestine and brain of E18.5 B6-ROSA26↔129/Sv chimeric-transgenic and normal chimeric mice using the RNeasy kit (Qiagen, Santa Clara, CA). RNA was also purified from 2 cm segments harvested from the middle third of the small intestine, and from the intact brain of P42 animals. Oligo dT-primed cDNA was generated and mutant Rac1 mRNAs were identified by PCR using two different sets of primers, both of which recognize Rac1 sequences and sequences from exon 2 of hGH (5′-AAATACCTGGAGTGCTCGGC-3′ and 5′-GACAGTGTTTGACG-AAGCGA-3′; 5′-GGCAGAGCAGGCCAAAAGCC-3′ and 5′-GAA-TGGTTGGGAAGGCACTG-3′). Thermocycling conditions were as follows: (i) 95°C for 1 minute (denaturation); (ii) 60°C for 1 minute (annealing), and (iii) 72°C for 2 minutes (extension) for 30 cycles.

#### Immunoblotting

Proteins from the small intestine and brain of E18.5 Rac1Leu61 chimeric-transgenic mice and age matched normal chimeras were extracted by placing these tissues in a solution containing 8 M urea, 0.19 M Tris-HCl pH 6.8, 1% SDS, and 1% β-mercaptoethanol. Tissues were disrupted by passage through an 18-gauge needle, and insoluble material was removed by centrifugation at 12,000 ***g*** for 5 minutes. Protein concentrations in the resulting supernatants were determined according to Bradford (1976).

Protein was also isolated from the small intestinal epithelium of normal adult (P42) chimeras. To do so, the epithelium was separated from the underlying mesenchyme using a protocol adapted from Weiser (1973). Epithelial and mesenchymal proteins (50 μg/sample) were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were pre-treated for 1 hour at 24ºC with blocking buffer (PBS containing 5% powdered nonfat milk), and then probed for 1 hour, at 24ºC with rabbit anti-human/mouse Rac1 (raised against the C terminus of the protein; final dilution in blocking buffer = 1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Duplicate blots were also probed with rabbit anti-human RhoA (1:1000, Santa Cruz Biotechnology), or with rabbit antibodies raised against the C-terminal 11 residues of all actins (1:2000, Sigma, St. Louis, MO). Antigen-antibody complexes were visualized with horseradish peroxidase-conjugated donkey anti-rabbit Ig using protocols and reagents provided in the Super Signal West Femto Detection kit (Pierce Chemical, Rockford, IL).

### Histochemistry

Small intestine whole mounts (Wong et al., 1998) were prepared from P42 normal chimeras and B6-ROSA26↔129/Sv-Rac1Asn17 chimeric-transgenic mice as follows. The small intestine was removed, flushed gently with ice-cold PBS, opened along its mesenteric side, pinned villus-side up on wax sheets, and fixed in periodate-lysine-paraformaldehyde (PLP) for 1 hour at 24°C. The whole mount was then washed three times in PBS, incubated in a solution containing 20 mM DTT, 20% ethanol, and 150 mM Tris-HCl (pH 8.0) for 45 minutes at 24°C to disrupt any adherent mucus, and washed once again in PBS. The epithelium was then genotyped by incubating the preparation overnight at 4°C in PBS containing 2 mM X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside), 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, and 2 mM $MgCl_2$ (final pH, 7.6). After the epithelium was photographed, the whole mount was embedded in 2% agar, cut in half along its cephalocaudal axis, and placed in tissue cassettes so that histologic sections could be prepared parallel to the cephalocaudal axis and perpendicular to the crypt-villus axis.

For E18.5 chimeras, intestines were removed en bloc, submerged in ice-cold PBS, and the mesentery was removed using a stereoscope and a pair of fine forceps. The tissue was then fixed in PLP for 2 hours at 24°C, washed in PBS, and stained as above, except that the staining solution was supplemented with 0.01% deoxycholate and 0.02% IGEPAL CA-630 (Sigma) to improve X-gal penetration. Fixed and stained intestines were then embedded in 2% agar and divided into 2 mm wide 'donuts' by cutting perpendicular to the cephalocaudal axis. Donuts from each intestine were placed in tissue cassettes so that their original cephalocaudal relationships could be preserved.

Tissue samples were embedded in paraffin wax and 5 μm thick serial sections were prepared. Sections were stained with nuclear fast red, hematoxylin and eosin, periodic acid Schiff (PAS), Alcian blue, or phloxine and tartrazine (Luna, 1968). Adjacent, unstained sections were used for immunohistochemical studies (see below).

### Single and multi-label immunohistochemistry

PLP-fixed tissue sections were de-waxed with xylene, rehydrated, and pre-treated for 15 minutes at room temperature with blocking buffer (1% bovine serum albumin, 0.3% Triton X-100, and 1mM $CaCl_2$ in PBS). Sections of E18.5 or P42 small intestine were stained with the following antisera: (i) rabbit anti-human/mouse Rac1 (Santa Cruz Biotechnology; final dilution in blocking buffer = 1:100); (ii) mouse anti-c-myc epitope (clone 9E10.2; Calbiochem; see Evan et al., 1985; 1:100); (iii) rabbit anti-rat liver fatty acid binding protein (L-FABP; Sweetser et al., 1988; 1:1000); (iv) rabbit anti-rat intestinal fatty acid binding protein (I-FABP; Cohn et al., 1992; 1:1000-1:10,000); (v) rabbit antiserum against residues 4-35 of cryptdin-1 (cross reacts with cryptdins 1, 2, 3, and 6; see Selsted et al., 1992 and Garabedian et al., 1997; generously supplied by Michael Selsted, University of California, Irvine; 1:500); (vi) rabbit anti-secreted phospholipase A2 (Mulherkar et al., 1991; Garabedian et. al., 1997; kindly provided by Rita Mulherkar, Cancer Research Institute, Tata Memorial Center, Bombay, India; 1:40000); (vii) fluorescein isothiocyanate (FITC)-labeled mouse monoclonal antibodies to β-actin (clone AC-40; Sigma; 1:400); (viii) rabbit antibodies raised against amino acids 768-781 of mouse/human β-catenin (Sigma; 1:1000); or (ix) monoclonal rat antibody to E-cadherin (Sigma; 1:1000). Prior to incubation with antibodies to Rac1, c-myc, or β-catenin, sections were microwaved at 50% power in a 1.5 kW machine for 15 minutes in 100 mM sodium citrate, pH 6.0 (for antigen retrieval). Prior to incubation with antibodies to β-actin, antigen was retrieved by treating sections with 0.1% chymotrypsin (prepared in 0.1% $CaCl_2$) for 10 minutes at 37°C.

Cycling intestinal epithelial cells in E16.5 or E18.5 chimeras were labeled in S-phase by injecting their Swiss-Webster foster mothers intraperitoneally with an aqueous solution of 5′-bromo-2′-deoxyuridine (BrdU, 120 mg/kg) and 5′-fluoro-2′-deoxyuridine (12 mg/kg; Sigma). Chimeric mice labeled at E18.5 were killed 1.5 hours later for analysis of cell proliferation. Mice labeled at E16.5 were killed 2 days later for analysis of cell migration. Intestines from both groups of chimeric animals were fixed in PLP, stained with X-gal as above, and then post-fixed in Bouin's solution for 6 hours. Sections were prepared and incubated with goat anti-BrdU (Cohn et al., 1992; 1:2000).

Antigen-antibody complexes were detected with indocarbocyanine (Cy3)- or FITC-conjugated donkey anti-rabbit, anti-mouse, or anti-goat Ig (1:500, Jackson ImmunoResearch Laboratories). Nuclei were stained with bis-benzimide (50 ng/ml PBS).

Sections of PLP-fixed and X-gal stained chimeric intestine were also incubated with a series of FITC- or biotin-tagged lectins (all at a final concentration of 5 μg/ml blocking buffer; Falk et al., 1994). The lectin panel consisted of the following members: (i) *Dolichos biflorus* agglutinin (Sigma; carbohydrate specificity = GalNAcα-containing glycans); (ii) *Helix pomentia* agglutinin (Sigma; α-GalNAc/GalNAcβ4Gal-glycans); (iii) *Artrocarpus integrifolia* agglutinin (Jacalin-1; E.Y. Laboratories, Inc., San Mateo, CA; Galα6Gal/Galβ3GalNAc); (iv) cholera toxin B-subunit (Sigma, GalNAcβ4(Neu5Acα2,3)Galβ); (v) *Maackia amurensis* (E. Y. Laboratories; Neu5Acα2,3Galβ4Glc/GlcNAc); and (vi) *Ulex*

*europeaus* agglutinin-1 (Sigma; Fucα1,2Galβ). Bound biotinylated lectins were detected using horseradish peroxidase-conjugated avidin, VIP substrate, and protocols provided by their manufacturer (Vector Labs, Burlingame, CA). The lineage-specific, differentiation-dependent, and cephalocaudal patterns of reactivity of these lectins with the mouse intestinal epithelium have been described previously (Falk et al., 1994; Hermiston and Gordon, 1995a,b; Hermiston et al., 1996).

## RESULTS

### Rationale for using chimeric mice to test the function of Rac1 in the intestinal epithelium

The mouse intestine establishes and maintains complex cephalocaudal differences in the differentiation programs of its component lineages, the organization of its mucosal immune system, and the composition of its microflora (Falk et al., 1998). This spatial complexity makes it imperative that audits of gene function pay attention to cellular location and environment. One way to control for this complexity is to use chimeric mice produced by injection of genetically manipulated 129/Sv embryonic stem cells into C57Bl/6-ROSA26 (B6-ROSA26) blastocysts (Wong et al., 1998). Genotyping of the intestines of these mice is straightforward. The B6-ROSA26-derived intestinal epithelium produces *E. coli* β-galactosidase (*lacZ*) throughout the lifespan of the mouse and can be visualized by staining whole-mount preparations of gut, or histologic sections, with X-gal. The 129/Sv ES cell-derived epithelium is *lacZ*-negative (Fig. 1A,B). Crypts are monoclonal in the small intestine of adult chimeras: i.e. they are populated with either B6-ROSA26 or 129/Sv epithelial cells, but not a mixture of both (Fig. 1B). Each villus is supplied by several crypts. Therefore, a chimeric mouse offers the opportunity to compare epithelial cell phenotypes in adjacent villi that are supplied exclusively by 129/Sv ES cell-derived crypts, or exclusively by B6-ROSA26-derived crypts. Some villi will be supplied by both types of crypts. These 'polyclonal' villi contain juxtaposed columns of normal B6-ROSA26 cells and genetically manipulated 129/Sv cells (Fig. 1A,B). A single polyclonal villus in a chimeric mouse represents a well-controlled experiment where the effects of a genetic manipulation on proliferation, migration, differentiation, or death can be ascertained simply by comparing cells of each genotype at a given location along the length of the crypt-villus axis.

In late fetal life, before crypts have formed, the intervillus epithelium of chimeric mice contains a polyclonal population of stem cells. Therefore, the fetal intervillus epithelium and its associated nascent villi can contain intermingled populations of B6 or 129/Sv cells that can be compared (Fig. 1C).

### Studies of normal chimeras indicate that Rac1 is induced as 129/Sv and B6-ROSA26 intestinal epithelial cells undergo terminal differentiation

Previous northern blot analyses indicated that Rac1 mRNA is expressed in most organs of the adult mouse (Moll et al., 1991). The cellular patterns of Rac1 expression in the developing and adult mouse small intestinal epithelium have not been reported. Therefore, we began our study by addressing this point in normal adult chimeras generated by introducing non-manipulated ('wild type') 129/Sv ES cells into B6-ROSA26 blastocysts. Epithelium was recovered from the middle third of the small intestine (jejunum) of P42 chimeras. When western blots of total epithelial cell proteins were probed with antibodies specific for Rac1, a single immunoreactive protein of the expected mass (25 kDa) was detected (Fig. 2A). Rac1 was present in the intact small intestine of E18.5 chimeras but at a considerably lower level than in adults (Fig. 2A) ($n$=2 mice surveyed/developmental time point).

The same antibody preparation was used to define the cellular patterns of Rac1 accumulation. Immunohistochemical surveys disclosed that in normal P42 chimeras, Rac1 is not detectable in epithelial cells located in the middle and upper thirds of crypts where relatively undifferentiated cycling

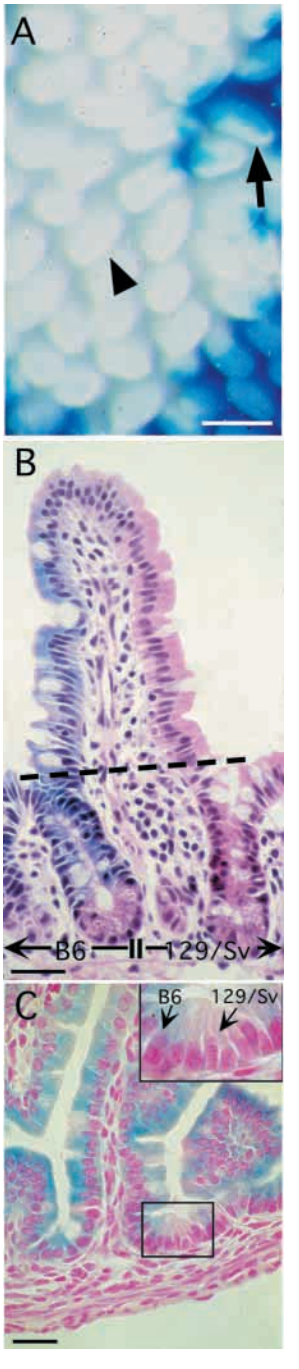


**Fig. 1.** Small intestine in adult and E18.5 B6-ROSA26↔29/Sv chimeric mice. (A) Whole mount preparation of jejunum from a normal adult chimeric mouse. The intestine has been opened and stained with X-gal. The 129/Sv-derived epithelium does not express β-gal and is white, while the B6-ROSA26-derived epithelium contains β-gal and appears blue. The arrowhead points to a villus that is only supplied by 129/Sv crypts. The arrow points to a striped polyclonal villus supplied by B6-ROSA26 and 129/Sv crypts. (B) Hematoxylin and eosin-stained section from a X-gal-stained whole mount of adult jejunum. The dashed line denotes the junction between this polyclonal villus and the two crypts that supply it with epithelial cells. The B6-ROSA26 crypt is monoclonal: i.e. it is composed of a wholly β-gal-positive population of epithelial cells. The monoclonal 129/Sv crypt only contains β-gal-negative epithelial cells. Cells from each crypt migrate up the villus in coherent columns. (C) Section of jejunum from an E18.5 B6-ROSA26↔129/Sv chimera, stained with X-gal and nuclear fast red. A portion of the intervillus epithelium is boxed. The inset shows a high power view of this region, which is composed of a mixture of β-gal-positive B6-ROSA26 cells, and β-gal-negative 129/Sv cells. Bars (A) 235 μm; (B,C) 25 μm.

epithelial cells reside. The finding applied to crypts positioned in the proximal, middle and distal thirds (duodenum, jejunum, ileum) of the small intestine (*n*=15 mice surveyed). Rac1 levels rise abruptly as epithelial cells migrate from the upper crypt to the base of their associated villi. Levels do not change appreciably as cells complete their migration to the villus tip. Rac1 is not only prominent throughout the villus epithelium but also at the crypt base where mature Paneth cells are located (Fig. 2B). In each region of the intestine, there was no appreciable difference between the cellular pattern of Rac1 expression in adjacent 129/Sv and B6-ROSA26 crypt-villus units, or between the 129/Sv and B6 components of polyclonal villi. Control experiments revealed that in each region of the small intestine, villus epithelial and Paneth cell staining could be blocked by pre-treating Rac1 antibodies with the peptide used for their generation (Fig. 2C). Together, these results indicate that Rac1 accumulation correlates with terminal differentiation of small intestinal epithelial cells in *adult* chimeric mice.

Although the very sensitive detection methods employed for western blot analysis revealed Rac1 in the late fetal small intestine, we were unable to reliably detect the protein in E16.5-18.5 intervillus or villus epithelium (or mesenchyme) using the immunohistochemical techniques employed for adult intestine (*n*=10 normal fetal chimeras/time point; e.g. Fig. 2D,E). However, Rac1 levels rose appreciably as gut morphogenesis proceeded. The protein was readily detectable in the villus epithelium by P3. In nascent P3 crypts, only a small subset of cells belonging to the goblet lineage were Rac1-positive (Fig. 2F). This cohort of goblet cells was lost, beginning at P7, as crypt morphogenesis proceeded to completion by the third postnatal week (data not shown).

### Generation of B6-ROSA26↔129/Sv chimeras that express Rac1Leu61 in their 129/Sv intestinal epithelium

We reasoned that if Rac1 functioned as an effector of intestinal epithelial differentiation, rather than a marker of differentiation, then expression of a constitutively active Rac1 in the epithelium of nascent or fully formed crypts could result in 'precocious' differentiation within this proliferative compartment. Of course, this would require that the cells contain other components of a Rac1-dependent signaling pathway that mediates differentiation.

We selected a previously characterized (Lamarche et al., 1996) constitutively active human Rac1 mutation (Gln61→Leu) with an N-terminal c-myc tag for this gain-of-function experiment. (Note, the amino acid sequences of the orthologous human and mouse Rac 1 proteins are identical; Moll et al., 1991). 129/Sv ES cells were stably transfected with

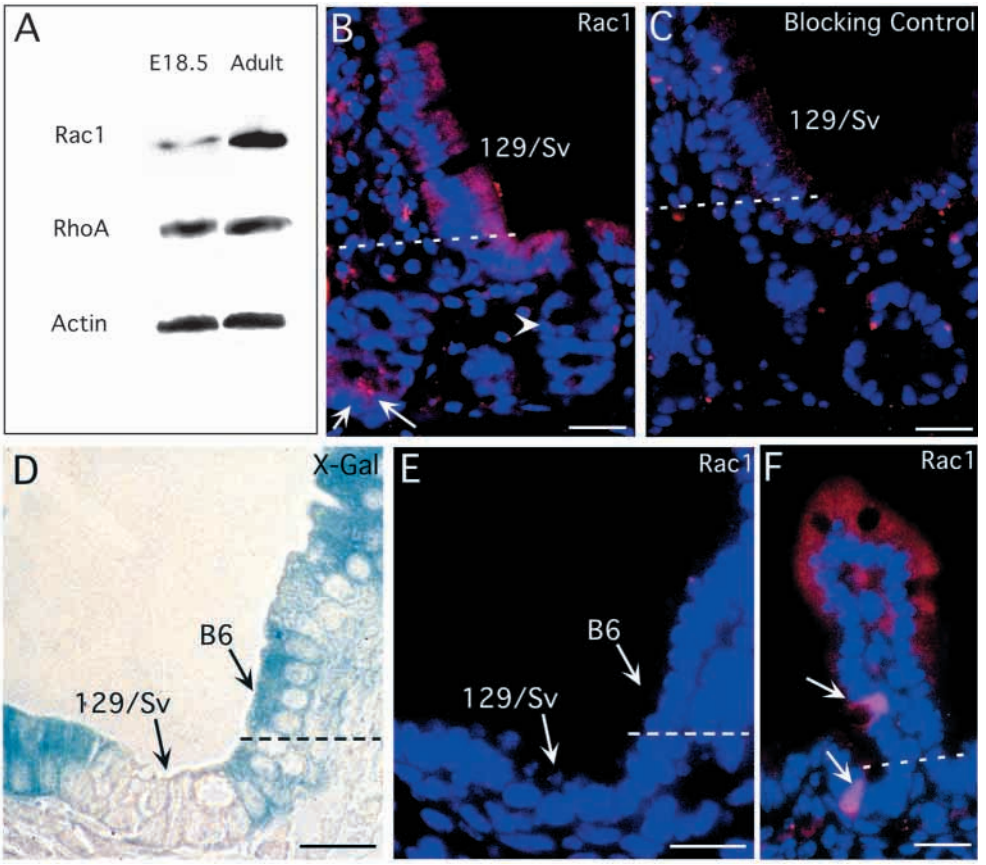


**Fig. 2.** Rac1 expression in the developing and adult jejunal epithelium. (A) Immunoblot analysis of total cellular proteins (50 μg/lane) isolated from jejunal epithelial cells from a 'normal' P42 B6-ROSA26↔129/Sv chimeric mouse, and the intact small intestine of an E18.5 chimeric mouse. Duplicate blots were probed with antibodies to Rac1 and RhoA. The blot probed with Rac1 was stripped and re-probed with antibodies to actin. (B) Section of jejunum from a normal adult chimeric mouse stained with rabbit anti-mouse Rac1, Cy3-conjugated donkey anti-rabbit Ig, and bis-benzimide (to label nuclei dark blue). Rac1-positive epithelial cells (red) are distributed throughout the villus epithelium, and in Paneth cells positioned at the base of a crypt (arrows). A dashed line has been placed at the crypt-villus junction. Note that epithelial cells located in the mid-portion of the crypt (proliferative zone) do not contain detectable levels of immunoreactive Rac1 (e.g. see the crypt with an arrowhead; this crypt has been sectioned tangentially so its base and associated Paneth cell population are not evident). (C) Blocking control, performed on a section adjacent to the one shown in B. The section was treated with the same reagents as in B, except that Rac1 antibodies were pre-incubated with excess peptide antigen. (D,E) Section from the middle of the small intestine of a normal E18.5 chimeric mouse. (D) X-gal staining allows genotyping of B6-ROSA26 and 129/Sv epithelial cells. (E) Same section after staining with the reagents used in B. (F) Rac1 expression in the villus epithelium of a P3 B6-ROSA26↔129/Sv chimera. A subset of goblet cells in the nascent crypt and lower portion of the villus have high levels of immunoreactive protein (arrows). Rac1 is readily detectable in villus enterocytes at this stage of development. Bars, 25 μm.

a recombinant DNA consisting of nucleotides –596 to +21 of a fatty acid binding protein gene (*Fabp*l) linked to an open reading frame encoding c-myc-Rac1Leu61. These *Fabpl* elements were chosen because previous studies had shown that other *Fabpl*-reporter transgenes are expressed throughout the intervillus and villus epithelium. Highest levels of expression occur in the jejunum. Expression commences by E15, is sustained after crypt-villus units have formed, and occurs in all four epithelial lineages throughout the course of their differentiation (e.g. Hermiston and Gordon, 1995b; Hermiston et al., 1996; Wong et al., 1998).

Six cloned, stably transfected *Fabpl*-Rac1Leu61 ES cell lines were injected (individually) into B6-ROSA26 blastocysts (10-15 ES cells/blast). Three of the clones produced high percentage adult chimeras (30-50% 129/Sv by coat color). However, these chimeras did not show expression of the *Fabpl*-Rac1Leu61 transgene, as determined by RT-PCR of P42 small intestinal RNA (data not shown). The three remaining *Fabpl*-Rac1Leu61 ES cell lines produced high percentage chimeras (defined by glucose 6-phosphate isomerase assay) that died within a day after birth ($n$=2-4 litters of mice examined/ES cell line). None of these mice had any obvious histopathologic changes in their organs, and the cause of their death remains unclear.

We examined B6-ROSA26↔129/Sv-Rac1Leu61 chimeras, generated from each of the three Rac1Leu61 lines that produced neonatal mortality, at E18.5. Sixty nine of 71 chimeric-transgenic mice (97%) surveyed at this stage of development were alive and free of any gross deformities. This level of viability was identical to that of normal chimeras (33 of 34 mice). RT-PCR analysis of small intestinal RNA showed the expected size product of *Fabpl*-Rac1Leu61 in each of these chimeric-transgenic 'lines' ($n$=2 mice surveyed/line) (Fig. 3A). The RT-PCR product was not present in RNA prepared from their brain, or in RNA isolated from the small intestine and brain of E18.5 normal chimeras (Fig. 3A).

Production of Rac1Leu61 was verified by immunoblot analysis. A single 25 kDa reactive species was present in the small intestines of E18.5 chimeric-transgenic mice. The level of immunoreactive protein was several-fold higher than the level of endogenous Rac1 in age-matched normal chimeric mouse intestine (Fig. 3B). This difference is consistent with our engineered forced expression of the RacLeu61 mutation.

X-gal plus antibodies to either Rac1 or its N-terminal c-myc epitope tag were used to stain adjacent serial sections of the mid-portion of the small intestine of E18.5 chimeric-transgenic mice. The same results were obtained with both antibodies: Rac1 was present in the cytoplasm of 129/Sv intervillus and villus epithelial cells and absent from adjacent B6-ROSA26 epithelial cells (see Fig. 3C,D and compare with Fig. 2D,E).

### Expression of Rac1Leu61 causes precocious differentiation of Paneth cells and enterocytes within the proliferative compartment of the E18.5 small intestinal epithelium

E18.5 chimeric-transgenic mice generated from two of the three *Fabpl*-Rac1Leu61 ES cell lines were analyzed in detail. Animals produced from both lines had the same phenotype.

During late fetal life, the Paneth cell and enterocytic lineages can be used as sensitive reporters of precocious differentiation in the intervillus epithelium. As noted in the Introduction, mature Paneth cells do not normally appear until P7-P14 (Bry et al., 1994). Surveys of serial sections of E18.5 B6-ROSA26↔129/Sv-*Fabpl*-Rac1Leu61 intestine, stained with hematoxylin and eosin and X-gal, revealed 129/Sv cells with large apical eosinophilic granules scattered throughout the intervillus epithelium (Fig. 4A). These granules reacted with antibodies raised against residues 4-35 of one of the most abundant intestinal defensins, cryptdin-1 (Fig. 4B). These granules also reacted with antibodies specific for a secreted phospholipase A2 encoded by the *Pla2g2a* gene (Mulkerhar et

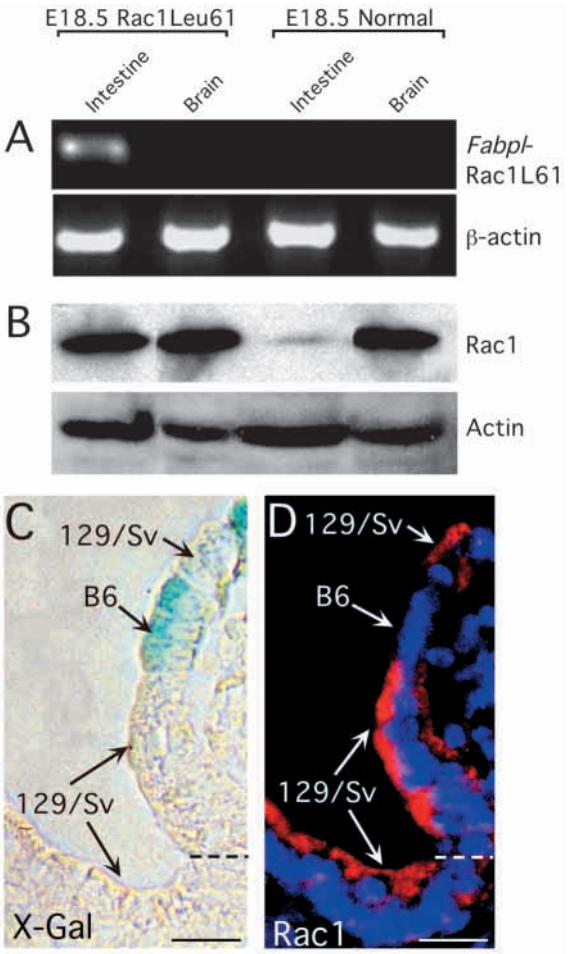


**Fig. 3.** Forced expression of Rac1Leu61 in the 129/Sv intestinal epithelium of chimeric mice. (A) RT-PCR analysis of RNAs isolated from the small intestine and brain of an E18.5 chimeric-transgenic mouse, and an E18.5 normal chimera. The top panel shows that the mRNA product of *Fabpl*-Rac1Leu61 is only present in the small intestine of the chimeric-transgenic mouse. The bottom panel shows similar levels of the RT-PCR product of β-actin mRNA in all samples. (B) Immunoblot analysis containing extracts of the indicated tissues (50 μg protein/lane). The top panel presents results obtained after probing an immunoblot with antibodies to Rac1. Bottom panel: the immunoblot was stripped and re-probed with antibodies to β-actin. (C,D) Immunohistochemical evidence of Rac1Leu61 expression in the 129/Sv jejunal intervillus and villus epithelium of an E18.5 chimeric-transgenic mouse. Both panels show the same section. The section was genotyped with X-gal (C). In D, Rac1Leu61 has been visualized in red with rabbit anti-Rac1 and Cy3-donkey anti-rabbit Ig. Nuclei are stained dark blue with bis-benzimide. Bars in C,D, 25 μm.

al., 1993; Harwig et al., 1995) (data not shown). Large apical granules containing these two protein products are characteristic features of mature Paneth cells (Cheng, 1974a; Bry et al., 1994; Garabedian et al., 1997). Cells with these features were not detectable in the B6-ROSA26 intervillus or villus epithelium of E18.5 chimeric-transgenic mice ($n$=30). In addition, they were absent from the 129/Sv and B6-ROSA26 intestinal epithelium of age-matched normal chimeras ($n$=20).

The enterocytic lineage can also be readily scored for precocious differentiation in the E18.5 intervillus epithelium. In normal chimeras, 129/Sv and B6-ROSA26 intervillus epithelial cells do not contain detectable levels of two homologous cytoplasmic fatty acid binding proteins, I-FABP and L-FABP. These proteins only appear as differentiating enterocytes move to the base of nascent villi (Fig. 5A,C). In contrast, there was a large population of L- and I-FABP-positive 129/Sv intervillus epithelial cells in the intestines of all E18.5 chimeric-transgenic mice surveyed ($n$=5-8 animals/line) (Fig. 5B,D). Adjacent B6-ROSA26 intervillus epithelial cells did not contain detectable levels of the FABPs (Fig. 5B,D). The effect of forced expression of Rac1Leu61 on these proteins was limited to the intervillus epithelium: there were no appreciable changes in the steady state levels of either FABP in 129/Sv villus enterocytes (reference controls = adjacent B6-ROSA26 cells and 129/Sv jejunal villus enterocytes in normal chimeras).

Goblet and enteroendocrine cells are present in the intervillus and villus epithelium of normal E18.5 chimeras and, thus, are not sensitive reporters of precocious differentiation at this stage of development. Nonetheless, after staining X-gal-marked sections with (i) antibodies to Rac1, c-myc, and chromogranin A, (ii) Alcian blue and periodic acid Schiff, or (iii) lectins that recognize glycans produced by members of these two lineages, we concluded that Rac1Leu61 does not impede goblet or enteroendocrine cell differentiation (data not shown).

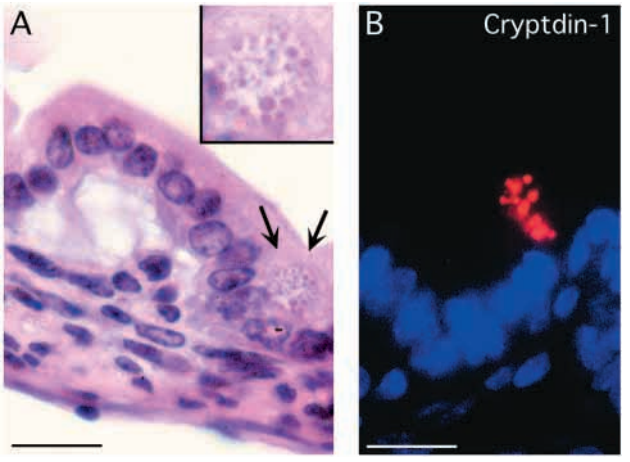


**Fig. 4.** Expression of a constitutively active Rac1 causes precocious differentiation of Paneth cells. (A) Hematoxylin and eosin-stained section of jejunum from an E18.5 Rac1Leu61 chimeric-transgenic mouse showing a Paneth cell with large eosinophilic granules (arrows) in a region of wholly of 129/Sv intervillus epithelium. The inset provides a higher power view of these granules. (B) Adjacent section stained with rabbit anti-cryptdin-1, Cy3-donkey anti-rabbit Ig and bis-benzimide, reveals that these large apical Paneth cell granules contain cryptdins. Bars, 25 μm.

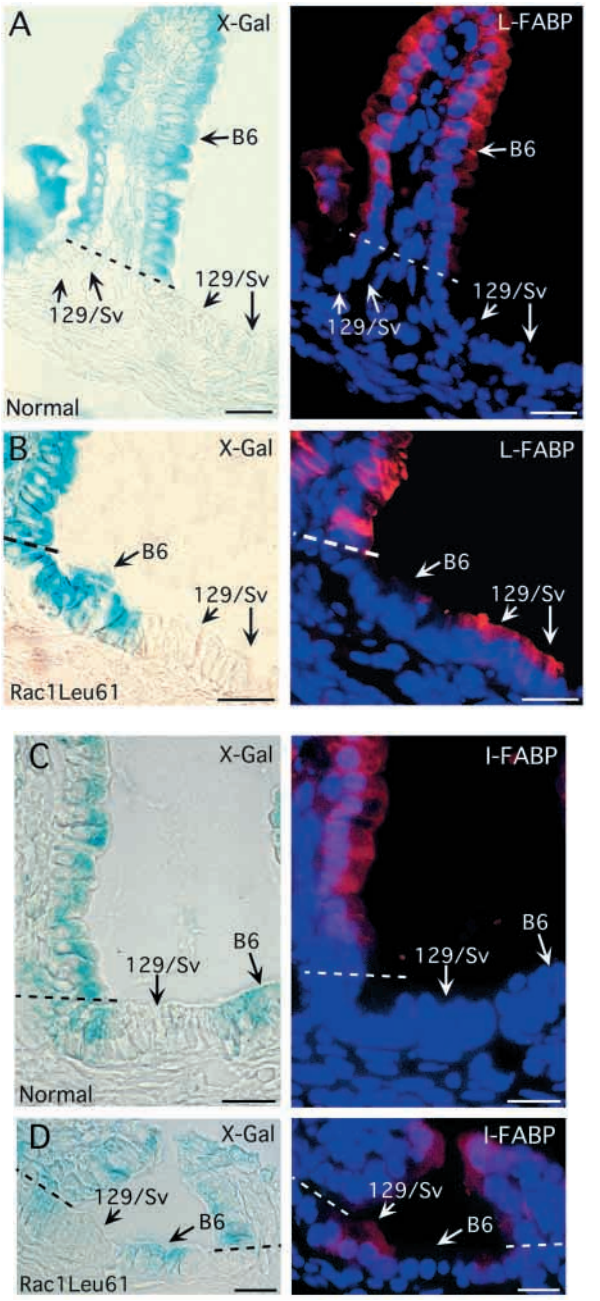


**Fig. 5.** Forced expression of Rac1Leu61 causes premature expression of enterocyte-specific proteins. Sections of X-gal-stained E18.5 jejunum from a normal chimeric mouse (A,C), and an age-matched chimeric-transgenic animal (B,D). For each animal, a given section was genotyped with X-gal and then stained with either rabbit anti-L-FABP (A,B) or rabbit anti-I-FABP (C,D), Cy3-conjugated donkey anti-rabbit Ig, and bis-benzimide. In the normal chimeric mouse, L-FABP and I-FABP are only detectable in differentiating villus enterocytes, regardless of genotype. The intervillus epithelium (below the dashed line) is negative for each protein, irrespective of genotype. In the Rac1Leu61 chimeric-transgenic mouse, both L-FABP and I-FABP show staining within the villus epithelium. However, both of these proteins are also expressed in 129/Sv (but not B6-ROSA-26) intervillus epithelial cells. Bars, 25 μm.

### Rac1Leu61 does not block proliferation or migration in the E18.5 small intestinal epithelium

We considered two possible mechanisms that may contribute to the effect of Rac1Leu61 on enterocytic and Paneth cell differentiation. First, the mutation inhibits proliferation in the intervillus epithelium, allowing cells to differentiate. Second, the mutation does not affect proliferation but impedes migration, thereby giving cells additional time to execute their terminal differentiation before they arrive on nascent villi. If cell non-autonomous mechanisms act to normally prohibit differentiation in the intervillus compartment, then either of the two postulated mechanisms would require that Rac1Leu61 allows cells to override, or ignore, differentiation-restrictive signals produced in the compartment.

To address the issue of proliferative status, E18.5 Rac1Leu61 chimeras were exposed in utero to BrdU for 1.5 hours to mark cells in S-phase. Subsequently, sections of their jejunum were stained with antibodies to BrdU (Cohn et al., 1992). No discernible differences were found in the number of cycling BrdU-positive cells in the 129/Sv- and B6-ROSA26 components of the intervillus epithelium (Fig. 6A) (*n*=3-4 chimeric-transgenic mice surveyed/ES cell line plus 5 normal chimeric mice). Because cycling 129/Sv and B6-ROSA26 epithelial cells are intermingled in various proportions within the polyclonal intervillus epithelium, a more detailed quantitative assessment of the ratios of BrdU-positive to -negative cells of each genotype was not feasible.

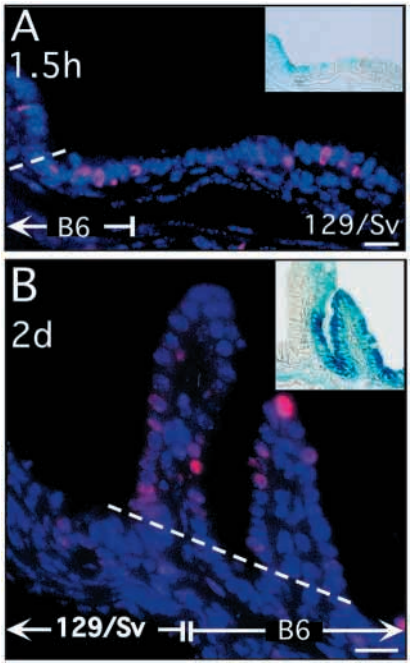


**Fig. 6**. Forced expression of Rac1Leu61 in the intervillus epithelium does not block proliferation or subsequent cell migration up the villus. (A) An E18.5 Rac1Leu61 chimeric-transgenic mouse was labeled in utero with BrdU 1.5 hours before being killed. A section of jejunum was genotyped with X-gal (see inset) and then stained with goat anti-BrdU, Cy3-conjugated donkey anti-goat Ig, and bis-benzimide. The number of BrdU-positive cells marked in S-phase is equivalent in both the 129/Sv and B6-ROSA26 intervillus epithelium. (B) To examine the migration of Rac1Leu61-expressing cells, an E18.5 chimeric-transgenic mouse was labeled in utero with BrdU 48 hours before being killed. Sections were stained as in A. Both 129/Sv and B6-ROSA26 epithelial cells have migrated from the intervillus epithelium up the polyclonal villus (see the inset for X-gal genotyping). Bars, 25 μm.

To assess the effects of Rac1Leu61 on cell migration, we took advantage of two observations. RT-PCR assays established that at E16.5 *Fabpl*-Rac1Leu61 is expressed in the small intestine of chimeric-transgenic mice. In addition, 1.5 hours after intraperitoneal administration of BrdU to pregnant mothers, S-phase cells are only rarely encountered in the villus epithelium of E16.5 and E18.5 chimeric-transgenic or normal chimeric mice (data not shown). Therefore, we were able to examine the migratory behavior of 129/Sv Rac1Leu61-producing intervillus cells by giving a single injection of BrdU to the pregnant mothers of E16.5 chimeric-transgenic fetuses, and then analysing the fetuses 2 days later. Migration was defined by noting the distribution of BrdU-positive cells along the intervillus-villus axis. Cells were genotyped by staining adjacent sections with X-gal. The results revealed that Rac1Leu61 does not block the ability of 129/Sv cells to move up the villus (*n*=2 mice/ES cell line) (Fig. 6B).

### The effects of Rac1Leu61 on the intracellular distribution of β-actin in intervillus and villus epithelial cells

Rac1Leu61 can have varied effects on the shape, mobility, and actin cytoskeleton of cultured epithelial cells, depending upon the cell line studied, and the culture conditions employed (e.g. Braga et al., 1997; Takaishi et al., 1997; Jou and Nelson, 1998; Sander et al., 1998). We characterized the effects of Rac1Leu61 on the actin cytoskeleton of intestinal epithelial cells by staining adjacent sections of jejunum, harvested from E18.5 normal chimeras and chimeric-transgenic mice, with X-gal and antibodies to β-actin. There were no appreciable differences in the intracellular distribution of actin between *intervillus*129/Sv-Rac1Leu61 and B6-ROSA26 epithelial cells. Differences in the actin cytoskeleton were apparent by the time cells migrated to the villus epithelium. In normal E18.5 chimeras, the bulk of the actin cytoskeleton is localized at the apex of 129/Sv and B6-ROSA26 jejunal villus enterocytes, which constitute the majority of the epithelial population. Much smaller amounts are present at cell-cell and cell-substratum junctions (Fig. 7A). In chimeric-transgenic mice, Rac1Leu61 expression was associated with a modest decrease in apical actin (Fig. 7B) (*n*=5 mice surveyed/ES cell line).

Other markers of enterocytic polarization were not affected in 129/Sv-Rac1Leu61 villus epithelial cells. For example, there were no detectable differences in the intensity of staining of adherens junction proteins (E-cadherin, β-catenin) between adjacent 129/Sv and B6-ROSA26 enterocytes (data not shown; *n*=5 chimeric-transgenic mice/ES cell line; 5 normal chimeras).

### A loss-of-function experiment supports conclusions made from the gain-of-function experiment

Thr17→Asn substitution in Rac1 (Rac1Asn17) yields a dominant negative mutation (Ridley et al., 1992). B6-ROSA26↔129/Sv-*Fabpl*-Rac1Asn17 chimeric-transgenic mice were created by injecting each of six cloned, stably transfected ES cell lines into B6-ROSA26 blastocysts. Unlike the situation with the constitutively active mutation, three *Fabpl*-Rac1Asn17 ES cell clones gave rise to adult chimeric-

transgenic mice that expressed the transgene (e.g. inset to Fig. 8A). All three 'lines' of mice produced from these ES cell clones had a similar abnormal phenotype, although in two lines the changes were more pronounced. This phenotype was characterized by alterations in the morphology of jejunal crypt-villus units, an inhibition of differentiation without a change in proliferation, and a slowing of epithelial migration. These abnormalities were not associated with increased lethality, or with any statistically significant alterations in adult body weight compared to age-matched normal chimeras.

### Crypt-villus units have abnormal morphology

Surveys of jejunal whole mounts prepared from members of two of the three lines of P42 Rac1Asn17 mice ($n \geq 3$/line) showed that 129/Sv villi were 1.5-2 times wider than adjacent normal B6-ROSA26 villi (Fig. 8A). This effect was most prominent in the distal half of the jejunum where >75% of 129/Sv villi were widened. All of these widened villi were supplied by at least one elongated 129/Sv crypt, although not all 129/Sv crypts that were associated with a widened villus had this abnormal morphology. The elongated crypts extended into the core of the villus, emerging at various locations along its length (Fig. 8B,C). Widened villi and elongated crypts were not present in the B6-ROSA26 component of the proximal or distal jejunum, nor were they evident in either genotypic component of the normal chimeric gut. A quantitative comparison of proliferation in juxtaposed elongated 129/Sv Rac1Asn17 crypts and normal B6 crypts revealed no significant differences in the number of M-phase cells.

As noted above, Rac1 is normally induced at the crypt-villus junction. Therefore, we hypothesized that the 'elongated' phenotype was due to Rac1Asn17-mediated inhibition of epithelial differentiation and hence an expansion (extension) of the upper crypt into what would normally be the base of a villus. Cells at the crypt-villus junction should be particularly susceptible to the effects of the dominant negative mutation since levels of endogenous Rac1 are lower in this epithelial population compared to more differentiated cells positioned on the villus. Our hypothesis was supported by the following observations. As enterocytes move from the upper portions of normal 129/Sv and B6-ROSA26 crypts onto villi, three prominent changes occur: I- and L-FABP are induced, levels of apical actin increase, and the concentration of Fucα1,2Galβ-glycans in apical membranes rises (Fig. 8E,G,I). I- and L-FABP-positive cells were absent in the upper halves of elongated Rac1Asn17 crypts (e.g. Fig. 8D). In addition, these crypt cells had low levels of apical actin and Fucα1,2Galβ-glycans (Fig. 8F,H).

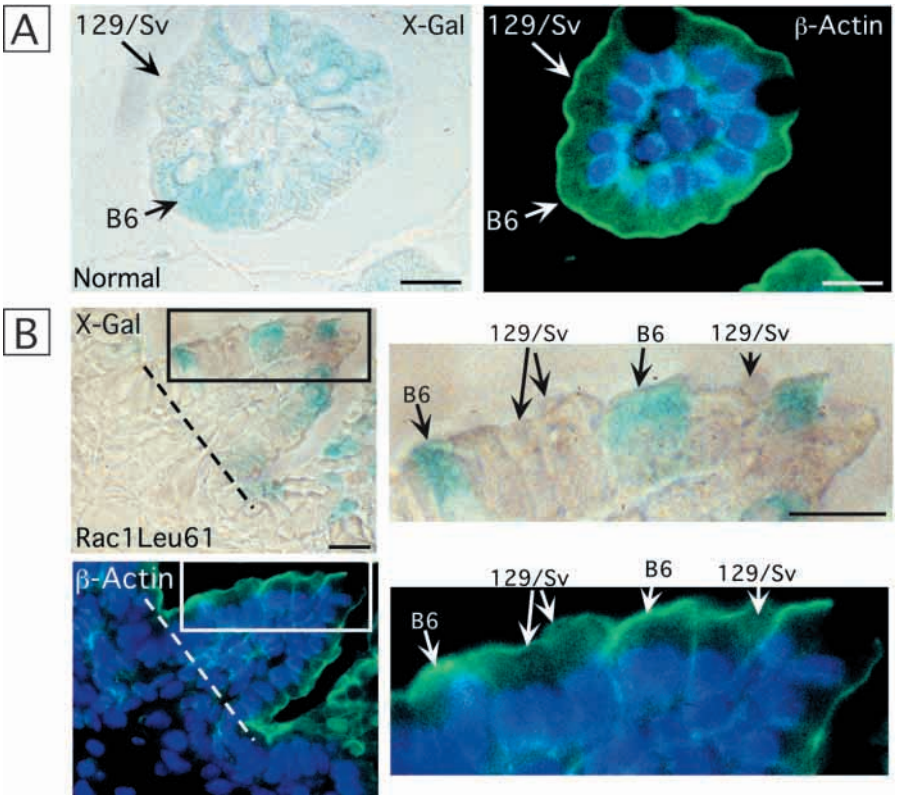


**Fig. 7.** Forced expression of Rac1Leu61 decreases the apical actin cytoskeleton in 129/Sv villus epithelial cells. Sections of jejunum from an E18.5 normal chimera (A) and a chimeric-transgenic mouse (B). Each section was genotyped with X-gal and then stained with FITC-conjugated mouse antibodies to β-actin, and bis-benzimide. (A), villi sectioned perpendicular to the intervillus-villus axis. There is equivalent staining of apical actin in 129/Sv and B6-ROSA26 epithelial cells. (B), 129/Sv villus epithelial cells in the Rac1Leu61 chimeric-transgenic mouse have decreased apical actin staining compared to juxtaposed B6-ROSA26 cells. This is most clearly seen in the enlarged views of the boxed area. Bars, 25 μm.

### Rac1Asn17 inhibits differentiation without altering proliferation

Rac1Asn17 had other effects on epithelial cell differentiation. There was an increase in intermediate cells and a pronounced decrease in mature goblet cells. These changes were evident in architecturally distorted crypt-villus units located in the *distal* jejunum, as well as in normal appearing crypt-villus units located in the *proximal* jejunum.

Lineage ablation experiments, conducted in transgenic mice, have indicated that intermediate cells are bi-potent precursors of a subset of goblet and Paneth cells (Garabedian et al., 1997). Intermediate cells possess apical electron-dense core granules intermediate in size between those in granule goblet cells and in young Paneth cells (Merzel and Leblond, 1969; Troughton and Trier, 1969). Intermediate cell granules contain anti-microbial cryptdins, the secreted phospholipase A2 encoded by *Pla2g2a*, and small amounts of mucin (Garabedian et al., 1997).

Quantitative surveys of P42 *normal* chimeras ($n$=4) established that intermediate cells were restricted to 129/Sv (and B6) jejunal crypts. Moreover, there were no significant genotypic differences in the number of these cells (data not shown). In all three lines of Rac1Asn17 chimeric-transgenic mice, intermediate cells were more numerous in the 129/Sv epithelium, and extended from

the crypt to villus (*n*=4-5 mice/line (Fig. 9A,B). Like intermediate cells in normal chimeras, members of this expanded population of *Fabpl*-Rac1Asn17 intermediate cells contained cryptdins and the product of *Pla2g2a* (Fig. 9C, plus data not shown).

The expansion of 129/Sv intermediate cells was accompanied by a marked decrease in the number of mature 129/Sv goblet cells, and a less prominent reduction in Paneth cells. The decrease in mature goblet cells was defined using members of our lectin panel, and by staining with Alcian blue (e.g. Fig. 9D; compare with the normal chimera in Fig. 9E). These results suggest that intermediate cell differentiation is blocked by Rac1Asn17. However, since intermediate cells may not be the only precursor of goblet cells, it is possible that the pronounced loss of members of this lineage reflects Rac1Asn17-mediated inhibition of differentiation of other progenitors.

In contrast to its effect on the goblet and Paneth cell lineages, Rac1Asn17 did not produce a detectable alteration in the number, or crypt-villus distribution, of chromogranin A-positive enteroendocrine cells (*n*=6 mice from both lines).

The effect of Rac1Asn17 on epithelial differentiation was not associated with changes in proliferation. Proliferation was measured by examining X-gal- and hematoxylin and eosin-stained sections containing normal appearing 129/Sv and B6-ROSA26 crypts affiliated with 50 proximal jejunal polyclonal villi (*n*=two P42 chimeric-transgenic mice from 2 lines). The number of M-phase cells was similar irrespective of genotype

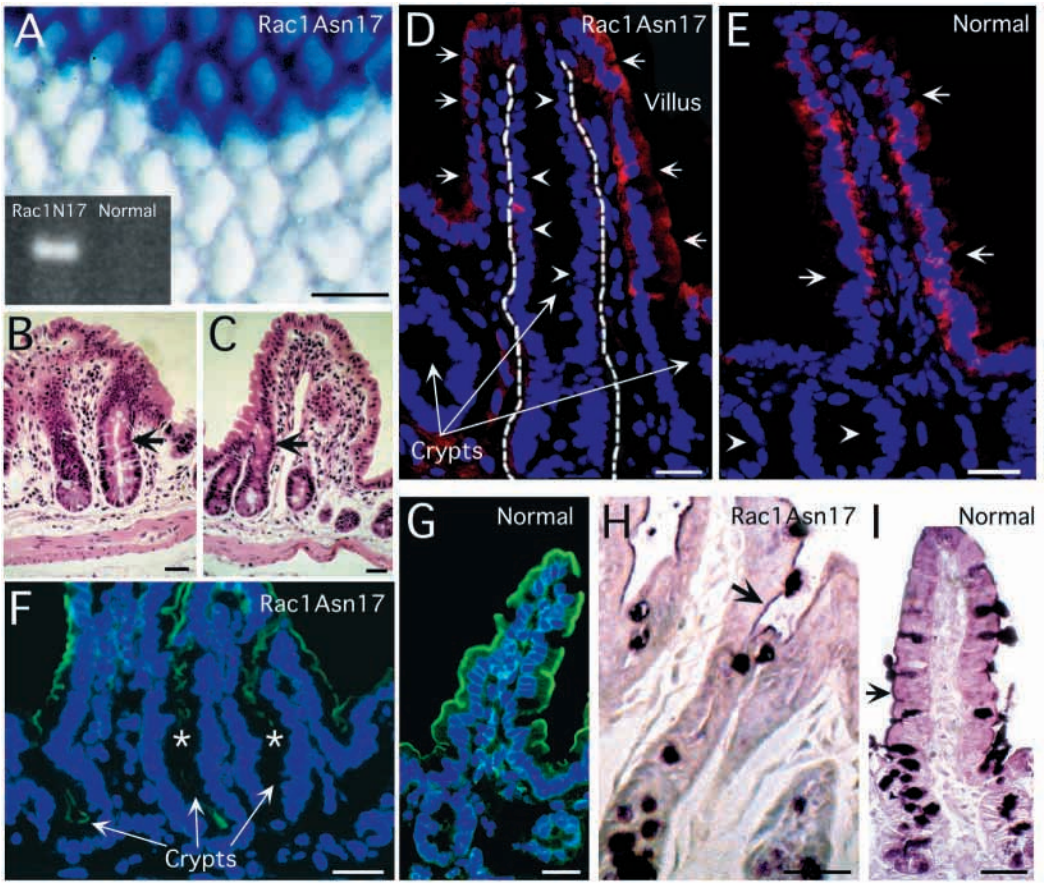


**Fig. 8**. Expression of a dominant negative Rac1Asn17 in adult chimeric-transgenic mice affects the morphology of crypt-villus units. (A) Whole-mount preparation of X-gal stained distal jejunum from a P42 Rac1Asn17 chimeric-transgenic mouse. 129/Sv villi are wider than adjacent B6-ROSA26 villi. The inset presents an RT-PCR analysis of RNAs isolated from the jejunums of P42 chimeric-transgenic and normal chimeric mice. A 250 bp PCR product, generated from the mRNA transcript of *Fabpl*-Rac1Asn17, is present in RNA recovered from the chimeric-transgenic animal. (B,C) Hematoxylin and eosin-stained sections from a Rac1Asn17 mouse showing widened 129/Sv distal jejunal villi with elongated crypts (examples indicated by arrows). (D) I-FABP-stained section of a widened villus with a central elongated crypt extending throughout its core (outlined with the dashed lines). I-FABP (red) is limited to villus enterocytes (arrows). Epithelial cells lining the elongated crypt do not contain detectable levels of this cytoplasmic protein (e.g. arrowheads). Normal appearing crypts flank the widened villus. (E) Distal jejunal crypt-villus unit from a normal chimera showing induction of I-FABP expression as enterocytes emerge from crypts. (F) Section from a single widened 129/Sv-Rac1Asn17 villus stained with FITC-conjugated antibodies to β-actin (green) and bis-benzimide. Two elongated crypts, each indicated by an asterisk, penetrate the core of the villus. A normal appearing crypt is located to the left. Apical actin only becomes prominent as epithelial cells migrate to the upper portions of the elongated crypts. (G) Distal jejunal crypt-villus unit from a normal chimera processed as in (F). (H) Higher power view of a crypt located in a widened 129/Sv villus. The section has been stained with *Ulex europeaus* agglutinin 1 (UEA-1). Fucα1,2Galβ glycans recognized by the lectin become apparent in the apical membranes of enterocytes (e.g. arrow), as they emerge from the elongated crypt. (I) Distal jejunal crypt-villus unit from a normal chimeric mouse, stained as in H. The arrow points to Fucα1,2Galβ glycans present in the apical membrane of a villus enterocyte. Bar in A 235 μm; in all other panels, 25 μm.

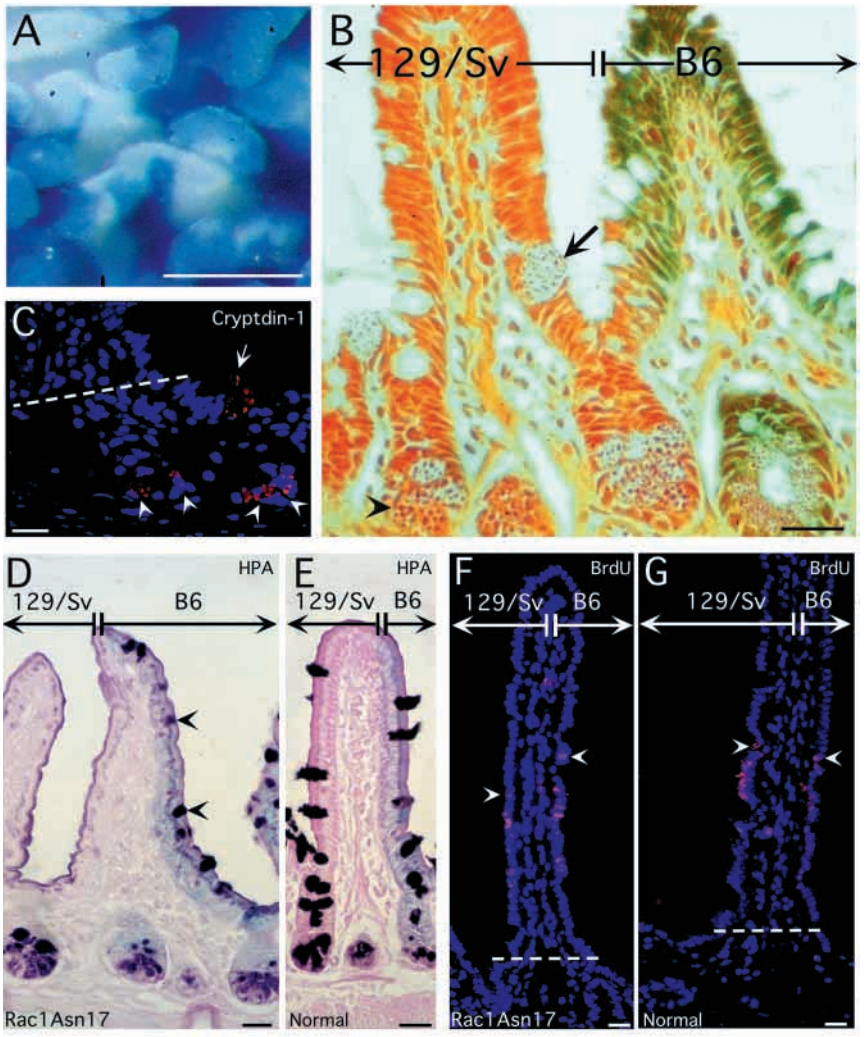


**Fig. 9.** The dominant negative Rac1Asn17 mutation inhibits epithelial differentiation and slows enterocytic migration. (A) Whole-mount preparation of X-gal stained proximal jejunum from a chimeric-transgenic mouse showing a polyclonal villus with coherent columns of juxtaposed 129/Sv and B6-ROSA26 cells. (B) Phloxine- and tartrazine-stained section of the proximal jejunum from a P42 Rac1Asn17 chimeric-transgenic animal. The arrow points to an intermediate cell located at the base of a 129/Sv villus. These cells contain apical tartrazine-positive granules (arrow) that are noticeably smaller than the granules of mature Paneth cells located at the base of crypts (arrowhead). (C) Section of jejunum from a Rac1Asn17 chimeric-transgenic mouse, incubated with rabbit anti-cryptdin-1, Cy3-conjugated donkey anti-rabbit Ig, and bis-benzimide. The entire area shown in this photograph was genotyped as 129/Sv, with X-gal. Intermediate cells express cryptdins (arrow) as do Paneth cells (arrowhead). (D) Staining with *Helix pomentia* agglutinin (HPA) discloses a marked loss of goblet cells in the 129/Sv component of this polyclonal villus (arrowheads indicate goblet cells). (E) HPA-stained section prepared from the jejunum of a normal chimera (age matched with the mouse in D). There are no appreciable differences in the number of goblet cells in the juxtaposed 129/Sv and B6-ROSA26 epithelium of this polyclonal villus. (F,G) Forced expression Rac1Asn17 in 129/Sv epithelial cells slows cell migration. A P42 Rac1Asn17 chimeric-transgenic mouse (F), and an age-matched normal chimera (G), were pulse labeled with BrdU and killed 48 hours later. Sections of their X-gal stained proximal jejunums were incubated with goat anti-BrdU, Cy3-donkey anti-goat Ig and bis-benzimide. In the normal chimeric mouse, B6-ROSA26 and 129/Sv epithelial cells, tagged with BrdU (red) in the crypt during S-phase, have migrated equivalent distances up the polyclonal villus during the 48 hour period (see arrowheads). In contrast, 129/Sv-*Fabpl*-Rac1Asn17 enterocytes have not moved as far up the polyclonal villus as enterocytes in the adjacent band of B6-ROSA26 epithelium. Bar in A, 235 μm; in all other panels, 25 μm.

(73-85 cells/100 sectioned 129/Sv Rac1Asn17 crypts; 78-80 cells/100 sectioned B6-ROSA26 crypts). The number was also similar to values obtained from 129/Sv and B6-ROSA26 proximal jejunal crypts present in age-matched normal chimeras (82-84 M-phase cells/100 sectioned crypts/genotype). No M-phase cells were encountered in the 129/Sv-Rac1Asn17 or B6 villus epithelial compartments of polyclonal villi.

### Rac1Asn17 produces a slowing of cell migration

Recent studies of cultured rat embryo fibroblasts have shown that Rac1Asn17 inhibits protrusive activity/migration in a monolayer wound closure assay. The effect is correlated with the ability of Rac1Asn17 to reduce lamellipodia formation (Nobes and Hall, 1999). We were able to examine the effects of the dominant negative mutation on epithelial migration in vivo by surveying adjacent sections prepared from the same proximal jejunal polyclonal crypt-villus units that had been used to quantitate proliferation. We chose these proximal jejunal units because of their normal morphology. The lack of architectural complexity simplified our task of accurately assessing whether Rac1Asn17 altered cell movement.

Mice harboring these polyclonal villi had received a single intraperitoneal injection of BrdU 48 hours prior to being killed. Sections containing polyclonal villi were incubated with BrdU antibodies plus bis-benzimide (to visualize all nuclei). The positions of 129/Sv epithelial cells that had migrated the greatest distance up a given villus were scored relative to the positions of adjacent B6-ROSA26 cells that had migrated the greatest distance up the same villus. For example, if the leading 129/Sv epithelial cell was 5 cell diameters above the leading B6-ROSA26 cell, it would be assigned a number of +5. If the leading 129/Sv cell was 5 cell diameters below the leading B6-ROSA26 cell, it would be assigned a number of –5.

Surveys of polyclonal villi ($n$=50/mouse; 2 chimeric-transgenic mice) revealed that after 48 hours, the leading 129/Sv-Rac1Asn17 cells had moved to a position that averaged 6.4 cell diameters below the leading B6-ROSA26 cells (mean values for each mouse = –6.5 and –6.3) (e.g. Fig. 9F). Intestinal epithelial cells move at a rate of 5-10 μm/hour (Heath, 1996). Therefore, a 6 cell-diameter difference should correspond to an approx. 6 hour time difference. Of course, we can not

formally rule out the possibility that Rac1Asn17 produces its effect on migration by delaying the initiation of upward movement, rather than by reducing the *speed* of movement.

Surveys of X-gal-stained jejunum whole mounts disclosed that the alteration in distance migrated per unit time was not accompanied by a perturbation in the orderliness of cell migration. Polyclonal villi were composed of coherent columns of wholly 129/Sv-Rac1Asn17 cells and wholly B6-ROSA26 cells. The borders between these columns were sharply demarcated and there was no intrusion of cells of one genotype into cellular columns of the opposite genotype (Fig. 9A).

Two control experiments were performed to help interpret the results of the migration assay. First, analysis of a comparable number of polyclonal villi from normal chimeras revealed no significant difference in the relative positions of the leading, BrdU-positive 129/Sv and B6-ROSA cells (mean values = −0.4 and −0.2, for the two mice analyzed; e.g. Fig. 9G). When migration is defined in this way, we found that the difference between the movement of normal 129/Sv and Rac1Asn17 129/Sv villus enterocytes was statistically significant ($P<0.001$). Second, although our quantitative assessment of proliferation in crypts supplying the polyclonal villi ruled out reduced cell production in 129/Sv-Rac1Asn17 crypts as a cause of reduced cell migration, we still had to rule out increased cell loss. Therefore, hematoxylin and eosin-stained sections that had been used to quantitate the number of M-phase cells in crypts were reviewed so that apoptosis could be scored using well defined morphologic criteria (Hall et al., 1994; Wong et al., 1998). The results established that Rac1Asn17 did not produce a significant change in basal apoptosis: there were 9-22 apoptotic cells per 100 sectioned Rac1Asn17 crypts versus 7-19 apoptotic cells per 100 sectioned (adjacent) B6-ROSA26 crypts.

Epithelial movement along the crypt-villus axis is viewed by some as a ‘passive’ process driven solely by mitotic pressure generated in the crypts (reviewed by Heath, 1996). Data obtained from these chimeric-transgenic mice indicate that intestinal epithelial cell movement may be an active process orchestrated, at least in part, by the epithelial cells themselves through Rac1-mediated pathways. Since Rac1Asn17 does not affect the orderliness of migration, it appears that the level of inhibition of Rac1 activity achieved in these chimeras results in a partial blockage of epithelial traffic flow without an associated disruption in the functional organization of the underlying ‘roadbed’.

## DISCUSSION

Three observations described in this report provide in vivo evidence that Rac1 participates in a pathway that affects epithelial differentiation in the small intestine. First, in the *normal* developing and adult mouse, Rac1 is induced as post-mitotic cells execute their terminal differentiation program. Second, forced expression of a constitutively active Rac1 mutation induces precocious differentiation of members of the Paneth cell and enterocytic lineages. Third, forced expression of a dominant negative Rac1 results in an inhibition of epithelial differentiation. These effects on differentiation occur without an alteration in proliferation, and do not generalize to juxtaposed B6-ROSA26 epithelial cells.

Studies using cultured cells have assigned a variety of functions to the Rac subgroup of Rho GTPases. The challenge has been to determine which of these functions are expressed in specified cell populations in vivo and how function is affected by various physiologic, or pathophysiologic, states. The stem cell hierarchy of the self-renewing mouse small intestinal epithelium makes it particularly well suited for using genetic mosaic analysis to assess the in vivo role of Rac GTPases in regulating proliferation, differentiation and migration. Chimeric mice can be generated that contain easily identifiable and juxtaposed cohorts of genetically manipulated 129/Sv ES cell-derived and normal B6-ROSA26 blastocyst-derived cells. These cells can be compared and contrasted at various locations along the small intestine’s cephalocaudal and crypt-villus axes. By transfecting ES cells with gain-of-function or loss-of-function mutations under the control of regulatory elements that function at specified locations along these axes, it is possible to perform an internally controlled experiment which is restricted to a subset of the 129/Sv gut epithelium.

Activation of Rac in the intervillus epithelium of the fetal mouse gut represents the first reported example that we are aware of where precocious differentiation has been engineered in the proliferative compartment of the developing intestine without an accompanying general inhibition of the cell cycle. The one other report of precocious differentiation in the fetal gut involved mice homozygous for a null allele of the T-cell factor-4 gene (Korinek et al., 1998a). *Tcf-4* encodes a HMG box transcription factor that mediates Wnt signaling in the small intestinal epithelium (Korinek et al., 1998b). Loss of Tcf-4 causes a complete block in proliferation in the intervillus epithelium. The intervillus epithelium of these knockout mice is populated with cells that have the same morphologic appearance as ‘differentiated’ post-mitotic villus epithelial cells (Korinek et al., 1998a).

Interestingly, Ramalho-Santos et al. (2000) have shown that *Indian hedgehog* (*Ihh*) is expressed in the intervillus region in E18.5 mice. Their analysis of E18.5 $Ihh^{-/-}$ fetuses indicate that loss of Indian hedgehog leads to a reduction in proliferation (manifested by reduced villus size) *and* a loss of differentiation (reduced numbers of enteroendocrine cells). These changes are not accompanied by detectable alterations in Tcf4.

In adult chimeras, the junction between the upper crypt and lower villus served as a very sensitive reporter of the effects of the dominant negative Rac1Asn17 on differentiation. As noted in *Results*, levels of Rac1 rise abruptly as epithelial cells pass through this junction, making them vulnerable to the impact of a dominant negative mutation. Inhibition of Rac1 appears to delay the differentiation of post-mitotic cells in the upper crypt, resulting in a marked elongation of crypts, and a distortion of villus architecture. Normally, as cells move from the upper crypt to the lower villus, their actin cytoskeleton undergoes reorganization and a variety of genes involved in terminal differentiation are induced. The ability of Rac1Asn17 to impede actin re-organization and to modulate gene transcription at the crypt-villus junction is consistent with the known effects of this molecule in cultured cells. The inability of Rac1Asn17 to produce sustained alterations in cell polarity as enterocytes complete their migration up the villus is likely due to the fact that levels of endogenous Rac1 normally increase as these cells exit the crypt.

Results obtained from in vitro or in vivo experiments using dominant negative mutations are subject to questions about whether the level of expression was sufficient to *fully* interfere with the function of the endogenous protein, and whether the effect of the mutation was restricted to the target protein of interest. Despite these caveats, the results of expressing the dominant negative Rac1Asn17 complement the results obtained with the constitutively active Rac1Leu61: they both enforce the idea that Rac1 signaling is critical for programming epithelial differentiation.

Our findings support the notion that expression of Rac1 mutations perturbs the proper entry and/or processing of (unspecified) extracellular cues that normally help define the state of epithelial differentiation in vivo. This idea is consistent with the formulation that differentiation of intestinal epithelial cells is regulated in large part by cell non-autonomous mechanisms, and that position-dependent cues are received during the course of cellular migration along crypt-villus units (see Introduction). We do not know whether the constitutively active or dominant negative Rac1 perturbs receipt or processing of signals imported from the mesenchyme underlying the small intestinal epithelium, or from adjacent epithelial cells. We do know from the chimeric system that the effect of the constitutive active mutation on differentiation is not 'exported' to juxtaposed normal B6-ROSA26 cells. This suggest that the protein acts directly *within* expressing cells, and not by re-sculpting the local extracellular environment so as to generate instructions sufficient to enforce differentiation of non-expressing cells.

We believe that the real challenge in understanding the molecular mechanisms involved in Rac1-mediated regulation of intestinal epithelium is to first identify upstream components of its signaling pathway(s). Numerous in vitro studies of cultured cell lines have emphasized how extracellular context (milieu) can influence cellular responses to Rac GTPase-mediated pathways (e.g. Sander et al., 1998). The intestine establishes and maintains a remarkable degree of spatial diversity in its structure and function. In such a system, it is critical that Rac1-mediated signaling be considered in the context of where epithelial cells are in space and time. Therefore, it will be important to define how potential upstream signaling molecules, derived from mesenchymal (Kaestner et al., 1997), and/or epithelial populations (Lefebvre et al., 1999), or the microflora (Hooper et al., 1999), are distributed along crypt-villus units.

We thank David O'Donnell, Maria Karlsson, and Sabrina Wagoner for expert technical assistance and Melissa Wong for her many helpful suggestions. This work was supported by grants from the National Institutes of Health (DK30292 and DK37960).

## REFERENCES

**Aspenström, P.** (1999). Effectors of the Rho GTPases. *Curr. Opin. Cell Biol.* **11**, 95-102.

**Bjerknes, M. and Cheng, H.** (1981a). The stem-cell zone of the small intestinal epithelium. I. Evidence from Paneth cells in the adult mouse. *Am. J. Anat.* **160**, 51-63.

**Bjerknes, M. and Cheng, H.** (1981b). The stem-cell zone of the small intestinal epithelium. III. Evidence from columnar, enteroendocrine, and mucous cells in the adult mouse. *Am. J. Anat.* **160**, 77-91.

**Bjerknes, M. and Cheng, H.** (1999). Clonal analysis of mouse intestinal epithelial progenitors. *Gastroenterology* **116**, 7-14.

**Bradford, M. M.** (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 254-259.

**Braga, V. M. M., Machesky, L. M., Hall, A. and Hotchin, N. A.** (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol.* **137**, 1421-1431.

**Bry, L., Falk, P., Huttner, K., Ouellette, A., Midvedt, T. and Gordon, J. I.** (1994). Paneth cell differentiation in the developing intestine of normal and transgenic mice. *Proc. Natl. Acad. Sci. USA* **91**, 10335-10339.

**Calvert, R. and Pothier, P.** (1990). Migration of fetal intestinal intervillus cells in neonatal mice. *Anat. Rec.* **227**, 199-206.

**Cheng, H., Merzel, J. and Leblond, C. P.** (1969). Renewal of Paneth cells in the small intestine of the mouse. *Am. J. Anat.* **126**, 507-525.

**Cheng, H.** (1974a). Origin, differentiation, and renewal of the four main epithelial cell types in the mouse small intestine. II. Mucous cells. *Am. J. Anat.* **141**, 481-502.

**Cheng, H.** (1974b). Origin, differentiation, and renewal of the four main epithelial cell types in the mouse small intestine. IV. Paneth cells. *Am. J. Anat.* **141**, 521-536.

**Cheng, H. and Leblond, C. P.** (1974a). Origin, differentiation, and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cells. *Am. J. Anat.* **141**, 461-480.

**Cheng, H. and Leblond, C. P.** (1974b). Origin, differentiation, and renewal of the four main epithelial cell types in the mouse small intestine. III. Entero-endocrine cells. *Am. J. Anat.* **141**, 503-520.

**Cheng, H. and Leblond, C. P.** (1974c). Origin, differentiation, and renewal of the four main epithelial cell types in the mouse small intestine. V. Unitarian theory of the origin of the four epithelial subtypes. *Am. J. Anat.* **141**, 537-561.

**Cohn, S. M., Simon, T. C., Roth, K. A., Birkenmeier, E. H. and Gordon, J. I.** (1992). Use of transgenic mice to map *cis*-acting elements in the intestinal fatty acid binding protein (*Fabpi*) that control its cell lineage-specific and regional patterns of expression along the duodenal-colonic and crypt-villus axes of the gut epithelium. *J. Cell Biol.* **119**, 27-44.

**Coso, O. A., Chiariello, M., Yu, J.-C., Teramoto, H., Crespo, P., Xu, N., Miki, T. and Gutkind, J. S.** (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* **81**, 1137-1146.

**Eaton, S., Auvinen, P., Luo, L., Jan,Y. N., and Simons, K**. (1995). CDC42 and Rac1 control different actin-dependent processes in the *Drosophila* wing disc epithelium. *J. Cell Biol.* **131**, 151-164.

**Evan, G. I., Lewis, G. K., Ramsey, G. and Bishop, J. M.** (1985). Isolation of monoclonal antibodies specific for human *c-myc* proto-oncogene product. *Mol. Cell. Biol.* **5**, 3610-3616.

**Falk, P., Roth, K. A. and Gordon, J. I.** (1994). Lectins are sensitive tools for defining the differentiation programs of mouse gut epithelial cell lineages. *Am. J. Phys.* **266**, G987-G1003.

**Falk, P., Hooper, L. V., Midtvedt, T. and Gordon, J. I.** (1998). Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol. Mol. Biol. Rev.* **62**, 157-1170.

**Garabedian, E. M., Roberts, L. J., McNevin, M. S. and Gordon, J. I.** (1997). Examining the role of Paneth cells in the small intestine by lineage ablation in transgenic mice. *J. Biol. Chem.* **272**, 23729-23740.

**Hall, A.** (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-514.

**Hall, P. A., Coates, P. J., Ansari, B. and Hopwood, D.** (1994). Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis. *J. Cell Sci.* **107**, 3569-3577.

**Harwig, S. S., Tan, L., Qu, X., Cho, Y., Eisenhauer, P. B. and Lehrer, R. I.** (1995). Bactericidal properties of murine intestinal phospholipase A2. *J. Clin. Invest.* **95**, 603-610.

**Heath, J. P.** (1996). Epithelial cell migration in the intestine. *Cell Biol. Int.* **20**, 139-146.

**Hermiston, M. L. and Gordon, J. I.** (1995a). In vivo analysis of cadherin function in the mouse small intestinal epithelium: essential roles in adhesion, maintenance of differentiation, and regulation of programmed cell death. *J. Cell Biol.* **129**, 489-506.

**Hermiston, M. L. and Gordon, J. I.** (1995b). Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science* **270**, 1203-1207.

**Hermiston, M. L., Wong, M. H. and Gordon, J. I.** (1996). Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and

provides evidence for nonautonomous regulation of cell fate in a self-renewing system. *Genes Dev.* **10**, 985-996.

**Hill, C. S., Wynne, J. and Treisman, R.** (1995). The Rho family GTPases RhoA, Rac1, and Cdc42Hs regulate transcriptional activation by SRF. *Cell* **81**, 1159-1170.

**Hooper, L. V., Xu, J., Falk, P. G., Midvedt, T., and Gordon, J. I.** (1999). A molecular sensor that allows a gut commensal to control its nutrient foundation in a competitive ecosystem. *Proc. Natl. Acad. Sci. USA* **96**, 9833-9838.

**Hordijk, P. L., ten Klooster, J. P., van der Kammen,R. A., Michiels, F., Oomen, L. C. and Collard, J. G.** (1997). Inhibition of invasion of epithelial cells by Tiam1-Rac signaling. *Science* **278**, 1464-1466.

**Jou, T. S. and Nelson, W. J.** (1998). Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. *J. Cell Biol.* **142**, 85-100.

**Jou, T. S., Schneeberger, E. E. and Nelson, W. J.** (1998). Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. *J. Cell Biol.* **142**, 101-115**.**

**Kaestner, K. H., Silberg, D. G., Traber, P. G., and Schultz, G.** (1997). The mesenchymal winged helix transcription factor *Fkh6* is required for the control of gastrointestinal proliferation and differentiation. *Genes Dev.* **11**, 1583-1595.

**Kaufmann, N., Wills, Z. P. and van Vactor, D.** (1998). *Drosophila* Rac1 controls motor axon guidance. *Development* **125**, 453-461.

**Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J. and Parise, L. V.** (1997). Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* **390**, 632-636.

**Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J. and Clevers, H.** (1998a). Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nature Genet.* **19**, 379-383.

**Korinek, V., Barker, N., Willert, K., Molenaar, M., Roose, J., Wagenaar, G., Markman, M., Lamers, W., Destree,O. and Clevers, H.** (1998b). Two members of the Tcf family implicated in Wnt/β-catenin signaling during embryogenesis in the mouse. *Mol. Cell. Biol.* **18**, 1248-1256.

**Lamarche, N., Tapon, N., Stowers, L., Burbelo, P. D., Aspenström, P., Bridges, T., Chant, J. and Hall, A.** (1996). Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. *Cell* **87**, 519-529.

**Lefebvre, O., Sorokin, L., Kedinger, M. and Simon-Assmann, P.** (1999). Developmental expression and cellular origin of the laminin α2. α4, and α5 chains in the intestine. *Dev. Biol.* **210**, 135-150.

**Lores, P., Morin, L., Luna, R., and Gacon, G.** (1997). Enhanced apoptosis in the thymus of transgenic mice expressing constitutively activated forms of human Rac2 GTPase. *Oncogene* **15**, 601-605.

**Luna, L. G.** (1968). *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology* (ed), New York: McGraw-Hill.

**Luo, L., Hensch, T. K., Ackerman, L., Barbel, S., Jan, L. Y. and Jan, Y. N.** (1996). Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. *Nature* **379**, 837-840.

**Mackay D. J. and Hall, A.** (1998). Rho GTPases. *J. Biol. Chem.* **273**, 20685-20688.

**Merzel, J., and Leblond, C. P.** (1969). Origin and renewal of goblet cells in the epithelium of the mouse small intestine. *Am. J. Anat.* **124**, 281-305.

**Minden, A., Lin, A., Claret, F. -X., Abo, A. and Karin, M.** (1995). Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* **81**, 1147-1157.

**Moll, J., Sansig, G., Fattori, E. and van der Putten, H.** (1991). The murine *rac1* gene: cDNA cloning, tissue distribution and regulated expression of rac1 mRNA by disassembly of actin microfilaments. *Oncogene* **6**, 863-866.

**Mulherkar, R., Desai, S. J., Rao, R. S., Wagle, A. S. and Deo, M. G.** (1991). Expression of enhancing factor gene and its localization in mouse tissues. *Histochemistry* **96**, 367-370.

**Mulherkar, R., Rao, R. S., Wagle, A. S., Patki, V. and Deo, M. G.** (1993). Enhancing factor, a Paneth cell specific protein from mouse small intestine: predicted amino acid sequence from RT-PCR amplified cDNA and its expression. *Biochem. Biophys. Res. Comm.* **195**, 1254-1263.

**Nagy, A. and Rossant, J.** (1992). Production of completely derived ES cell-derived fetuses. In: *Gene Targeting, a Practical Approach* (ed. A. L. Joyner), pp. 147-180. Oxford: Oxford University Press.

**Nobes, C. D. and Hall, A.** (1995). Rho, rac and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia and filopodia. *Cell* **81**, 53-62.

**Nobes, C. D. and Hall, A.** (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement. *J. Cell Biol.* **144**, 1235-1244.

**Olson, M. F., Ashworth, A. and Hall, A.** (1995). An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. *Science* **269**, 1270-1272.

**Ouellete, A. J.** (1997). Paneth cells and innate immunity in the crypt microenvironment. *Gastroenterology* **113**, 1779-1784.

**Ramalho-Santos, M., Melton, D. A. and McMahon, A. P.** (2000). Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* **127**, 2763-2772.

**Ridley, A. J. and Hall, A.** (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-399.

**Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A.** (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-410.

**Ridley, A. J., Comoglio, P. M. and Hall, A.** (1995). Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac and Rho in MDCK cells. *Mol. Cell. Biol.* **15**, 1110-1122.

**Roberts, A. W., Kim, C., Zhen, L., Lowe, J. B., Kapur, R., Petryniak, B., Spaetti, A., Pollock, J. D., Borneo, J. B., Bradford, G. B., Atkinson, S. J., Dinauer, M. C. and Williams, D. A.** (1999). Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity* **10**, 183-196.

**Sander, E. E., van Delft, S., ten Klooster, J. P., Reid, T., van der Kammen, R. A., Michiels, F., and Collard, J. G.** (1998). Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. *J. Cell Biol.* **143**, 1385-1398.

**Schmidt, G. H., Winton, D. J. and Ponder, B. A.** (1988). Development of the pattern of cell renewal in the crypt-villus unit of chimaeric mouse small intestine. *Development* **103**, 785-790.

**Schmidt, G. H., Wilkinson, M. M. and Ponder, B. A.** (1985). Cell migration pathway in the intestinal epithelium: an *in situ* marker system using mouse aggregation chimeras. *Cell* **40**, 425-429.

**Selsted, M. E., Miller, S. I., Henschen, A. H. and Ouellette, A. J.** (1992). Enteric defensins: antibiotic peptide components of intestinal host defense. *J. Cell Biol.* **118**, 929-936.

**Steven, R., Kubiseski, T. J., Zheng, H., Kulkarni, S., Mancillas, J., Ruiz Morales, A., Hogue, C. W., Pawson, T. and Culotti, J.** (1998). UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in *C. elegans*. *Cell* **92**, 785-795.

**Sugihara K., Nakatsuji, N., Nakamura, K., Nakao, K., Hashimoto, R., Otani, H., Sakagami, H., H., Kondo, H., Nozawa, S., Aiba, A. and Katsuki, M.** (1998). Rac1 is required for the formation of three germ layers during gastrulation. *Oncogene* **17**, 3427-3433.

**Sulciner, D. J., Irani, K., Yu, Z. X., Ferrans, V. J., Goldschmidt-Clermont, P. and Finkel, T.** (1996). Rac1 regulates a cytokine-stimulated, redox-dependent pathway necessary for NF-kappaB activation. *Mol. Cell. Biol.* **16**, 7115-7121.

**Sweetser, D. A., Birkenmeier, E. H., Hoppe, P. C., McKeel, D. W. and Gordon, J. I.** (1988). Mechanisms underlying generation of gradients in gene expression within the intestine: an analysis using transgenic mice containing fatty acid binding protein-human growth hormone fusion genes. *Genes Dev.* **2**, 1318-1332.

**Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H. and Takai, Y.** (1997). Regulation of cell-cell adhesion by rac and rho small G proteins in MDCK cells. *J. Cell Biol.* **139**, 1047-1059.

**Takano, H., Komuro, I., Oka, T., Shiojima, I., Hiroi, Y., Mizuno, T. and Yazaki, Y.** (1998). The Rho family G proteins play a critical role in muscle differentiation. *Mol. Cell. Biol.* **18**, 1580-1589.

**Troughton, W. D. and Trier, J. S.** (1969). Paneth and goblet cell renewal in mouse duodenal crypts. *J. Cell Biol.* **41**, 251-268.

**Weiser, M. M.** (1973). Intestinal epithelial cell surface membrane glycoprotein synthesis. *J. Biol. Chem.* **248**, 2536-2541.

**Wilson, C. L., Heppner, K. J., Labosky, P. A., Hogan, B. L. M. and Matrisian, L. M.** (1997). Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin. *Proc. Natl. Acad. Sci. USA* **94**, 1402-1407.

**Wilson, C. L., Ouellette, A. J., Satchell, D. P., Ayabe, T., Lopez-Boado, Y. S. and Stratman, J. L., Hultgren, S. J., Matrisian, L. M. and Parks, W. C.** (1999). Regulation of intestinal alpha-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* **286**, 113-117.

**Wong, M. H., Rubinfeld, B. and Gordon, J. I.** (1998). Effects of forced expression of an $NH_2$-terminal truncated β-catenin on mouse intestinal epithelial homeostasis. *J. Cell Biol.* **141**, 765-777.