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Hepatocyte nuclear factor 1α and β control terminal differentiation and cell fate commitment in the gut epithelium

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SUMMARY

The intestinal epithelium is a complex system characterized by massive and continuous cell renewal and differentiation. In this context, cell-type-specific transcription factors are thought to play a crucial role by modulating specific transcription networks and signalling pathways. Hnf1α and β are closely related atypical homeoprotein transcription factors expressed in several epithelia, including the gut. With the use of a conditional inactivation system, we generated mice in which *Hnf1b* is specifically inactivated in the intestinal epithelium on a wild-type or *Hnf1a*$^{-/-}$ genetic background. Whereas the inactivation of *Hnf1a* or *Hnf1b* alone did not lead to any major intestinal dysfunction, the concomitant inactivation of both genes resulted in a lethal phenotype. Double-mutant animals had defective differentiation and cell fate commitment. The expression levels of markers of all the differentiated cell types, both enterocytes and secretory cells, were affected. In addition, the number of goblet cells was increased, whereas mature Paneth cells were missing. At the molecular level, we show that Hnf1α and β act upstream of the Notch pathway controlling directly the expression of two crucial components: *Jag1* and *Atoh1*. We demonstrate that the double-mutant mice present with a defect in intestinal water absorption and that Hnf1α and β directly control the expression of *Slc26a3*, a gene whose mutations are associated with chloride diarrhoea in human patients. Our study identifies new direct target genes of the Hnf1 transcription factors and shows that they play crucial roles in both defining cell fate and controlling terminal functions in the gut epithelium.

KEY WORDS: Gut epithelium, Hnf1, Commitment, Differentiation, Mouse

INTRODUCTION

The intestinal epithelium is a complex and dynamic system characterized by a remarkably high degree of cell renewal that requires a tightly controlled coordination of proliferation, migration, differentiation and cell death. In fact, the epithelial cells that are lost from the tip of the villi need to be replaced at a constant and equal rate by cell proliferation in the crypts of Lieberkühn. This leads to a continuous vectorial cell migration from the stem cell compartment at the base of the crypt to the tip of the villus, which is composed of differentiated cells. Villi are populated by three differentiated cell types comprising enterocyte, enteroendocrine and goblet cells, whereas a fourth cell type, the Paneth cell, resides at the bottom of the crypts in the small intestine (reviewed by Sancho et al., 2003; van der Flier and Clevers, 2009).

Several studies have indicated that the Wnt pathway drives the proliferation and maintenance of pluripotent intestinal progenitor cells that, in turn, employ the Notch pathway to make the binary choice between absorptive or secretory cell lineage differentiations. If the Notch pathway is activated in a progenitor cell, it will express Hes1 and differentiate into enterocytes. Conversely, if a precursor cell expresses Atoh1 instead, it will differentiate into one of the three secretory cell lineages – enteroendocrine, goblet or Paneth cells (Jensen et al., 2000; Yang et al., 2001). Cell-type-specific transcription factors might play a crucial role in this dynamic system by modulating the expression of effectors of signalling pathways that in turn are specifically activated or repressed in a cell-type-specific manner. In addition, they could also direct the expression of cell-specific genes that contribute to the function of terminally differentiated cells. In this context, the study of the function of transcription factors offers a unique perspective to understand these complex developmental processes.

Hepatocyte nuclear factor 1α and β (*Hnf1a* and *Hnf1b*, formerly known as *Tcf1* and *Tcf2*) genes encode for two closely related dimeric divergent homeobox proteins. Initially discovered in liver, Hnf1 proteins were subsequently shown to be expressed in the epithelia of several organs including pancreas, kidney and intestine. During mouse development, Hnf1β is first expressed in the extra-embryonic visceral endoderm and the primitive gut, whereas Hnf1α is expressed later during hepatic, pancreatic, renal and intestinal organogenesis in combination with Hnf1β (Cereghini, 1996). The study of the phenotype of mice carrying mutations in these genes has shown that Hnf1α and β play important roles in the function and/or differentiation of epithelia. In fact, *Hnf1a*-deficient mice (*Hnf1a*$^{-/-}$) are born normally, but suffer from hepatic, pancreatic and renal functional defects (Pontoglio et al., 1996; Pontoglio et al., 1997; Pontoglio et al., 1998; Lee et al., 1998; Pontoglio et al., 2001). By contrast, germ-line *Hnf1b*-deficiency is embryonic lethal because of

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defective differentiation of extra-embryonic visceral endoderm (Barbacci et al., 1999; Coffinier et al., 1999). Inactivation of *Hnf1b* demonstrated that this factor plays a crucial role in the differentiation/morphogenesis of the pancreas (Haumaitre et al., 2005), liver (Lokmane et al., 2008) and bile ducts (Coffinier et al., 2002), and in the maintenance of renal tubular structures (Gresh et al., 2004). In humans, patients carrying mono-allelic mutations in *HNF1A* or *HNF1B* suffer from maturity onset diabetes of the young (MODY type 3 and 5, respectively) and renal dysfunctions. Furthermore, bi-allelic inactivation of *Hnf1a* is frequently observed in hepatic adenomas (Bluteau et al., 2002). All of these observations indicate that HNF1α and β are involved in the differentiation program of epithelial structures in several organs including liver, kidney and pancreas. Interestingly, these two proteins are also expressed in the intestinal epithelium but little is known about their function in this organ. To date, several studies have demonstrated that these transcription factors activate the expression of a number of intestinal genes including *Fabp1*, *LPH*, *Cftr* and *Glc-6-Pase*, which are expressed in differentiated enterocytes (Mouchel et al., 2004; Gautier-Stein et al., 2006; Bosse et al., 2007). An in vitro study demonstrated that Hnf1α and β control the transcription of *Dpp4*, another marker of differentiated enterocytes (Erickson et al., 2000). Hnf1α and β bind DNA with the same sequence specificity and can form homo- or heterodimers. The gene duplication event at the origin of the Hnf1 family had been selected during evolution with the emergence of the first vertebrates. The fact that these two genes share the same DNA binding specificity and overlapping expression pattern, together with their ability to form heterodimers, suggests that these proteins could play complementary roles. The phenotypic characterization of single- and double-gene inactivation provides a very helpful strategy to understand the dual nature of their genetic program.

In the present study, we investigated the role of Hnf1α and β in the intestinal epithelium. Although the single *Hnf1b* mutant does not present any severe dysfunction, mice with the intestinal inactivation of *Hnf1b* in a context deficient for *Hnf1a* (double mutant $Hnf1a^{-/-}; Hnf1b^{\Delta intestine}$) survive only few weeks after deletion. We demonstrate here that Hnf1α and β participate in both cell fate commitment and differentiation programs in the intestine. In fact, we observed an increase in the number of goblet cells, a decrease in mRNA levels of several terminal differentiated markers of both enterocytes and secretory cell types and a lack of mature Paneth cells. We further demonstrate that Hnf1α and β control the expression of *Jag1*, *Atoh1* and *Cdx2* genes involved in cell commitment and differentiation.

Finally, we demonstrate that the double-mutant mice show a defect in intestinal water absorption that might be responsible for their death. We show that Hnf1α and β directly control the expression of *Slc26a3*, a gene whose mutations are responsible for a life-threatening form of congenital chloride diarrhoea in human patients. These studies identify crucial direct target genes of the Hnf1 transcription factors and elucidate their function in the gut epithelium.

MATERIALS AND METHODS

Generation of intestine-specific *Hnf1b* knockout mice and animal treatment

The generation of $Hnf1b^{flox/flox}$ and Vil-CreER^{T2} mice was previously described (Coffinier et al., 2002; el Marjou et al., 2004). All experiments involving animals were carried out in accordance with French government regulations. *Cre* recombinase was activated by a single injection of tamoxifen (TAM) solution (50 mg/kg; Inalco). We crossed the $Hnf1a^{+/+}; Hnf1b^{flox/flox}$ and Vil-CreER^{T2} mouse lines in order to obtain the single *Hnf1b* mutant mouse model following tamoxifen injection. The $Hnf1a^{+/+}; Hnf1b^{flox/flox}$ animals without the *Cre* recombinase transgene were used as control animals. Then, we crossed $Hnf1a^{+/-}; Hnf1b^{flox/flox}$ and Vil-CreER^{T2} in order to obtain the concomitant loss of Hnf1α and β in the intestinal epithelium ($Hnf1a^{-/-}; Hnf1b^{\Delta intestine}$). The $Hnf1a^{-/-}; Hnf1b^{flox/flox}$ animals without the *Cre* recombinase transgene were our single *Hnf1a* mutant animals. We intraperitoneally injected mice between 4 and 5 weeks after birth with a single dose of tamoxifen and we analyzed the intestinal phenotype of double-mutant mice 2 weeks after injection. All of the mouse strains indicated were treated with the same protocol. Mice were sacrificed by cervical dislocation and the intestine collected.

Real-time PCR analysis

Intestinal segments from animals were dissected and washed with ice-cold PBS. For small intestine samples, segments (2 cm) from each of the intestinal regions (duodenum, jejunum and ileum) were mixed and homogenized in Trizol. Total RNA was isolated with Trizol reagent (Life Technologies) followed by purification on RNeasy columns (Qiagen). Complementary DNA (cDNA) synthesis was performed according to manufacturer's instructions (SuperScript Kit; Invitrogen). For RT-PCR, cDNA and primers were mixed with SYBR Green RT-PCR Master Mix (Applied Biosystems) and then assayed in an ABI Prism 7000 Sequence Detection System according to manufacturer's instructions. The relative level of each mRNA was calculated using the standard curve method and normalized to the corresponding *Gapdh* RNA levels. Five independent RNA samples were used for each group (e.g. one group was $Hnf1a^{-/-}; Hnf1b^{\Delta intestine}$ mice) and triplicate reactions of each sample were used to derive the normalized expression level for each gene. The average normalized expression levels were used to determine the average expression level within a group and for statistical comparisons between groups (n=5 for each group). ANOVA and t-tests were performed to measure variations in gene expression between groups.

Oligonucleotides

The primers for each gene analyzed were designed with Primer Express software (Applied Biosystems) and are listed in Table S1 in the supplementary material.

Tissue sample preparation and immunohistochemistry

The intestinal tract was dissected as a whole and gently washed with cold PBS to remove any faecal content. The small intestine was rolled up as a 'Swiss roll' and fixed in paraformaldehyde 4% for 1 hour at room temperature. Specimens were then washed in cold PBS and included in agarose 4%. They were sectioned with vibratome (100 μm). For the detection of Hnf1α, we used also tissues embedded in Optimum Cutting Temperature compound (Tissue-Teck), frozen and sectioned on a cryostat at 8 μm. For the detection of Hnf1β, immunohistochemistry was performed on a paraffin wax-embedded rolled intestine. Antigen retrieval was performed by incubating the tissue sections in 10 mM sodium citrate, pH 6.0, for 15 minutes at 95°C. For immunohistochemistry, sections were sequentially incubated with biotinylated secondary antibodies (Jackson ImmunoResearch), peroxidase-conjugated streptavidin and DAB substrate (both Vector Labs). The following antibodies were used: rabbit anti-Hnf1α-TC284 (1:500) (Chouard, 1997), monoclonal anti-Hnf1β (1:100; clone 3.12) (Chouard et al., 1997), rabbit anti-ezrin (a gift from D. Louvard, Institut Curie, Paris), rabbit anti-chromogranin A+B (1:50; Progen), UEA1-FITC (1:50; Sigma), rabbit anti-lysozyme (1:200; DakoCytomation).

In situ hybridization

In situ hybridization (ISH) was carried out on 7 μm slices of the paraffin-embedded specimens. Partial Atoh1 cDNA was obtained by RT-PCR using the primers Atoh1F_IS (CCGTCAAAGTACGGGAACAG) and Atoh1R_IS (GAGTAACCCCCAGAGGAAGC), and then subcloned into TOPO cloning vector (Invitrogen). Digoxigenin-labelled RNA probe was prepared by in vitro transcription with the Digoxigenin RNA Labeling Kit (Roche, France) using T7 or Sp6 RNA polymerases. Sections were incubated overnight at 68°C in prehybridization buffer containing 200 ng/ml of digoxigenin-labelled RNA probe. Immunodetection of the hybridized probe was carried out using an anti-digoxigenin antibody (1:2000, Roche).

Chromatin immunoprecipitation (ChIP)

Nuclei were prepared from small intestine of control and *Hnf1a*$^{-/-}$; *Hnf1b*$^{\Delta intestine}$ mice. Chromatin preparation and ChIP were performed as previously described (Gresh et al., 2004). ChIP experiments were performed with rabbit anti-Hnf1α-TC284 (1:500, Chouard, 1997) or rabbit anti-Hnf1β (1:200, clone H-85, Santa Cruz Biotechnology) antibodies. Primers were designed using PrimerExpress 2.0 software. For primer sequences, see Table S1 in the supplementary material. Quantification of precipitated DNA fragments was carried out on an ABI Prism 7000 Sequence Detection System using SYBR Green in triplicates. Relative fold-enrichment of DNA fragments was calculated using the following formula: $(ChIP_{target}/ChIP_{normalizer})/(input_{target}/input_{normalizer})$. As normalizer, we used a DNA fragment lacking any Hnf1 site, located in the first intron of the aortic smooth muscle alpha actin 2 (*Acta2*) gene.

RESULTS

Generation of *Hnf1* mutant mice and *Hnf1* expression profile in the intestinal epithelium

To study the function played by Hnf1β in adult intestinal epithelium, we crossed mice carrying a floxed allele of *Hnf1b* (Coffinier et al., 2002) with a strain carrying a tamoxifen-inducible *Cre* recombinase specifically expressed in intestinal epithelium (Vil-CreER^{T2} mice) (el Marjou et al., 2004). In this strain, *Cre* recombinase activity can be induced in all the cell types, including intestinal stem cells, by intraperitoneal injections of tamoxifen, leading to a permanent inactivation of floxed alleles in all intestinal epithelial cells even several weeks after deletion (el Marjou et al., 2004). Our results showed that, after recombination, the loss of Hnf1β expression in the intestine (Hnf1β$^{\Delta intestine}$) did not elicit any overt phenotype, at least over a period of 20 weeks, in spite of a drastically decreased expression of Hnf1β. Conversely, we have previously demonstrated that the loss of Hnf1α (owing to a germ-line-null inactivation) results in liver, kidney and pancreatic dysfunction, whereas the intestine did not present with any particularly severe phenotype (Pontoglio et al., 1996; Shih et al., 2001). The lack of any prominent intestinal phenotype in any of the single-mutant mice raised the possibility that Hnf1α and β could compensate for each other when inactivated independently. As both transcription factors bind to the same DNA sequences, they could play complementary roles during intestinal cell commitment and differentiation. To address the extent of the possible redundancy between these two transcription factors, we analyzed the intestinal phenotype of *Hnf1a*$^{-/-}$; *Hnf1b*$^{\Delta intestine}$ mice (double-mutant mice). In our present study, we principally focused our attention on the small intestine because our Cre inactivation system was particularly efficient in deleting *Hnf1b* in this compartment (el Marjou et al., 2004).

In both the single- and the double-mutant mice, *Hnf1b* expression levels were dramatically compromised (90% reduction, Fig. 1A). Conversely, the lack of *Hnf1a* did not affect *Hnf1b* mRNA levels in the small intestine of single Hnf1α mutant mice (Fig. 1A). Similarly, the lack of *Hnf1b* did not affect *Hnf1a* mRNA levels in the small intestine of *Hnf1b*$^{\Delta intestine}$ mice (see Fig. S1A in the supplementary material). Previous expression studies have revealed that both Hnf1α and β are expressed along the crypt-villus axis in the gut epithelium. These studies were performed both at the protein and mRNA level for Hnf1α (Boudreau et al., 2002; Fang et al., 2006; Serfas and Tyner, 1993) but only at the mRNA level for Hnf1β (Fang et al., 2006; Serfas and Tyner, 1993). Moreover, it was reported that Hnf1α protein levels increased after the suckling weaning transition (Boudreau et al., 2002), whereas in another study, its mRNA decreased during this transition phase (Fang et al., 2006). Thus, owing to the differences observed in expression and to the lack of Hnf1β protein profile, we decided to investigate the Hnf1α and β protein expression patterns in detail at the histological level in 4- to 5-week-old mice. This temporal phase corresponded to the window of time in which we induced Hnf1β inactivation. Immunofluorescence with antibodies specific for Hnf1α revealed that this protein was expressed in all of the nuclei along the crypt-villus axis in the small intestine (Fig. 1B). Depending on the experimental conditions employed, the cells at the very bottom of the crypts gave signals of different intensity. In particular, on vibratome sections (100 μm; Fig. 1B) this signal was much paler than that on sections obtained with a cryostat microtome (see Fig. S1B in the supplementary material). Similarly, immunohistochemistry analysis revealed that Hnf1β was expressed along the crypt-villus axis in the small intestine (Fig. 1D). The specificity of the staining was confirmed by the absence of any signal in mutant mice (Fig. 1C,E; see also Fig. S1C in the supplementary material). This demonstrated that our double-mutant mouse model is completely inactivated for both Hnf1 transcription factors in the gut epithelium. To assess the extent of the tissue-

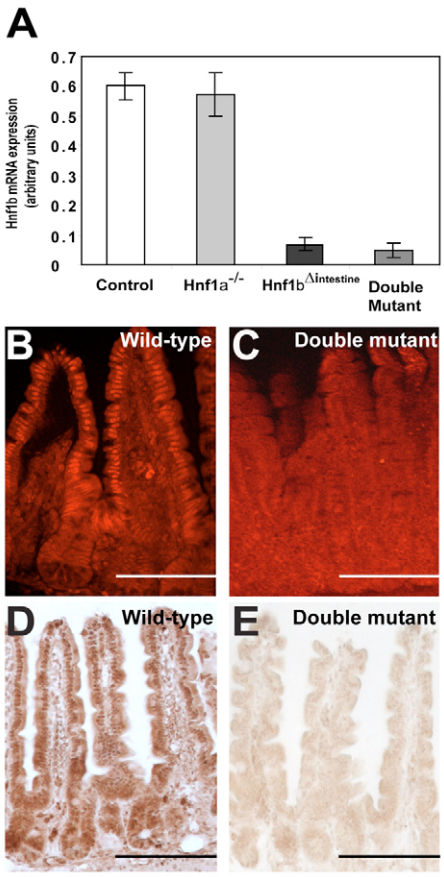


Fig. 1. Expression profile of Hnf1α and β in the intestinal epithelium. (**A**) Expression levels of *Hnf1b* mRNA in *Hnf1a*$^{-/-}$, *Hnf1b*$^{\Delta intestine}$ and *Hnf1a*$^{-/-}$; *Hnf1b*$^{\Delta intestine}$ double mutants. *Hnf1b* expression was not affected in the small intestine of *Hnf1a*$^{-/-}$ mice. *Hnf1b* expression was dramatically reduced (90%) in single *Hnf1b*$^{\Delta intestine}$ mice and double-mutant mice. (**B**,**C**) Indirect immunofluorescence detection of Hnf1α in small intestine of wild-type (B) and double-mutant (C) mice on jejunum sections. Staining shows a nuclear localization of Hnf1α all along the crypt-villus axis in wild type. (**D**,**E**) Immunohistochemistry analysis for Hnf1β expression revealed the same expression pattern all along the crypt-villus axis in wild type. No signal was observed in double-mutant mice (C,E). Scale bars: 100 μm.

specific inactivation of *Hnf1b*, we performed PCR to amplify the recombined allele as previously described (Coffinier et al., 2002). We analyzed several tissues and recombination was observed only in the intestine (see Fig. S1D in the supplementary material). Our results showed that the loss of Hnf1β after a single injection of tamoxifen in *Hnf1a*$^{-/-}$; *Hnf1b*$^{flox/flox}$ mice resulted in a rapid demise of the animals. More than 50% of the double-mutant animals died within 2 weeks after the deletion, whereas only a few of them (less than 10%) survived to 8 weeks (see Fig. S1E in the supplementary material). Thus, we tried to unravel the cause for the rapid death of double-mutant animals.

Hnf1 controls enterocyte terminal differentiation

To assess the function of Hnf1α and β in the gut epithelium we monitored cell fate commitment and differentiation programs. Upon histological analysis, small intestine of double-mutant animals appeared with a normal crypto-villar architecture (Fig. 1C,E; Fig. 2C). Immunofluorescence with anti-ezrin antibodies (specific for enterocytes) showed a normal signal uniformly localized at the brush border membranes of enterocytes all along the villus both in control (Fig. 2A) and single- or double-mutant mice (Fig. 2B,C). In order to assess the degree of differentiation of the intestinal epithelium, we decided to investigate the expression of markers of enterocyte differentiation. Dpp4, an aminopeptidase localized at the brush border of enterocytes, was shown to be activated by Hnf1α and β in transfection studies (Erickson et al., 2000). When we analyzed the expression levels of *Dpp4*, we observed that the mRNA level of the enzyme was strongly downregulated in double-mutant animals (80% reduction) and significantly downregulated in single Hnf1α mutant mice (60% reduction; Fig. 2D), indicating that this gene is an in vivo target gene of Hnf1 and that the differentiation program of enterocytes could be altered in our mouse mutant. In addition, *Cdx2*, a transcription factor involved in intestinal development and enterocyte maturation (Suh and Traber, 1996; Mutoh et al., 2005; Grainger et al., 2010), was downregulated in double-mutant mice (37% reduction) and slightly downregulated in single Hnf1α mutant mice (16% reduction; Fig. 2D). To investigate the relationship between Cdx2 and Hnf1 transcription factors, we took advantage of an in silico approach based on a previously described position-weight matrix (Tronche et al., 1997) (M.P. and S.G., unpublished). This approach revealed that *Cdx2* contained a putative binding site for Hnf1 that is well conserved in several vertebrate species. This site, located more than 10 kb upstream of the transcription site, is indeed bound by Hnf1α by chromatin immunoprecipitation (ChIP; Fig. 2E). These results confirm that Hnf1 transcription factors do have a role in the control of enterocyte differentiation and identify a new in vivo target gene.

Hnf1 double-mutant mice have abnormal cell fate commitment and defective terminal differentiation in secretory cell lineages

Normally, goblet cells are present in a rostrocaudal gradient all along the length of the small intestine with the highest level in the ileum. Histological sections stained with UEA1, a lectin specific for mucin-expressing cells, revealed an increased number of goblet cells all along the length of the small intestine in double-mutant mice. We observed an increased number of goblet cells both in the ileum (Fig. 3F compared with 3D) and in the duodenum (Fig. 3C compared with 3A and 3G). By contrast, no significant difference was observed in the single Hnf1α (Fig. 3B compared with A; Fig. 3E compared with 3D) or Hnf1β intestinal single mutant (data not shown). To determine whether the goblet lineage abnormalities in mice

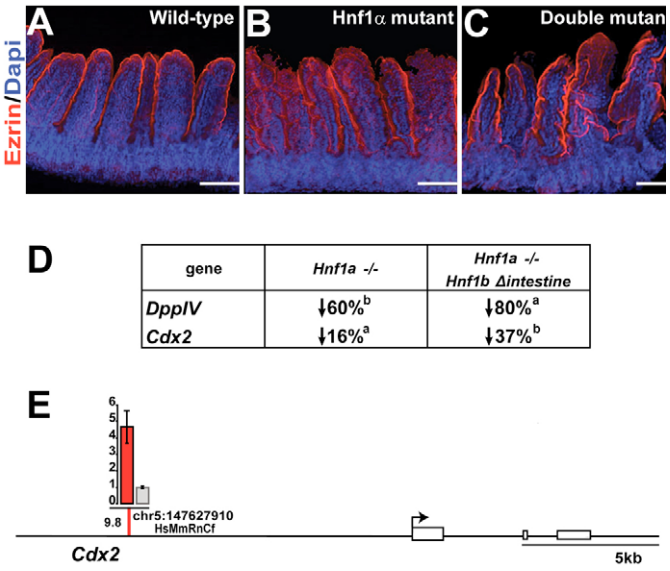
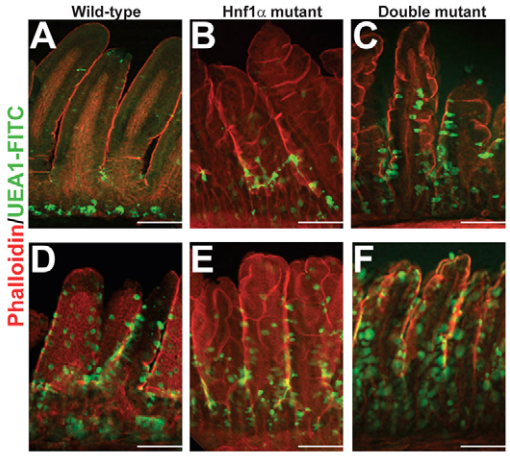
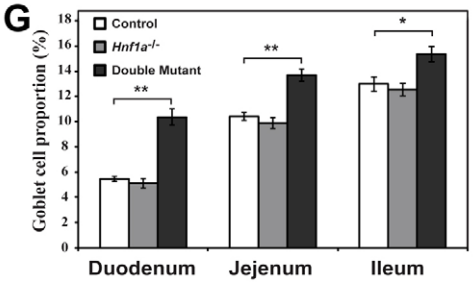


Fig. 2. Analysis of enterocytes in the intestinal epithelium. (**A-C**) Immunofluorescence detection marker for enterocytes in wild-type (A), *Hnf1a*$^{-/-}$ (B) and double-mutant animals (C). Staining for ezrin, a specific marker for enterocytes, indicates that it is normally expressed in wild-type and mutant mice in sections of the ileum. (**D**) Gene expression of two intestinal genes in *Hnf1a*$^{-/-}$ and double-mutant mice. Values are the ratios (wild-type/*Hnf1a*$^{-/-}$ and wild-type/double-mutant mice) of normalized expression levels. Negative values indicate reduced gene expression in mutant intestines compared with wild type. Significant differences in expression levels are indicated ($^{a}P<0.05$; $^{b}P<0.01$). (**E**) Predicted in silico Hnf1 binding site in the *Cdx2* gene was tested in ChIP experiments. Representative scheme of the Hnf1 binding site selected for the degree of conservation and the statistical HMM score. The relative binding for the DNA fragment upon immunoprecipitation of *Hnf1a* and *Hnf1b* is represented. PCR experiments were performed in triplicates and the standard errors of these quantifications are shown as error bars. The chromosomal position of the site is indicated, as well the species where the site had been found conserved. Cf, *Canis familiaris*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*. Histograms depicted in red and grey represent chromatin immunoprecipitation enrichments for Hnf1α and Hnf1β, respectively. Scale bars: 50 μm.

were due to abnormal proliferation, we performed double immunostaining with proliferative anti-Histone-H3 phosphorylated on Ser10 and cell-specific UEA1 markers. However, our results did not reveal any significant difference in the extent of proliferation of this cell type (data not shown). The expression levels of *Klf4*, a transcription factor involved in differentiation of goblet cells, and of *Muc3*, a goblet-specific gene, were moderately upregulated in double-mutant mice (1.2- and 2.0-fold, respectively; Fig. 3H). This paralleled the significant increase of goblet cells observed in the epithelium. On the contrary, other specific markers for goblet cells such as *Muc2*, *gob-4* (*Agr2* – Mouse Genome Informatics) and *gob-5* (*Clca3* – Mouse Genome Informatics) were strongly downregulated in *Hnf1a*$^{-/-}$; *Hnf1b*$^{\Delta intestine}$ mice (75%, 64% and 81% reduction, respectively; Fig. 3H). This indicated that goblet cells, although present in excess, failed to completely activate their cell-type specific genetic program or they basically reduced gene transcription to be able to normalize with the increased cell number. These alterations in cell fate commitment and/or differentiation were observed only when both factors were inactivated. Moreover, the



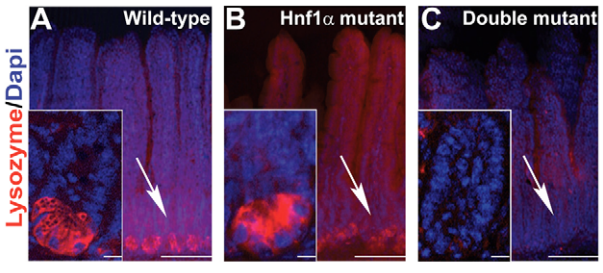


H

gene	*Hnf1a -/-*	*Hnf1a -/- Hnf1b Δintestine*
Klf4	1.1	**1.2**[a]
Muc3	**1.2**[a]	**2.0**[d]
Muc2	1.0	**↓75%**[c]
Gob4	1.0	**↓64%**[b]
Gob5	**↓50%**[b]	**↓81%**[b]
Elf3	1.0	1.0
Hnf4a	1.0	1.1

Fig. 3. Analysis of goblet cells in the intestinal epithelium. (**A-F**) Staining with UEA1, a marker for goblet cells, showed that goblet cells were normally present in *Hnf1a*$^{-/-}$ mice (B,E) compared with wild-type animals (A,D) but their number was increased in double-mutant mice (C,F). The increased number was observed both in the duodenum (A-C) and in the ileum (D-F). (**G**) Quantification of goblet cells number in single *Hnf1a*$^{-/-}$ and double-mutant mice. Cell-type proportion is represented by the percentage calculated on the total number of epithelial cells. Error bars represent standard errors (n=3 animals per genotype). The number of goblet cells was statistically significantly increased in respect to the control animals, particularly in duodenum. *, P<0.001; **, P<0.0001; ANOVA test. (**H**) Gene expression of genes that control goblet cell differentiation in *Hnf1a*$^{-/-}$ and double-mutant mice. Expression analysis was performed on a mix of small intestine segments from each of the intestinal regions (duodenum, jejunum and ileum). Values are the ratios (wild-type/ *Hnf1a*$^{-/-}$ and wild-type/double-mutant mice) of normalized expression levels. Negative values indicate reduced gene expression in mutant intestines compared with wild-type. Significantly different expression levels are indicated in bold. [a]P<0.05; [b]P<0.01; [c]P<0.001; [d]P<0.0001. Scale bars: 100 μm.

expression of Elf3, a transcription factor involved in both enterocyte and goblet cell differentiation (Ng et al., 2002), was not affected (Fig. 3H). Hnf4α, a transcription factor essential for development of the colon, regulates goblet-cell maturation (Garrison et al., 2006). Interestingly, Hnf1α and Hnf4α have been described to regulate each other (Odom et al., 2004). To assess the possible contribution



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gene	*Hnf1a -/-*	*Hnf1a -/- Hnf1b Δintestine*
Cryp1	1.0	**↓82%**[d]
Lyzs	↓9%	**↓87%**[d]
Gfi1	1.0	**↓67%**[c]
Sox9	1.0	1.1[a]
Msi-1	1.0	↓9%
cMyc	1.0	↓9%
Cycd1	↓9%	1.0
Atg16l1	1.0	1.0
Atg7	1.0	↓9%[a]

Fig. 4. Analysis of Paneth cells in the intestinal epithelium. (**A-C**) Immunodetection of lysozymes revealed an apparent loss of Paneth cells on jejunum sections of double-mutant mice (C) compared with *Hnf1a*$^{-/-}$ mice (B) or the controls (A). Arrows indicate the bottom of the crypts in sections of jejunum. Magnification of the crypts is shown in the insets. (**D**) Gene expression of genes that control Paneth cell differentiation in *Hnf1a*$^{-/-}$ and double-mutant mice. Values are the ratios (wild-type/ *Hnf1a*$^{-/-}$ and wild-type/double-mutant mice) of normalized expression levels. Negative values indicate reduced gene expression in mutant intestines compared with wild-type. Significantly different expression levels are indicated in bold. [a]P<0.05; [c]P<0.001; [d]P<0.0001. Scale bars: 100 μm; insets 5 μm.

of Hnf4α to the phenotype observed, we measured the expression levels of *Hnf4a* in our mutant animals. Both the single *Hnf1a* mutant and the double-mutant mouse showed no differences in the expression of Hnf4α (Fig. 3H). In our mutants, enteroendocrine cell differentiation appeared to occur normally as no difference in the number of chromogranin A-positive cells was observed in the small intestine of the double mutant compared with the control animals (data not shown). Conversely, a drastic defect in Paneth cell differentiation was observed as no apparent mature cells were visible at the bottom of the crypts of *Hnf1a*$^{-/-}$; *Hnf1b*$^{\Delta intestine}$ mice (Fig. 4C versus 4A). No significant difference in mature Paneth cells was observed in single Hnf1α or β mutant mice (Fig. 4B versus A; data not shown). Furthermore, markers specific for Paneth cells (*Cry1* and *Lyz2*) were strongly downregulated (respectively 82% and 87% reduction) in the small intestine of *Hnf1a*$^{-/-}$; *Hnf1b*$^{\Delta intestine}$ mice (Fig. 4D). This correlated with the observed drastic disappearance of immunoreactive positive cells at the bottom of the crypts of double-mutant mice. Interestingly, the transcription factor *Gfi1*, required for intestinal goblet and Paneth cell differentiation (Shroyer et al., 2005), was significantly reduced in double-mutant animals (67% reduction). On the contrary, *Sox9 and Msi1*, genes required for Paneth cell differentiation in the intestinal epithelium (Bastide et al., 2007; Mori-Akiyama et al., 2007; Murayama et al., 2009), were not affected in *Hnf1a*$^{-/-}$; *Hnf1b*$^{\Delta intestine}$ mice (Fig. 4D). It is known that the Paneth cell maturation program is controlled by the Wnt pathway in the crypt (Andreu et al., 2008; van Es et al., 2005a). However, when we verified the expression levels of *c-Myc* and *Cycd1* (two important Wnt target genes), we did not detect differences in their levels (Fig. 4D), suggesting that this pathway is

A

gene	*Hnf1a -/-*	*Hnf1a -/- Hnf1b Δintestine*
Notch2	↓9%	**↓47%**[b]
Jag1	↓9%	**↓58%**[b]
Atoh1	**↓16%**[b]	**↓77%**[d]
Hes1	↓9%	**↓50%**[a]
Hes5	1.0	**↓63%**[c]
Hes6	1.0	**↓28%**[b]

B

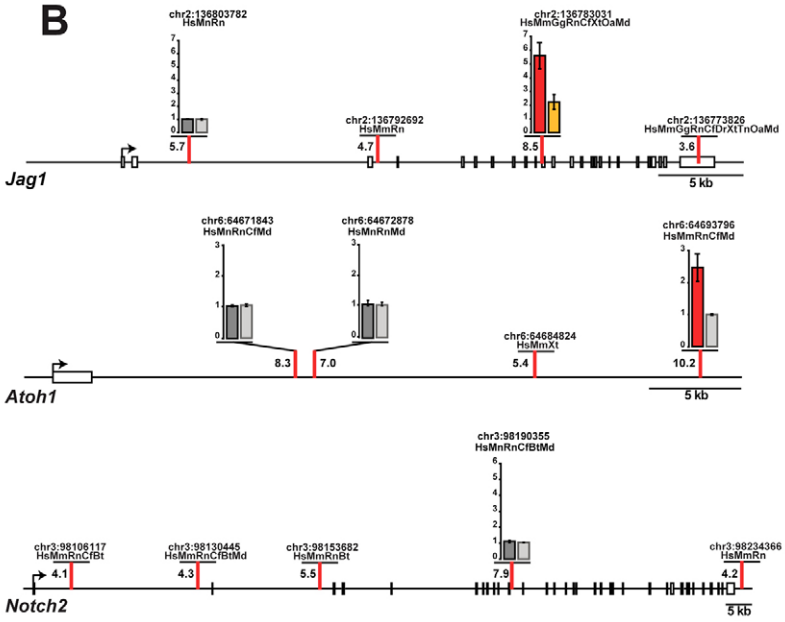

Fig. 5. Analysis of Notch pathway components in the intestinal epithelium. (**A**) Gene expression of Notch pathway component genes in *Hnf1a*$^{-/-}$ and double-mutant mice. Values are the ratios (wild-type/*Hnf1a*$^{-/-}$ and wild-type/double-mutant mice) of normalized expression levels. Negative values indicate reduced gene expression in mutant intestines compared with wild-type. Significantly different expression levels are indicated in bold. [a]P<0.05; [b]P<0.01; [c]P<0.001; [d]P<0.0001. (**B**) Predicted in silico Hnf1 binding sites in *Jag1*, *Atoh1* and *Notch2* genes were tested in ChIP experiments. Representative scheme of Hnf1 binding sites selected for their degree of conservation and their statistical HMM score. The relative enrichment for each DNA fragment upon immunoprecipitation of *Hnf1a* and *Hnf1b* is represented. PCR experiments were performed in triplicates and the standard errors of these quantifications are shown as error bars. The chromosomal position for each site is indicated, as well the species where the site had been found conserved. Bt, *Bos taurus*; Cf, *Canis familiaris*; Dr, *Danio rerio*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Md, *Monodelphis domestica*; Mm, *Mus musculus*; Oa, *Ornithorhynchus anatinus*; Rn, *Rattus norvegicus*; Xt, *Xenopus tropicalis*. Histograms depicted in red or yellow represent significant binding for Hnf1α or β, respectively, whereas histograms in grey represent nonsignificant enrichment of ChIP.

normally activated and that other genes not necessarily involved in the Wnt pathway might play a key role in the terminal differentiation process of Paneth cells. Paneth cell lifespan is regulated by autophagy, a mechanism in which several genes are involved (Cadwell et al., 2008; Cadwell et al., 2009). To exclude defects in autophagy, we analyzed the expression of two key genes for this process. Our results showed that *Atg16l1* and *Atg7* were not affected in the double-mutant mice (Fig. 4D), suggesting that the disappearance of Paneth cells in our model is probably due to a differentiation defect.

These results indicate that Hnf1α and β play a crucial role both in cell fate commitment represented by the increased number of goblet cells and in secretory cell-type-specific differentiation represented by the incomplete activation of cell-type-specific gene expression.

Hnf1α and β control components of the Notch pathway

Cell fate commitment during the differentiation of intestinal cells occurs in the crypts where both Hnf1α and β are expressed. The number of goblet cells was increased without an increased proliferation rate of goblet precursors suggesting that the two transcription factors might be mechanistically directly involved in cell fate choice. To address this point, we investigated the consequences of Hnf1α and β inactivation in the intestinal epithelium on the intricate transcriptional and signalling network governing the early cell fate decision of all four epithelial lineages found in the small intestine. We focused our attention on the expression of crucial regulators of cell fate commitment and particularly on the effectors of the Notch pathway, a pathway that has been demonstrated to control absorptive/secretory cell fate choice. In this respect, we considered those effectors that are normally expressed in intestine and whose genomic sequences showed a significant enrichment in evolutionarily conserved Hnf1 binding sites. Our results revealed that *Jag1*, *Atoh1* and *Notch2* genes were particularly enriched in putative Hnf1 binding sites that are well conserved in several vertebrate species (Fig. 5B). Indeed, expression of both *Notch2* and *Jag1* was downregulated (47% and 58% reduction, respectively) in *Hnf1a*$^{-/-}$*; Hnf1b*$^{\Delta intestine}$ mice (Fig. 5A). In a similar way, *Atoh1* was highly downregulated (77% reduction) in *Hnf1a*$^{-/-}$*; Hnf1b*$^{\Delta intestine}$ mice and slightly downregulated (16% reduction) in *Hnf1a*$^{-/-}$ mice. To assess the possible defective activation of Notch signalling, we tested the expression of the typical targets represented by the Hes genes. Our results showed that *Hes1*, *Hes5* and *Hes6* expression was downregulated (50%, 63% and 28% reduction, respectively; Fig. 5A).

To verify the possible direct involvement of Hnf1α and β in these transcriptional defects, we performed ChIP with anti-Hnf1α or anti-Hnf1β antibodies. In this way, we assessed the in vivo binding of Hnf1 proteins on several putative Hnf1 binding sites that we selected for their degree of conservation, their genomic position and their statistical Hidden Markov Model (HMM) score. In particular, we selected putative binding sites with HMM ≥5.7 and conserved in at least three species out of fourteen analyzed. Our results showed that sites in *Jag1* and *Atoh1* genes were bound in vivo in chromatin prepared from small intestine of wild-type animals (Fig. 5B). The specificity of our ChIP experiments was supported by the fact that no significant enrichment for all DNA fragments tested was observed in double-mutant animals (data not shown). In particular, our results showed that both Hnf1α and β could bind the site that we identified in the eleventh intron of the *Jag1* genomic sequence (Fig. 5B).

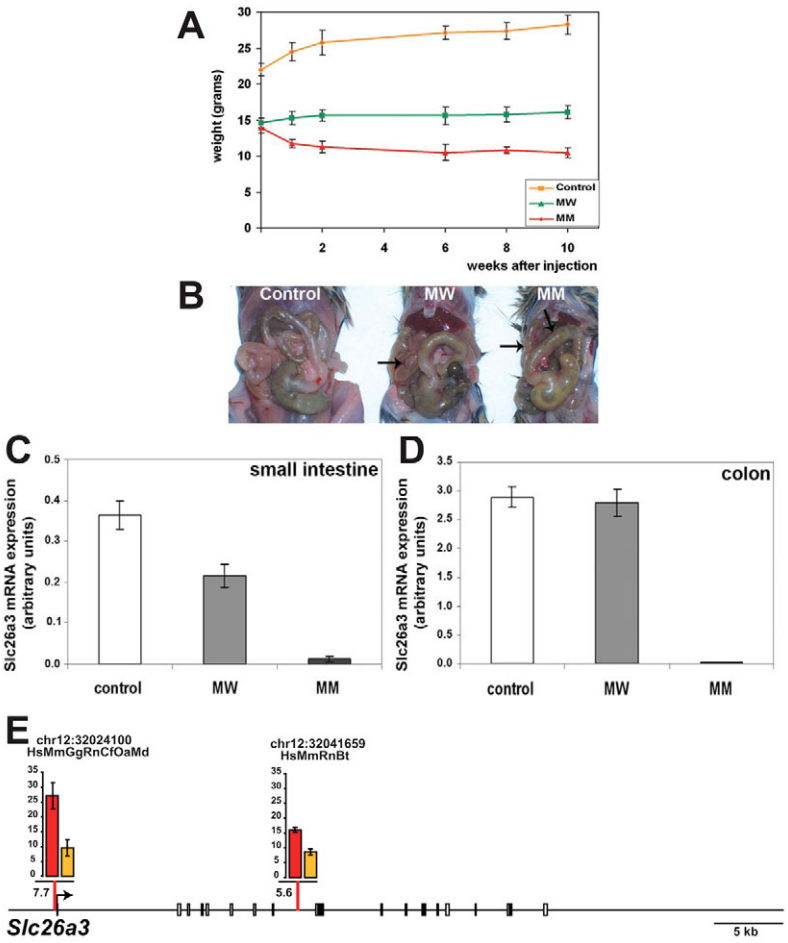

Fig. 6. Hnf1α and β control water absorption in the intestine. (**A**) Average weight values of control, *Hnf1a*$^{-/-}$ (MW) and *Hnf1a*$^{-/-}$*; Hnf1b*$^{\Delta intestine}$ mice (MM). (**B**) Dilatations are visible immediately after death and dissection. Arrows point to the small intestine in MW mice and to both the small intestine and the colon in MM mice. (**C**,**D**) Hnf1α and β directly control *Slc26a3* expression in the intestine. mRNA expression levels of *Slc26a3* in small intestine (C) and colon (D). *Slc26a3* expression was completely abolished in MM mice and slightly reduced in the small intestine of MW mice. (**E**) In vivo binding of Hnf1 proteins to *Slc26a3* sites by ChIP. The relative enrichment for each DNA fragment upon immunoprecipitation of Hnf1α and β is represented. PCR experiments were performed in triplicates and the standard errors of these quantifications are shown as error bars. The chromosomal position for each site is indicated, as well the species where the site had been found conserved. Bt, *Bos taurus*; Cf, *Canis familiaris*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Md, *Monodelphis domestica*; Mm, *Mus musculus*; Oa, *Ornithorhynchus anatinus*; Rn, *Rattus norvegicus*; Xt, *Xenopus tropicalis*. Histograms depicted in red or yellow represent significant binding for Hnf1α or β, respectively whereas histograms in grey represent nonsignificant enrichment of ChIP.

Conversely, only Hnf1α (and not β) could bind a site located in *Atoh1* (30 Kb downstream to the transcriptional start site in the 3′ region of the gene; Fig. 5B). The putative Hnf1 binding site of *Notch2* that we tested was not bound in vivo (Fig. 5B); it is possible that other putative binding sites with a lower HMM score are bound in vivo by Hnf1α and/or β. Evolutionary conserved sites not bound in the intestine are probably involved in the control of these genes in other tissues.

It is known that *Hes1* negatively regulates *Atoh1* (Jensen et al., 2000; Yang et al., 2001). *Hes1* is expressed only in the crypt compartment, whereas *Atoh1* is expressed in progenitors of the secretory lineage, as well as in their corresponding fully differentiated cell types (Jenny et al., 2002; Pinto et al., 2003; van Es et al., 2005b; Yang et al., 2001). The analysis of *Atoh1* expression by in situ hybridization showed that it was dramatically decreased in the mature secretory cells of the villi in the double-mutant animals (see Fig. S2 in the supplementary material).

Hnf1 transcription factors control water absorption

The observed cell fate allocation and differentiation defects could not easily explain the rapid death observed in double-mutant animals. Interestingly, we observed that double-mutant mice presented with a sudden and drastic loss of body weight (20%) during the first week after deletion (Fig. 6A). This correlated with the appearance of signs of severe dehydration in double mutants compared with the single-mutant mice (*Hnf1a*$^{-/-}$) or control littermates (see Fig. S3 in the supplementary material). Remarkably, double-mutant animals presented with a significant distension of the intestine compared with wild-type mice. Loops of intestine appeared fluid-filled, with increased volume of the bowel contents (Fig. 6B). Hnf1α single-mutant mice presented with a slightly distended gut, with a milder phenotype compared with the double-mutant mice (Fig. 6B). Interestingly, a closer inspection of villi in the double mutants (Fig. 2C) showed that their structure appeared less turgid compared with that of control animals (Fig. 2A), suggesting that mutant villi might have defective water absorption capacity. Intestinal water transport is linked to solute transports. In this respect, the currently accepted model indicates that active ion transport sets up local osmotic gradients that, in turn, drive water absorption by local osmosis (reviewed by Loo et al., 2002). Hnf1α and β could directly activate the transcription of specific transporter/exchanger genes involved in intestinal water absorption. Among these genes, we focused our attention on *Slc26a3*, a gene whose mutations are associated to congenital chloride diarrhoea (OMIM 214700). This disease is manifested by enhanced chloride and water loss in stools (Hoglund et al., 1996). The encoded protein is a Cl^-/HCO_3^- exchanger driving NaCl absorption. The murine model of *Slc26a3* knockout recapitulates the human disease as inactivation of the gene resulted in chloride diarrhoea (Schweinfest et al., 2006). On the basis of these observations, we examined expression of *Slc26a3* in control, *Hnf1a*$^{-/-}$ and *Hnf1a*$^{-/-}$*; Hnf1b*$^{\Delta intestine}$ mice. Interestingly, *Slc26a3* expression was completely abolished both in the small intestine and the colon of *Hnf1a*$^{-/-}$*; Hnf1b*$^{\Delta intestine}$ mice (Fig. 6C,D). In single-mutant *Hnf1a*$^{-/-}$ mice, *Slc26a3* expression was reduced (41% reduction) in the small intestine (but not in the colon) with respect to control animals

(Fig. 6C,D). We next tested if this gene could be directly controlled by Hnf1 proteins. Our in silico approach predicted the presence of two Hnf1 putative binding sites, particularly well conserved in several species, in the proximal promoter and in the eighth intron of *Slc26a3* genomic sequence (Fig. 6E). Our results showed that both Hnf1α and β did indeed bind those sites, indicating that these factors play, in concert, a direct and crucial role for *Slc26a3* gene expression.

DISCUSSION

The differentiation of the four distinct cell types that populate the villi is controlled by the interplay between signalling pathways and cell-type-specific transcription factors. In the present study, we demonstrate that Hnf1α and β contribute to both cell fate decision and terminal differentiation in the gut epithelium.

Hnf1α and β bind to identical DNA sequences indicating that they could play complementary roles in the organism. Our study shows that single $Hnf1a^{-/-}$ or $Hnf1b^{\Delta intestine}$ animals have no major intestinal phenotypes. However, the lack of both Hnf1α and β in the gut epithelium significantly affects this organ and causes a lethal phenotype.

$Hnf1a^{-/-}; Hnf1b^{\Delta intestine}$ mice die from severe dehydration in a matter of few days after inactivation. This dysfunction is linked to the complete suppression of the expression of *Slc26a3*, a gene encoding for an anion exchanger involved in intestinal water absorption. A murine model of *Slc26a3* gene inactivation showed that newborn pups cannot easily survive after birth (Schweinfest et al., 2006), similar to what happens with newborn children that carry null mutations in this gene (OMIM 214700). We have shown that Hnf1α and β directly control the expression of the *Slc26a3* gene, indicating that Hnf1α and β expression in the intestinal epithelium is essential for survival. Previous studies have demonstrated that the Hnf1 transcription factors activate, in vivo, the expression of a number of intestinal genes including *Fabp1*, *LPH*, *Cftr* and *Glc-6-Pase*, which are expressed in differentiated enterocytes (Bosse et al., 2007; Gautier-Stein et al., 2006; Mouchel et al., 2004). We have confirmed the previous in vitro studies on *Dpp4* as a target gene of Hnf1α and β, demonstrating that its levels in vivo were severely affected in our Hnf1 mouse models (Erickson et al., 2000). In addition, Cdx2, a transcription factor involved in enterocyte differentiation (Mutoh et al., 2005; Suh and Traber, 1996), is known to control the expression of *Muc2*, a marker of goblet cells (Yamamoto, 2003). In our studies, we showed that *Cdx2*, a gene harbouring an Hnf1 binding site conserved throughout evolution, is defectively expressed in double-mutant mice and is a direct target of Hnf1α. A complex regulatory mechanism was demonstrated for *Cdx2* expression (Benahmed et al., 2008; Boyd et al., 2009). Recently, it was demonstrated that *Cdx2* controls Hnf1α during embryonic development (Gao et al., 2009). Our data demonstrate that Hnf1 transcription factors partially contribute to *Cdx2* expression, suggesting that there is a reciprocal control between *Cdx2* and Hnf1α in the intestinal epithelium (Fig. 7).

In addition to the already known role of Hnf1s in controlling the transcription of enterocyte-specific genes, we demonstrate for the first time that they are also involved in production and differentiation of secretory cell lines. Inactivation of both Hnf1α and β gave rise to supernumerary goblet cells not associated with an increase in the proliferation rate of goblet cell precursors. Several studies have demonstrated that the major pathway involved in intestinal cell fate commitment is the Notch pathway (reviewed by Crosnier et al., 2006; Radtke and Clevers, 2005). Intestinal progenitors are committed through a binary cell fate choice. Activation of this pathway leads the

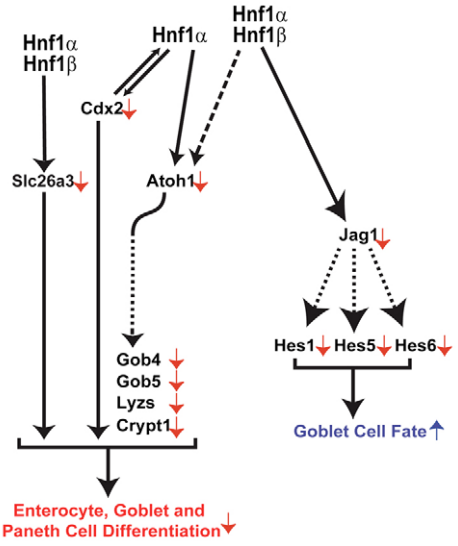

Fig. 7. The role played by Hnf1α and β in intestinal cell differentiation and cell fate commitment. The concerted action of Hnf1α and β directly activates the expression of *Slc26a3*, *Atoh1* and *Jag1*, which in turn act on both cell fate commitment and differentiation. Dashed lines indicate an indirect effect.

intestinal progenitors to differentiate into enterocytes. On the contrary, in the absence of the activation of the Notch pathway, intestinal progenitors express *Atoh1* and differentiate into one of the three secretory cell types (Jensen et al., 2000; Yang et al., 2001). Inactivation of Notch signalling resulted in the complete loss of proliferating crypt progenitors and their conversion into postmitotic goblet cells (van Es et al., 2005b). The molecular mechanisms of this conversion were recently elucidated. Notch-mediated Hes1 expression contributes to the maintenance of the proliferative crypt compartment of the small intestine by inhibiting the transcription of two cyclin-dependent kinase inhibitors (Riccio et al., 2008). Gain-of-function studies also demonstrated that Notch activity is required for the maintenance of proliferating crypt cells in the intestinal epithelium (Fre et al., 2005). Interestingly, we showed that Hnf1α and β can activate directly the expression of *Jag1*, a gene that encodes for a ligand of the Notch pathway and whose genomic sequence contains Hnf1 binding sites conserved throughout evolution that are bound by both Hnf1α and Hnf1β. In line with this observation, the expression of several effectors directly activated by Notch signalling, including *Hes1*, *Hes5* and *Hes6*, was downregulated in the double mutants. This should explain why our double-mutant mice had a slight, but significant, increased number of goblet cells, which could be ascribed to the specific downregulation of Notch signalling activity. One of the key genes controlling secretory cell lineage commitment is *Atoh1*. This gene is known to be under the direct repression of Hes1. When Notch signalling is inactive, *Atoh1* expression is increased. In spite of a partial inactivation of the Notch pathway, we unexpectedly observed a downregulation of *Atoh1* in our double-mutant mice. Interestingly, several Hnf1 binding sites were conserved throughout evolution in this gene and we showed that at least Hnf1α can bind in vivo a distal (30 Kb) downstream transcriptional control element of this gene. Previous studies have shown that a null mutation of *Atoh1* leads to a complete depletion of the secretory cell lineage in the intestine, indicating that this factor is required for specification of enteroendocrine, goblet and Paneth cells (Shroyer et al., 2007; Yang

et al., 2001). Surprisingly, in our double mutants, the reduction in *Atoh1* expression did not prevent secretory cell commitment. In fact, the proportion and differentiation of enteroendocrine cells was not affected and, conversely, the number of goblet cells was paradoxically increased in *Hnf1a*$^{-/-}$; *Hnf1b*$^{\Delta intestine}$ mice. This paradox could be explained by the fact that the expression of *Atoh1* is still maintained in the crypts. The residual expression of *Atoh1* in our model could be sufficient for the commitment of goblet and enteroendocrine cell fates. Notably, *Atoh1* is expressed in progenitors of the secretory lineage as well as in their corresponding fully differentiated cell types (Jenny et al., 2002; Pinto et al., 2003; van Es et al., 2005a; van Es et al., 2005b; Yang et al., 2001), suggesting that *Atoh1* could also play a role in postmitotic secretory cells. In our model, *Atoh1* appears to be completely abolished in the secretory cells of the villi but not in the crypts supporting the role of Hnf1 in the control of terminal differentiation (Fig. 7).

In our double-mutant mouse model, we detected the lack of mature Paneth cells at the bottom of the crypts. Some transcription factor inactivation models, including *Gfi1*$^{-/-}$ or *Sox9*$^{-/-}$, are characterized by a drastic defective differentiation of Paneth cells (Bastide et al., 2007; Shroyer et al., 2005). Interestingly, this defect is normally accompanied by colonization of the bottom of the crypts by proliferative cells. In our double-mutant animals, the apparent lack of Paneth cells did not result in this colonization. This would indicate that committed postmitotic Paneth cell precursors probably occupy their correct position at the bottom of the crypts but simply failed to complete their differentiation program. The expression levels of *c-Myc*, cyclin D1 (the key effectors of Wnt pathway) and *Sox9* (a mediator of the Wnt-dependent program) were not affected, suggesting that the defect of the differentiation program might be Wnt-independent. However, we did not find any difference in the expression levels of *Msi1*, a gene important for the Paneth cell Wnt-independent differentiation program, suggesting that other genes might be involved in this differentiation program.

Finally, we observed the defective activation of the Notch signalling linked to the decreased expression of *Jag1* (Fig. 7). This leads to an increased goblet cell fate commitment. Conversely, we observed defective differentiation of both goblet and Paneth cells as a considerable number of specific secretory markers were downregulated. We checked for the presence on Hnf1 binding sites in all the affected genes involved in terminal differentiation of goblet and Paneth cells. These genes did not present any overtly conserved Hnf1 binding sites. Thus, Hnf1 transcription factors appear to indirectly regulate terminal differentiation markers of secretory cell lineages, possibly through *Atoh1*. In addition, both Hnf1 transcription factors directly control *Slc26a3* a gene involved in intestinal water absorption. Thus, Hnf1α and β both directly and indirectly control terminal differentiation of several cell lineages in the gut epithelium and cell fate commitment through the direct regulation of intestinal key actors (Fig. 7).

In conclusion, Hnf1α and β transcription factors are crucial both for enterocytes and secretory cell lines in the gut epithelium.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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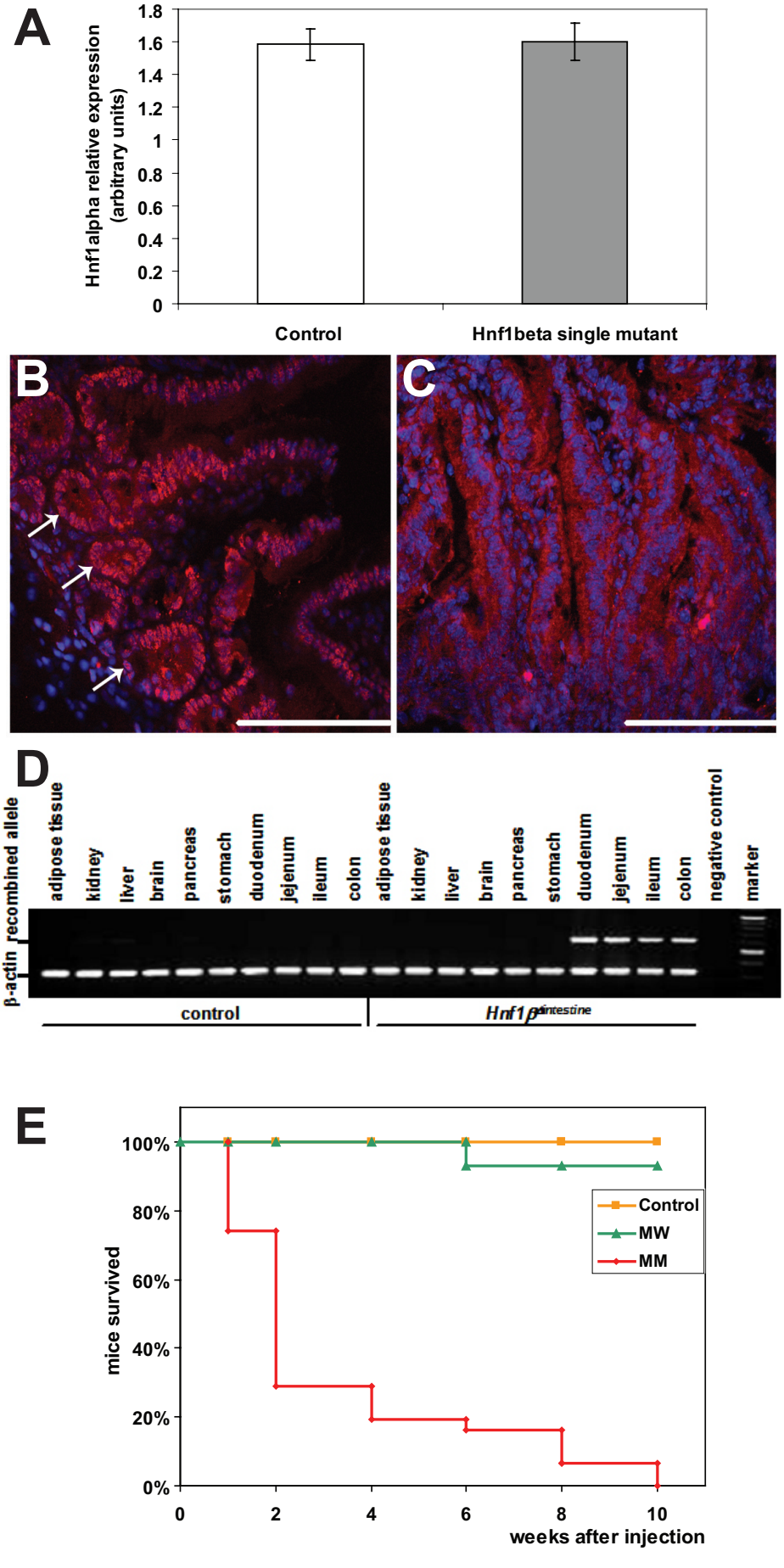
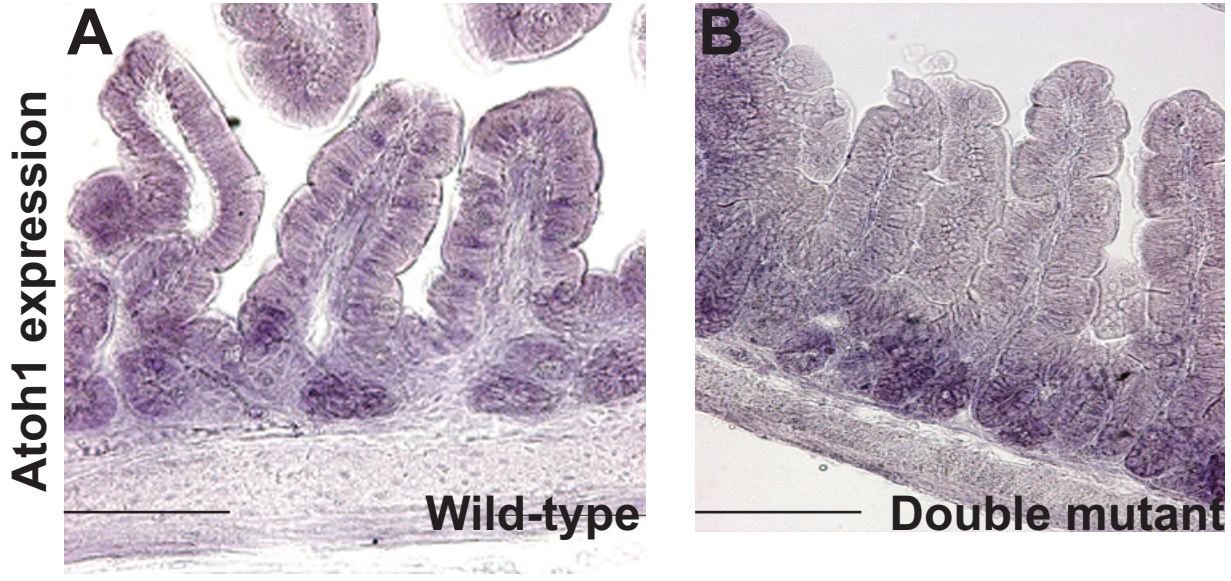

A
Hnf1alpha relative expression (arbitrary units)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Hnf1beta single mutant
B
C
D
recombined allele
β-actin
adipose tissue
kidney
liver
brain
pancreas
stomach
duodenum
jejenum
ileum
colon
adipose tissue
kidney
liver
brain
pancreas
stomach
duodenum
jejenum
ileum
colon
negative control
marker
control
Hnf1β intestine
E
100%
80%
60%
40%
20%
0%
mice survived
0
2
4
6
8
10
weeks after injection
Control
MW
MM


Atoh1 expression
A
Wild-type
B
Double mutant

Control

Hnf1a$^{-/-}$

Double Mutant

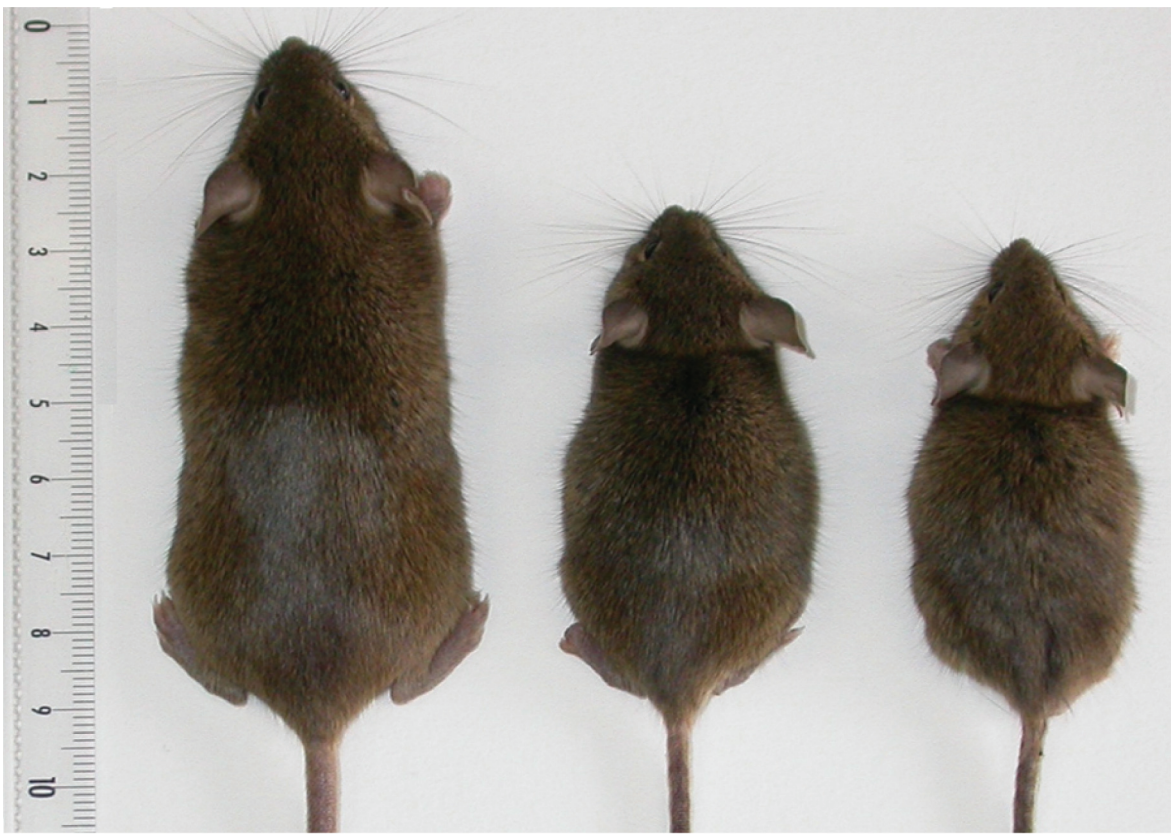


Table S1. List of the primers of the genes that have been analyzed in real-time PCR

Gene		Sequence
Hnf1b	Forward	ACAATCCCCAGCAATCTCAGAA
Hnf1b	Reverse	GCTGCTAGCCACACTGTTAATGA
Dpp4	Forward	GATTTGTGGACAGCAAGCGA
Dpp4	Reverse	ACCCTCCATATGACCAGCCC
Cdx2	Forward	TGGAGCTGGAGAAGGAGTTTCA
Cdx2	Reverse	TCCTCCTGATGGTGATGTATCG
Klf4	Forward	AGAGTTCTCATCTCAAGGCACACC
Klf4	Reverse	AAGGTTTCTCGCCTGTGTGAGT
Muc3	Forward	TGATGTCATCCTGAAGGCCA
Muc3	Reverse	TCCAGGTTTTTGACGACGGT
Muc2	Forward	ACCACTGTGATGCCAATGACA
Muc2	Reverse	TCGTGGCGCACAATAAGTGT
gob-4	Forward	CCAAGACAAGCAACAGACCCTT
gob-4	Reverse	GGCATTCGTCCAAGTGATGA
gob-5	Forward	AAGCAAACCACTCCCATGACA
gob-5	Reverse	TCCAATTTGCAGCAGTGAGAA
Cryp1	Forward	TCAAGAGGCTGCAAAGGAAGA
Cryp1	Reverse	ACCCTTTCTGCAGGTTCCATT
Lys2	Forward	ATGGCAAAACCCCAAGAGCT
Lys2	Reverse	GAGCACTGCAATTGATCCCA
Sox9	Forward	AAGTCGGTGAAGAACGGACAA
Sox9	Reverse	CAGCGCCTTGAAGATAGCATT
C-myc	Forward	TCCCTGAATTGGAAAACAACG
C-myc	Reverse	TGCTCGTCTGCTTGAATGGA
cyclin D1	Forward	AAGTTCATTTCCAACCCACCC
cyclin D1	Reverse	TGGAAAGAAAGTGCGTTGTGC
Notch2	Forward	CCCTGATCATCGTGGTGCT
Notch2	Reverse	AATGCGCAAGTTGGTGTGG
Jag1	Forward	ACTCGGAAGTGGAGGAGGATG
Jag1	Reverse	AGCGGACTTTCTGCTGGTGT
Atoh1	Forward	TATCCCGTCCTTCAACAACGA
Atoh1	Reverse	TGGTCATTTTTGCAGGAAGCT
Hes1	Forward	TCAACACGACACCGGACAAA
Hes1	Reverse	TTATTCTTGCCCTTCGCCTC
Hes5	Forward	GGTACAGTTCCTGACCCTGCA
Hes5	Reverse	CCGCTGGAAGTGGTAAAGCA
Hes6	Forward	GTCCCCAGAATCCCCATTGT
Hes6	Reverse	CAGGCACCCGGTTTAGTTCA
Slc26a3	Forward	AGAAGACGCACAGACATCACAAG
Slc26a3	Reverse	AACACCCTTTGAGATGGTCCAG
Gapdh	Forward	GCAAAGTGGAGATTGTTGCCA
Gapdh	Reverse	ATTTGCCGTGAGTGGAGTCAT
Msi1	Forward	GGCTTCGTCACTTTCATGGACCAGGCG
Msi1	Reverse	GGGAACTGGTAGGTGTAAC
Atg16l1	Forward	TGGGATATCCGGTCAGAGAG
Atg16l1	Reverse	GAGCAGCTCAGGAGCTCAGT
Atg7	Forward	ACCATGCAGGGAGCTAGAGA
Atg7	Reverse	CCACTGAGGTTCACCATCCT
Elf3	Forward	TTCTGACTCCGGTGGAAGTGAT
Elf3	Reverse	CCCTTCTTATAGTCAGGAAAGCCA
Gfi1	Forward	CATCTGCTCA TTCACTCGGACA
Gfi1	Reverse	GCTTTCTGCTATGAGTGATGAGGTT
Hnf4a	Forward	ATCACCTGGCAGATGATCGAA
Hnf4a	Reverse	AGGTTGTCAATCTTGGCCATG