# Regulation of Dab2 Expression in Intestinal and Renal Epithelia by Development

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## ABSTRACT

Disabled-2 (Dab2) is an intracellular adaptor protein proposed to function in endocytosis. Here, we investigate the intestinal and renal Dab2 expression versus maturation. Dab2 mRNA levels measured by RT-PCR are greater in the small than in the large intestine. Immunological studies localize Dab2 to the terminal web domain of the enterocytes and reveal the presence of a 96-kDa Dab2 isoform in the apical membrane of the jejunum, ileum, and renal cortex of the suckling and adult rat. A 69-kDa Dab2 isoform is only observed in the apical membranes of the suckling ileum. During the suckling period, the Dab2 mRNA levels measured in the enterocytes and crypts and those of the 96-kDa Dab2 isoform are greater in the ileum than in the jejunum. No segmental differences are observed in the adult intestine. In the intestine, the levels of Dab2 mRNA and those of the 96-kDa Dab2 isoform decrease to adult values at weaning, whereas in the kidney they increase with development. Weaning the pups on a commercial milk diet slows the periweaning decline in the levels of Dab2 mRNA in the crypts and of those of the 96-kDa isoform. This is the first report showing that the 96-kDa Dab2 isoform is expressed at the apical domain of rat small intestine, that

ontogeny regulates Dab2 gene expression in intestine and kidney and that retarding weaning affects intestinal Dab2 gene expression.

KEY WORDS: Dab2; EPITHELIUM; INTESTINE; ENTEROCYTES; KIDNEY; DEVELOPMENT

## INTRODUCTION

The small intestinal epithelium acts as a digestive/absorptive surface and as a barrier preventing luminal pathogens from gaining entry into the organism. It mainly consists of a morphological and functional polarized monolayer of columnar cells, the enterocytes, which perform net vectorial transepithelial transport of dietary nutrients. Macromolecules are taken up by the epithelial cells through invaginations of their apical membrane followed by vesicle formation, a process named endocytosis. Intestinal endocytosis occurs in different ways depending on the nature of the substrate that is taken up [see Keita and Sö derholm, 2010, for a review]. One route is via clathrin-mediated endocytosis, a highly specific receptor-mediated process, which is highly active during the suckling period to absorb the immunoglobulins (IgGs) and growth factors from the mother's milk [see Fujita et al., 2007, for a review]. At weaning the mammalian intestine gradually changes from a maternal milk diet to solid nutrition and the suckling intestinal absorptive cells gradually shift to adult absorptive cells. This shift conveys adaptive morphological and functional changes of the epithelium, such as the reduction of the apical endocytic membrane system [Fujita et al., 2007]. As a result, the epithelial permeability to macromolecules abruptly stops shortly at weaning [Fujita et al., 2007] and the adult enterocytes mainly absorb small molecules, albeit constitutive apical endocytosis occurs at a slow rate [Hansen et al., 2009; Keita and Sö derholm, 2010].

Disabled-2 (Dab2) is an intracellular adaptor protein recently implicated in epithelial clathrin-mediated endocytosis. It has a N-terminal binding domain that binds to a C-terminal cytoplasmatic domain of the lipoprotein receptor family members [Oleinikov et al., 2000; Morris and Cooper, 2001]. The C-terminal region of

Dab2 contains a domain that binds to myosin VI [Morris et al., 2002a] and its central region contains two motifs that bind to clathrin and to clathrin adaptor protein AP-2 [Morris and Cooper, 2001; Mishra et al., 2002]. The role of Dab2 in receptor-mediated endocytosis was first discovered in rat renal proximal tubule [Morris et al., 2002a; Nagai et al., 2005] and mice visceral endoderm [Maurer and Cooper, 2005] epithelia. In the kidney, Dab2 mediates the proximal tubule endocytosis of the proteins filtered in the glomeruli [Morris et al., 2002b] and in the visceral endoderm it mediates the endocytosis of trasferrin [Maurer and Cooper, 2005]. In both epithelia, Dab2 localizes to their terminal web domain, just below the microvilli [Maurer and Cooper, 2005; Nagai et al., 2005; Hosaka et al., 2009]. A recent report [Collaco et al., 2010] has shown the localization of Dab2 at the apical domain of the intestinal epithelium and has suggested that Dab2 mediates the apical endocytosis of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel.

Due to the physiological relevance of Dab2, the current study was designed to further study the presence of Dab2 in the rat intestine and to investigate whether Dab2 expression in intestine and kidney epithelia varies with ontogeny.

## MATERIALS AND METHODS

### CHEMICALS

Unless otherwise indicated, the reagents used in this study were from Sigma-Aldrich (Spain).

### ANIMALS AND DIETS

Twenty-day gestation fetuses, newborn (<1 day-postpartum), suckling (5- and 15-day-postpartum), weaning (21 day-postpartum), and adult (1, 2, and 5-month-old) Wistar rats were used. Adult rats were fed with a rat diet (Harlan Ibérica S.L.) *ad libitum* and had free access to water. For some experiments, 15 day-old rats were weaned onto either rat chow or dry commercial milk like mother's

(Cyntechnic Health Nutrition) and maintained under these experimental conditions up to either day 21 or 30.

The animals were anesthetized with a lethal intraperitoneal injection of pentobarbital (50 mg/kg). They were humanely handled and sacrificed in accordance with the European Council legislation 86/609/EEC concerning the protection of experimental animals.

## RELATIVE QUANTIFICATION OF REAL-TIME PCR

RT-PCR was performed on total RNA as Garcıa-Delgado et al. [2007]. The primers for Dab2 (antisense, cacatcacgagcaatgaagg and sense, caggttcaaaggtgatggtg) and the $Na^{þ}$/glucose cotransporter SGLT1 (antisense, gaccacttccgatgttactg and sense, cacccatccagtcc- tatgag) were chosen according to the rat cDNA sequences entered in Genbank and designed using PerlPrimer program. b-actin (antisense, acccacactgtgcccatcta and sense, cggaaccgctcattgcc) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; anti- sense, tgcaccaccaactgcttagc and sense, ggcatggactgtggtcatgag) served as reference genes for samples normalization. Analyses of real-time PCR were done using the comparative Ct method, with the Gene Expression Macro software supplied by BioRad.

## ISOLATION OF ENTEROCYTES, CRYPTS, AND MYOFIBROBLASTS

The jejunum and ileum were rapidly removed and washed with ice- cold saline solution. Enterocytes, crypt cells, and myofibroblasts were sequentially isolated. Enterocytes and crypts were isolated by a $Ca^{2þ}$-chelation technique [Weiser, 1973] as modified by Knickelbein et al. [1988]. Myofibroblasts were isolated following the method of Leeb et al. [2002] as described by Garcıa-Miranda et al. [2010]. Briefly, the tissues were cut into 2 cm segments and incubated in a shaking water bath at 378C for 15 min. The incubation buffer contained in mM: 96 NaCl, 27 $Na_3C_3H_5O(COO)_3$, 0.8 $KH_2PO_4$, 5.6 $Na_2HPO_4$, 1.5 KCl, 10 glucose and 0.5 dithiothreitol (DTT) pH 7.4 (buffer A) and was continuously gassed with 95%

$O_2$/5% $CO_2$. Following the 15 min incubation period, the pieces were incubated for 1 h in PBS containing, in mM: 1.5 EDTA, 10 glucose and 0.5 DTT (buffer B). The tissues were vortexed for 30 s to remove epithelial cells from the villi. Loosened epithelial cells were filtered through 60 mm nylon cloth and collected by centrifugation and resuspension in PBS. They constitute the enterocyte fraction. The tissues were incubated for another 10 min period in buffer B and the buffer discarded. Following a 20 min incubation period in buffer B, the tissues were vortexed for 5 min and the crypt cells collected by centrifugation and resuspension in PBS. The remaining tissue was rinsed and incubated for 30 min, in a shaking water bath at 378C, in PBS containing 1 mg/ml collagenase and 2 mg/ml hyaluronidase. The tissue pieces were vortexed for 30 s and the myofibroblasts were pelleted and resuspended in PBS. The isolated cells were used for immunostaining or frozen in liquid nitrogen and kept at —80ºCuntil used for RNA extraction. The cell enrichment of each intestinal fraction was evaluated as Garcıa-Miranda et al. [2010] by measuring the mRNA abundance of their specific markers: villin for enterocytes, $Na^þ/K^þ/2Cl^-$ cotransporter-1 for crypts and a-smooth muscle actin for myofibroblats (data not shown).

IMMUNOSTAINING ASSAYS

Immunostaining assays were performed on intact small intestine (10 mm cryosections) and on isolated intestinal cells, as Garcıa- Miranda et al. [2010]. The slides containing either the intestinal sections or the isolated enterocytes were incubated with a monoclonal anti-Dab2 antibody (BD Transduction Laboratories),

1:300 dilution, at 48C and overnight. Controls were carried out without primary antibody. Antibody binding was visualized with biotinylated antibodies, followed by immunoperoxidase staining using the Vectastain ABC peroxidase kit (Vector) and 3,3'- diaminobenzidine. The slides were rinsed, mounted, and photo- graphed with a Zeiss Axioskop 40 microscope.

### WESTERN BLOT ASSAYS

Apical membranes from the small intestine and renal cortex were obtained by $Mg^{2þ}$ precipitation method. Briefly, either intact intestine, isolated enterocytes or slices of kidney cortex were homogenized in a buffer (in mM: 100 mannitol, 5 EGTA, 15 Tris–HCl, pH 7) with the Ystral Polytron on setting 5 for 1 min. $MgCl_2$, up to a final concentration of 10 mM, was added to the homogenate. The suspension was gently stirred for 20 min and then centrifuged at 1,000*g* for 15 min. The resultant supernatant was centrifuged at 30,000*g* for 30 min and the resultant pellet was used for the Western blot assays. All the steps were carried out at 48C. Protein was measured by the method of Bradford [1976], using gamma globulin as the standard. SDS–PAGE was performed according to Laemmli [1970] on a 7.5% polyacrylamide gel as Peral et al. [2002]. A 1:1,000 dilution of the monoclonal anti-Dab2 antibody (BD Transduction Labora- tories) was used. The dilution of the secondary antibody (horseradish Peroxidase-conjugated anti-mouse IgG antibody) was 1:3,000. Anti-b-actin antibody was used to normalize bands density. The immunoreactive bands were viewed with a chemiluminescence procedure (GE Healthcare).

### STATISTICAL ANALYSIS

Data are presented As Mean SEM. In the figures the vertical bars representing the SEM are absent when they are less than symbol height. One-way ANOVA followed by Newman–Keuls' test was used for multiple comparisons (GraphPad Prism Program v5.0). Two-tailed Student's t-test was used for paired comparisons. Differences were set to be significant for $P < 0.05$.

## RESULTS

### Dab2 mRNA LEVELS IN THE SMALL AND LARGE INTESTINE

Real-time RT-PCR was carried out on total RNA extracted from the duodenum, jejunum, ileum, and colon of 15-day-old rats. The results are given in Figure 1. The

levels of Dab2 mRNA do not significantly vary along the duodenum and jejunum, they significantly decrease in the ileum and they further decrease in the large intestine.

## EXPRESSION OF Dab2 mRNA IN ISOLATED INTESTINAL CELLS

To localize the cells within the intestinal mucosa that express Dab2 mRNA, RT-PCR was carried out on total RNA isolated from intestinal fractions enriched either in enterocytes, crypts, or myofibroblasts and the results are summarized in Figure 2. In the 15 day-old rats, the highest levels of Dab2 mRNA are found in the enterocytes enriched fraction as compared to those measured in either crypts or myofibroblasts enriched fractions. The opposite is observed in the 30-day-old rat: the maximal Dab2 mRNA levels are found in myofibroblasts enriched fraction and the lowest in that of the enterocytes.

The results also show that Dab2 mRNA levels measured in both, enterocytes and crypt cells enriched fractions are lower in the 30 than in the 15 day-old rat, but those measured in the myofibroblasts enriched fraction increase from days 15 to 30 after birth by a factor of 14.

The abundance of Dab2 mRNA in the enterocytes is compared to that of the SGLT1 mRNA in the same cells. The results are given in Figure 2. In the 15 day-old rats the enterocyte levels of Dab2 mRNA are significantly greater than those of SGLT1 mRNA. As levels of SGLT1 mRNA do not significantly vary between the two ages tested, they are higher than those of Dab2 mRNA in the 30-day-old rats.

## LOCALIZATION OF Dab2 PROTEIN IN RAT SMALL INTESTINE

The localization of Dab2 protein in the intact small intestine and in isolated enterocytes of 5-day-old rats is shown in Figure 3. In the intestine, the specific signal produced by the anti-Dab2 antibody is seen in the villi but it is absent from the crypts (Fig. 3A). The signal is particularly strong at the terminal web domain of the cells lining the villus (Fig. 3B). Some labeling appears in the nucleus but this labeling does

not appear to be anti-Dab2 antibody specific because is also observed in the absence of the primary antibody (Fig. 3C). An anti-Dab2 antibody specific signal is not observed in the intestine of 30-day-old rats (data not shown).

In the isolated enterocytes the specific staining of Dab2 is concentrated in the supranuclear region of the cytosol, below the microvilli (Fig. 3D,E). In the absence of the primary antibody specific labeling is absent from the enterocytes (Fig. 3F).

EXPRESSION OF Dab2 GENE IN RAT SMALL INTESTINE AND KIDNEY DURING DEVELOPMENT

The effect of ontogeny on the abundance of Dab2 mRNA was examined by real-time RT-PCR assay. Total RNA was extracted from jejunum, ileum, and kidney cortex of rats of different ages. The results are given in Figure 4. In both jejunum and ileum, the abundance of Dab2 mRNA increases during the gestation and suckling periods and it peaks at day 15 after birth. Thereafter, it decreases by about three orders of magnitude, reaching nearly adult values by day 30 after birth. The relative abundance of Dab2 mRNA measured in kidney cortex increases with the age of the animal.

INTESTINAL AND RENAL Dab2 PROTEIN EXPRESSION VERSUS AGE

The abundance of Dab2 protein in the intestinal and renal epithelia was examined by Western assay. The epithelial apical membranes were obtained from jejunum, ileum, and renal cortex of 5 and 30 day-old rats. The anti-Dab2 antibody detects in the apical membrane of suckling enterocytes a polypeptide of approx. 96-kDa, which is more abundant in the ileum than in the jejunum. A second polypeptide band of 69-kDa is detected only in the apical membrane of ileum enterocytes. The time of exposure was 10 s.

The apical membranes obtained from 30-day-old enterocytes had to be exposed for at least 3 min to reveal the 96-kDa band. This indicates that the abundance of the 96-kDa polypeptide is lower in the 30-day-old intestine than in the suckling

intestine. This longer exposure time reveals two additional bands of 69 and 91 KDa, respectively. The abundance of these three bands is similar in jejunum than in ileum.

The antibody detects two polypeptides of 96- and 91-kDa in the kidney of the suckling rats and three of 96-, 91-, and 69-kDa in the kidney of 30-day-old rats. The intensity of the 96-kDa band increases with age.

EFFECT OF DIET ON INTESTINAL Dab2 GENE EXPRESSION

To investigate whether the periweaning-induced decrease in intestinal Dab2 mRNA levels is related to diet, the 15-day-old suckling rats were weaned onto either rat chow or commercial milk diet and maintained under these experimental conditions up to either day 21 or 30 after birth. Total RNA was extracted from enterocytes and crypts enriched fractions obtained from either the jejunum or ileum of 5, 15, 21, and 30 day-old rats and it was used for the RT-PCR assays. The results are summarized in Figure 6.

The results reveal that during the suckling period the levels of Dab2 mRNA measured in the cell fractions isolated from the ileum are significantly higher than those measured in the corresponding jejunal cell fractions. Regional differences are not observed after weaning. In both intestinal regions the Dab2 mRNA levels in enterocytes are similar to those in the crypts at day 5 after birth. The levels measured in enterocytes do not significantly change between days 5 and 15 after birth, whereas those in the crypts significantly decrease. In both intestinal cell types the abundance of Dab2 mRNA greatly decrease at weaning, being the decrease higher in the enterocytes than in the crypts. At days 21 and 30 after birth, Dab2 mRNA levels are significantly higher in the crypts than in the enterocytes.

Maintenance of the pups on a milk diet slows the weaning- induced decrease in Dab2 mRNA observed in the crypts, being the effect bigger in magnitude and time in the ileum than in the jejunum. The type of diet does not modify the weaning-

related decrease in the Dab2 mRNA levels observed in the enterocytes.

The abundance of Dab2 protein was measured, by Western analysis, in the apical membranes of jejunal and ileal enterocytes isolated from the 21-day-old rats that have been weaned onto either rat chow or commercial milk diet, as described above. The results are given in Figure 7 and reveal that, in both jejunum and ileum, the intensity of the 96-kDa band is significantly higher in the pups weaned onto commercial milk diet than in those weaned onto rat chow.

**DISCUSSION**

The current results demonstrate that rat small and large intestine express Dab2 and that small intestinal Dab2 gene expression is regulated by ontogeny and diet. The effect of development on the renal expression of Dab2 is opposite to that on the intestine.

Dab2 mRNA is more abundant in the small than in the large intestine. Before weaning, the Dab2 gene transcription in the enterocytes is quite significant, because it is even greater than that of SGLT1, a gene highly expressed by these cells. Immuno-histochemistry assays localize Dab2 to the terminal web domain, just below the brush-border of the enterocytes. Specific immunoreacting signal is neither in the crypts nor in the 30-day-old intact intestine. The apical location of Dab2 is consistent with its distribution in other polarized tissue such as the kidney cortex [Maurer and Cooper, 2005; Nagai et al., 2005; Hosaka et al., 2009] and visceral endoderm [Maurer and Cooper, 2005] and with a recent finding made in the intestine [Collaco et al., 2010]. Collaco et al. [2010], however, observed expression of Dab2 in the adult intestine by immunohistochemistry. The discrepancy between Collaco's and our observations may result from levels of Dab2 in the adult intestine too low to provide a visible signal in our tissue preparations. The Western results corroborate this point of view as the antibody detects

Dab2 on the apical membranes isolated from 30-day-old intestine, although the exposure time required

was longer to that needed when using apical membranes of 5-day- old intestine.

Alternative splicing of Dab2 gene produces different isoforms, with apparent molecular masses of 96- (long), 93- and, 67-(short) kDa in mouse [Xu et al., 1995] and human [Albertsen et al., 1996] and of 82- (long) and 59-(short) kDa in rat [Tseng et al., 1998]. Molecular weights for Dab2 isoforms of 100- and 75-kDa have been found in rat adrenal gland [Romero et al., 2007]. In the renal and visceral endoderm epithelia the long isoform has an apical location and is involved in receptor-mediated endocytosis [Maurer and Cooper, 2005; Hosaka et al., 2009]. The short isoform is thought to be a transcriptional activator because it localizes to the cytosol and nucleus [Tseng et al., 1998; Maurer and Cooper, 2005]. The current results corroborate the apical location of the long isoform (96-kDa) in the kidney cortex and reveals that the apical membrane of rat small intestine also expresses the 96-kDa isoform. Collaco et al. [2010] recently reported that Dab2 mediates the intestinal endocytosis of the CFTR. The apical location of the 96-kDa isoform suggests that this is the Dab2 isoform involved in intestinal endocytic processes. Another band of 69-kDa (short isoform) is only observed in the apical membrane of suckling ileum. Although the short isoform has a cytoplasmic location [Tseng et al., 1998; Maurer and Cooper, 2005], it retains endocytic motifs [Maurer and Cooper, 2005] that may explain its presence in the apical membranes of the suckling ileum. However, its intestinal role is not evident from the current observations.

The current results also suggest a role for Dab2 in the intestinal endocytosis of milk macromolecules, because the changes in the

Dab2 gene expression observed in the intestinal epithelia with development are consistent with the reported development-related changes in the intestinal endocytic apparatus [Wilson et al., 1991; Fujita et al., 2007]. Thus, the endocytic apparatus of the intestinal absorptive cells is assembled during the last 2–3 days of gestation [Wilson et al., 1991] and the fetal intestine has high levels of Dab2 mRNA. Secondly, the apical endocytic apparatus of the small intestine is greatly reduced at weaning, so that the intestinal permeability to milk macromolecules is terminated at day 21 after birth [Fujita et al., 2007] and we found that the intestinal levels of Dab2 mRNA and those of the 96-kDa protein decrease at weaning.

Consistent with a role of Dab2 in the intestinal endocytosis of milk macromolecules is that the renal Dab2 mRNA and protein levels increase with development. The opposite effects of maturation on intestinal and renal Dab2 gene expression agree with the physiological functions of the two organs. The renal endocytosis of the proteins filtered in glomeruli must remain active through life to prevent proteinuria whereas the intestine adapts to the shift from a maternal milk diet to solid nutrition during the weaning period.

Finally, the observation that Dab2 mRNA is more abundant in the small than in the large intestine of suckling rats further support the view that Dab2 may be involved in the endocytosis of milk macromolecules.

Signals involved in ‘‘telling’’ the intestine to turn on the correct transport systems at the correct time might be external (e.g., changes in dietary solute inputs associated with weaning) and internal. The latter are genetically to occur at a certain time, independent of external circumstances [Ferraris, 2001]. That weaning the pups to a commercial milk diet slows the weaning-related decrease in the levels of Dab2 mRNA in the crypts and of Dab2 protein in the enterocytes suggests that the periweaning changes in the intestinal Dab2 gene expression are in part related to the milk diet. Effects of nutrients on the postnatal gene expression shift and gene products processing, have been implicated on the intestinal expression of SGLT1 [Shirazi-Beechey et al., 1991; Freeman et al., 1993] and GLUT5 [Shu et al., 1998].

Weaning the pups onto a commercial milk diet reduces but it does not prevent the periweaning decrease in Dab2 gene expression. This could result from the lack of immunoglobulins, growth factors and other macromolecules in the commercial milk, otherwise present in the mother´s milk, which could affect Dab2 gene expression. Alternatively, it may indicate that the weaning-related decrease in Dab2 gene expression is also genetically programmed. The latter view is supported by the existence of diet-independent changes in the Dab2 mRNA levels, such as the high Dab2 mRNA levels found in the fetal intestine and the decrease in the abundance of Dab2 mRNA in the crypts during the suckling period.

Jejuno-ileal differences in the Dab2 gene expression are observed in the suckling rat but not in the adult. (i) The levels of Dab2 mRNA are higher in the enterocytes and crypts isolated from the suckling ileum than in those cells obtained from the suckling jejunum. These data contrast with those obtained using intact intestine, wherein the Dab2 mRNA levels are higher in the jejunum than in the ileum. The discrepancy may arise from the cell preparation used to obtain the mRNA: the Dab2 mRNA obtained from intact intestine originates from different cell types and we have observed that different mucosal cell types present different levels of Dab2 mRNA. (ii) The abundance of the 96-kDa isoform is greater in the ileum than in the jejunum. (iii) The 69-kDa isoform is only present in the suckling ileum. (iv) The effect of the commercial milk diet on Dab2 gene expression is bigger in magnitude and time in the ileum than in the jejunum. Jejuno-ileal differences in the processing of the milk macromolecules have been reported [Fujita et al., 2007]. The enterocytes of the suckling jejunum transport maternal antibodies into circulation without lysosomal degradation, whereas the enterocytes of the ileum endocytose, storage and digest macromolecules in the giant supranuclear lysosome [Fujita et al., 2007]. Further studies are required to find out whether the reported differences are related with those found in the current study.

In conclusion, this is the first study reporting that the 96-kDa Dab2 isoform localizes to the terminal web domain of the enterocytes, that Dab2 gene

expression is down-regulated in the intestine and up-regulated in the kidney by maturation and that diet regulates intestinal Dab2 gene expression.

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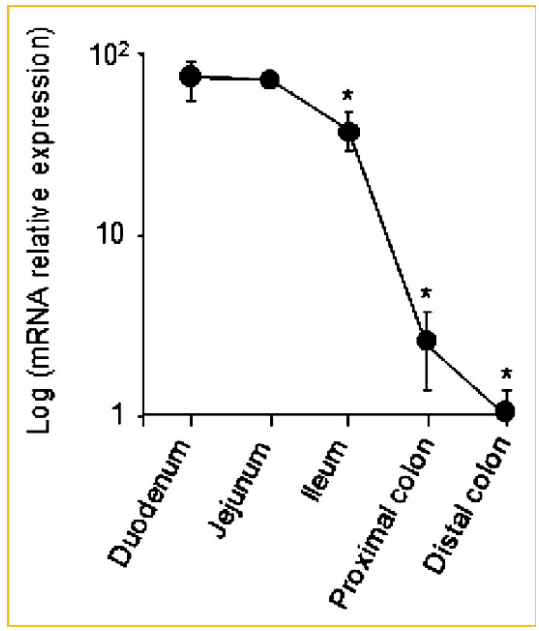


Fig. 1. Levels of Dab2 mRNA along the rat intestine. RT-PCR was performed on total RNA isolated from the duodenum, jejunum, ileum, proximal colon, and distal colon of 15-day-old rats. The mRNA levels measured in distal colon were set at 1. The values represent means SEM of arbitrary units of mRNA levels and are plotted on a logarithmic scale. Four animals were used. One-way ANOVA showed an effect of intestinal region on Dab2 mRNA levels ( $P < 0.001$). Newman–Keul's test: $^{\omega}P < 0.001$ as compared with duodenum

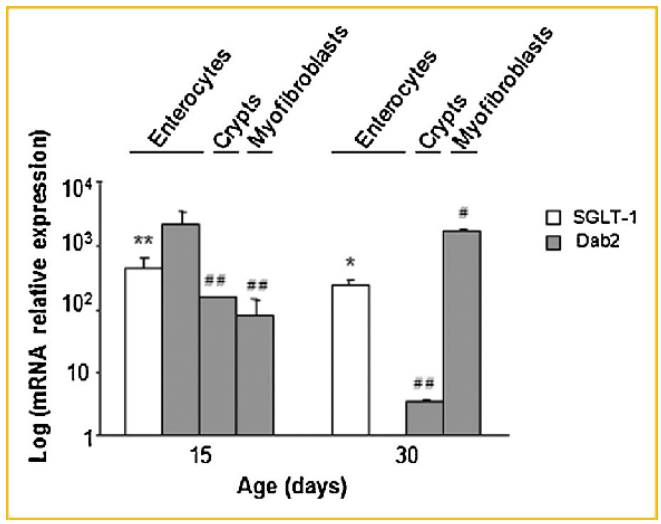


Fig. 2. Levels of the Dab2 mRNA in cells isolated from the small intestine. Intestinal fractions enriched in either enterocytes, crypts, or myofibroblasts were obtained from the small intestine of 15- and 30-day-old rats. RT-PCR was performed on total RNA obtained from each fraction. SGLT1 mRNA was only measured in enterocytes. The Dab2 mRNA levels measured in enterocytes of 30-day-old rat were set at 1. The values represent means SEM of arbitrary units of mRNA levels and are plotted on a logarithmic scale. Each value is the mean of four independent experiments. One-way ANOVA showed an effect of maturation on Dab2 mRNA levels in enterocytes, crypts and myofibroblasts ( $P < 0.001$). Newman–Keul's test: $^{\omega}P < 0.001$, $^{\omega\omega}P < 0.01$ Dab2 versus SGLT1 mRNA levels in enterocytes; $^{\#}P < 0.001$, $^{\#\#}P < 0.01$ as compared with the Dab2 mRNA levels of enterocytes in each age.

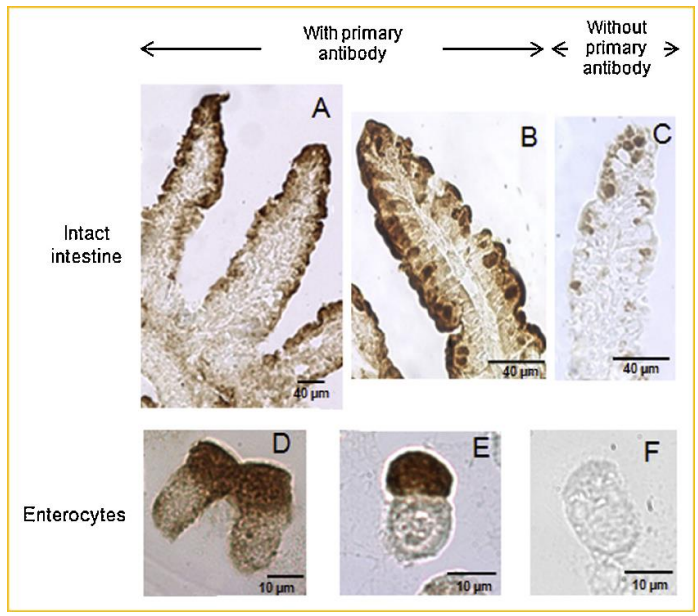


Fig. 3. Immunolocalization of Dab2 protein in rat small intestine. Cryosections (10 mm) of the ileum (panels A,B) and enterocytes isolated from jejunum (panel D) and ileum (panel E) were immunostained with anti-Dab2 antibody (BD Transduction Laboratories), 1:300 dilution. Five-day-old rats were used. In panels C (intact ileum) and F (enterocytes) incubation was carried out without primary antibodies. The photographs are representative of three different assays.

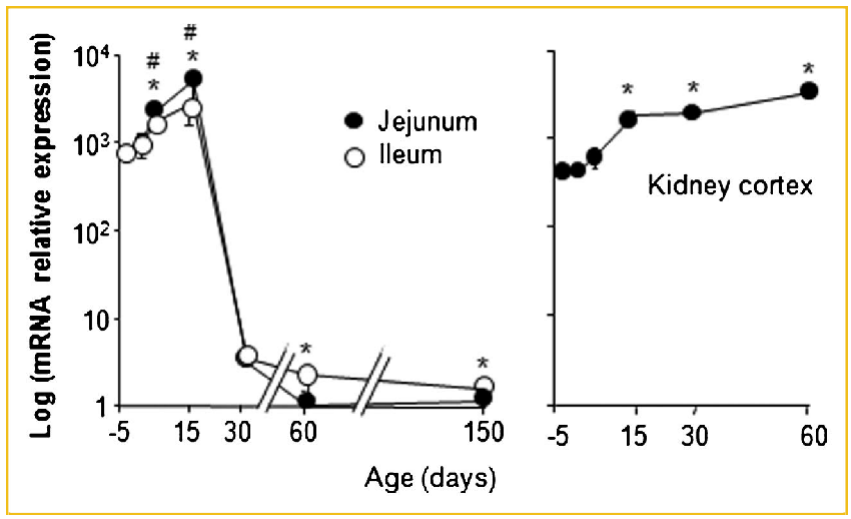


Fig. 4. Levels of intestinal and renal Dab2 mRNA during development. RT-PCR was performed on total RNA isolated either from jejunum, ileum, or kidney cortex of fetuses, newborn, 5, 15, 30, 60, and 150-day-old rats. The mRNA levels measured in jejunum of 2-month-old rats were set at 1. The values represent means SEM of arbitrary units of mRNA abundance and are plotted on a logarithmic scale. Each value is the mean of four independent experiments. One-way ANOVA showed an effect of maturation and segmental distribution on Dab2 mRNA levels ( $P < 0.001$). Newman-Keul's test: $^{\omega}P < 0.001$ as compared with fetuses; $^{\#}P < 0.05$ ileum versus jejunum.

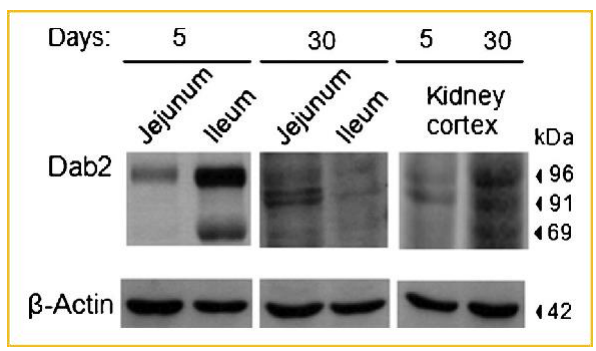


Fig. 5. Western blots of Dab2. Apical membranes were isolated from the jejunum, ileum, and renal cortex of 5- and 30 day-old rats. Thirty microgram of protein of intestinal membranes and 50 mg protein of renal membranes were loaded per lane. The blots were probed with a commercial monoclonal anti- Dab2 antibody (BD Transduction Laboratories) 1:1,000 dilution. The exposure time was 10 s for the intestinal membranes of 5-day-old rats and renal membranes of both ages and 3 min for the intestinal membranes of 30- day-old rats. The blot is representative of three Western blots.

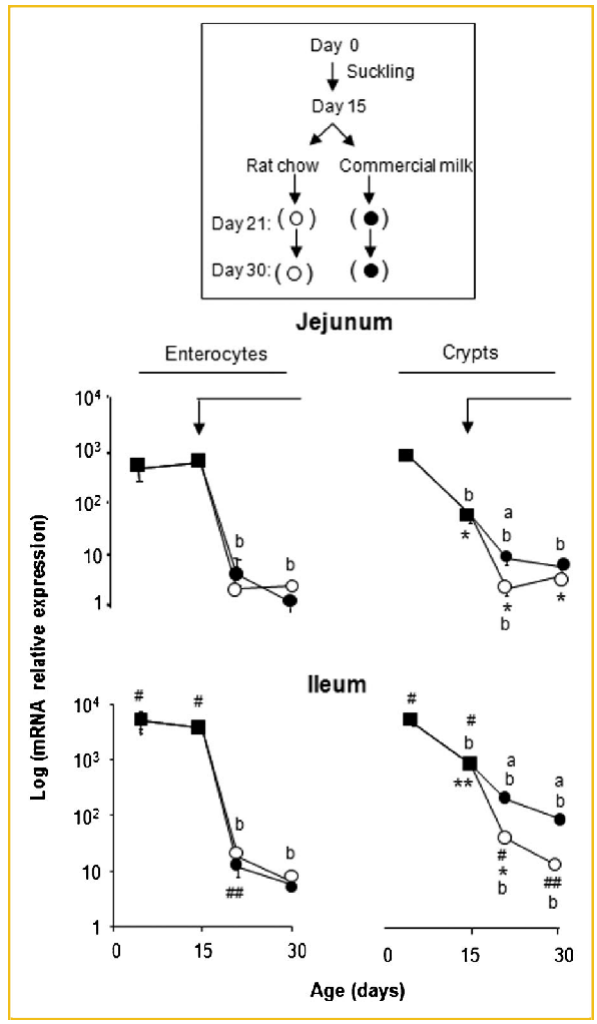


Fig. 6. Effect of the diet on intestinal Dab2 mRNA abundance. 15-day-old pups were weaned onto either rat chow or commercial milk diet as shown in the insert. Enterocytes- and crypts-enriched fractions were obtained from jejunum and ileum of 5, 15, 21, and 30 day-old rats. RT-PCR was performed on total RNA obtained from each fraction. The mRNA levels measured in jejunal enterocytes of 30-day-old rats weaned onto commercial milk were set at 1. The values represent means SEM of arbitrary units of mRNA levels and are plotted on a logarithmic scale. The treatment was repeated four times. One-way ANOVA showed an effect of maturation on enterocytes and crypts Dab2 mRNA ( $P < 0.001$) and of diet on crypts Dab2 mRNA ( $P < 0.001$). Newman-Keul's test: $^{\omega}P < 0.001$, $^{\omega\omega}P < 0.01$ crypts versus enterocytes in either jejunum or ileum; $^{\#}P < 0.001$, $^{\#\#}P < 0.01$ ileum versus jejunum; $^{a}P < 0.001$ comparisons between commercial milk and rat chow; $^{b}P <$ 0.001 as compared with 5-day-old rats.

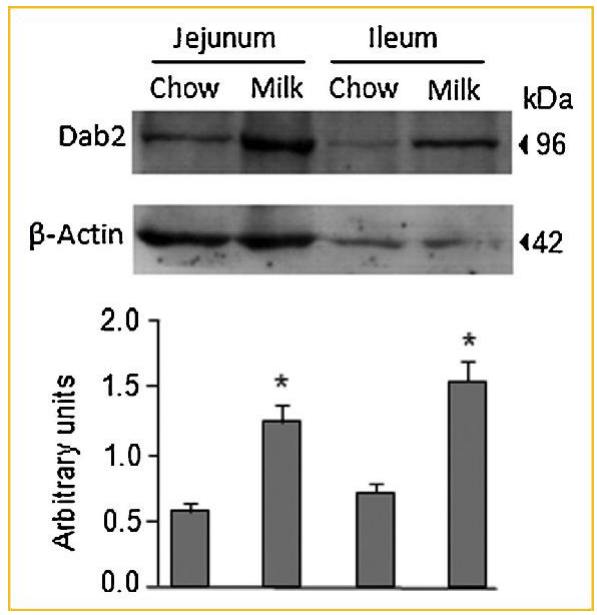


Fig. 7. Effect of the diet on intestinal Dab2 protein abundance. Apical membranes were obtained from enterocytes isolated from jejunum and ileum of the 21-day-old rats weaned onto either rat chow or commercial milk diet. Western assays were carried out as indicated in Figure 5. The exposure time was 30 s. Histograms represent the relative abundance of Dab2 protein in the apical membranes. Means SEM. Student's *t*-test $^{\omega}P < 0.001$, as compared with rat chow. The blot is representative of three Western blots.