

Shp2/MAPK signaling controls goblet/paneth cell fate decisions in the intestine

Julian Heuberger^a, Frauke Kosel^a, Jingjing Qi^a, Katja S. Grossmann^{a,1}, Klaus Rajewsky^b, and Walter Birchmeier^{a,2}

^aLaboratory of Signal Transduction in Development and Cancer and ^bImmune Regulation and Cancer, Max Delbrück Center for Molecular Medicine, 13125 Berlin, Germany

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In the development of the mammalian intestine, Notch and Wnt/ β -catenin signals control stem cell maintenance and their differentiation into absorptive and secretory cells. Mechanisms that regulate differentiation of progenitors into the three secretory lineages, goblet, paneth, or enteroendocrine cells, are not fully understood. Using conditional mutagenesis in mice, we observed that Shp2-mediated MAPK signaling determines the choice between paneth and goblet cell fates and also affects stem cells, which express the leucine-rich repeat-containing receptor 5 (Lgr5). Ablation of the tyrosine phosphatase Shp2 in the intestinal epithelium reduced MAPK signaling and led to a reduction of goblet cells while promoting paneth cell development. Conversely, conditional mitogen-activated protein kinase kinase 1 (Mek1) activation rescued the Shp2 phenotype, promoted goblet cell and inhibited paneth cell generation. The Shp2 mutation also expanded Lgr5+ stem cell niches, which could be restricted by activated Mek1 signaling. Changes of Lgr5+ stem cell quantities were accompanied by alterations of paneth cells, indicating that Shp2/MAPK signaling might affect stem cell niches directly or via paneth cells. Remarkably, inhibition of MAPK signaling in intestinal organoids and cultured cells changed the relative abundance of Tcf4 isoforms and by this, promoted Wnt/ β -catenin activity. The data thus show that Shp2-mediated MAPK signaling controls the choice between goblet and paneth cell fates by regulating Wnt/ β -catenin activity.

PTPN11 | Mek1DD | crypt | TCF7L2 | RTK

The intestinal epithelium of mammals consists of absorptive enterocytes and of three secretory cell types, paneth, goblet, and enteroendocrine cells, which are continuously replenished from stem cells that reside in niches in the lower parts of the crypts (1, 2). The secretory lineages differentiate from a common progenitor that emerges to occupy the +5 cell position above the stem cell niches (3). Goblet and paneth cells continue to share similar characteristics, whereas enteroendocrine cells develop separately through a divergent mechanism. During differentiation, paneth cells move back to the base of the crypts and become interspersed between the stem cells, whereas the other cell types migrate into the villi (1, 2). Paneth cells provide signals for the maintenance of stem cells, which are characterized by the expression of the stem cell marker Lgr5. Lgr5 is a receptor for R-spondins and participates in canonical Wnt signaling (4). Lgr5+ stem cells are reduced but not depleted when paneth cells are lacking (5, 6), which indicates that paneth cells are not the sole source of the signals that maintain stem cells.

Wnt/ β -catenin signaling, through cooperation of Wnt receptors and Lgr4/5 coreceptors, is essential in maintaining the intestinal epithelium and plays important roles in the generation of Lgr5+ stem cells (1). Canonical Wnt signaling also influences secretory cell lineages, because overexpression of the Wnt antagonist dickkopf1 (Dkk1) lead to a loss of all secretory cell types (7). However, Wnt/ β -catenin signaling has different effects on the development of the three secretory cell types: paneth cells require Wnt and leucine-rich repeat-containing receptor 4 (Lgr4) (8, 9); high Wnt activity interferes with goblet cell differentiation (10); and enteroendocrine progenitors are Wnt independent (11).

This indicates that additional mechanisms come into play in the differentiation of the secretory lineages. One candidate might be MAPK signaling, because conditional expression of oncogenic K-ras resulted in intestinal hyperplasia and was accompanied by altered goblet and paneth cell numbers (12).

The nonreceptor tyrosine phosphatase Shp2 mediates growth factor and cytokine signals and can regulate the activity of the Ras/Mek1/MAPK and other signaling pathways in development and disease (13, 14). In mice, a null mutation of *Shp2* interfered with the expansion of the trophoblast cell lineage and led to implantation deficits (15). Shp2 is also required for the development and maintenance of the nervous system, the kidney, and other organs (13, 14, 16, 17); its role in the maintenance of the intestinal epithelium is not fully investigated. Heterozygous mutation of Shp2 in an Egr mutant background resulted in the accumulation of desquamated intestinal epithelia (18). Shp2 and the transcription factor Stat3 are activated through the interleukin/gp130 receptor; however, mutation of the Shp2-binding site in gp130 has no major effects on intestinal development. Instead, it enlarges the proximal small intestine in aging mice and protects intestines from dextran-sulfate-induced colitis (19).

Here we used mouse genetics to demonstrate that Shp2 and Mek1/MAPK signaling control the choice between goblet and paneth cell fates. Ablation of *Shp2* promotes paneth cell expansion while reducing goblet cell formation. Conversely, activation of Mek1/MAPK promotes the generation of goblet cells at the expense of paneth cells. We provide evidence that

Significance

The mammalian intestine is covered by an extended villus epithelium that contains absorptive enterocytes and secretory cell types that are replenished from stem cells in the lower parts of crypts. Signals that control stem cells are known, but those that regulate differentiation of secretory lineages are less well understood. Here we used mouse genetics and intestinal organoid cultures to characterize the intricate signaling networks in the crypts that regulate the choice between secretory goblet and paneth cells and stem cells. MAPK suppression in the crypts increases Wnt/ β -catenin signaling and promotes paneth and stem cells. High MAPK signals in the crypts decreases paneth cells and stem cells and favors goblet cell properties. Mechanistically, MAPK regulates Wnt/ β -catenin signaling on the post-translational level.

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¹Present address: Deutsche Forschungsgemeinschaft (DFG), Kennedyallee 40, 53175 Bonn, Germany.

²To whom correspondence should be addressed. E-mail: wbirch@mdc-berlin.de.

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Shp2/Mek1/MAPK-mediated regulation of Wnt/ β -catenin signaling is crucial for the lineage decision by which goblet and paneth cells differentiate from a common progenitor cell type.

Results

Shp2 Is Essential for Goblet Cell Differentiation. We aimed to assess the role of Shp2/MAPK signaling in cell fate determination of the intestine. By conditional mutagenesis, we generated mutant mice that lacked *Shp2* in the intestine using *villinCre* (*Shp2*^{lox/lox}; *villinCre* and *Shp2*^{lox/+}; *villinCre*, hereafter called *Shp2* mutant and control, respectively) (*Material and Methods*) (16, 20). Remarkably, ablation of *Shp2* strongly reduced goblet cell numbers in the small intestine and the colon, as assessed by alcian blue staining (Fig. 1 *A* and *B*). The intervention was accompanied by a drop in mRNAs of *Spdef*, *Muc2*, and *Gob5*, which are typically expressed in goblet cells (21–23), as assessed by qRT-PCR (Fig. 1 *C* and *D*). Immunohistochemical analysis (IHC) of *Spdef* and *Gob5* proteins confirmed reduced goblet cell numbers (Fig. S1 *A* and *B*, quantification on the *Right*). The *Shp2* mutant phenotype was mildly mosaic: goblet cell numbers were reduced in a pronounced manner in large areas that displayed high levels of Cre protein and loss of *Shp2* expression (Fig. S1 *C–E*, *Right*); residual goblet cells only appeared in minor areas with very low or no Cre (Fig. S1 *C*, arrows), and with absence of *Shp2* expression (Fig. S1 *D*; yellow arrows point to *Shp2*-deficient villi and crypts without goblet cells). Enteroendocrine cells were not affected by the loss of *Shp2*, as assessed by IHC for ChromograninA (Fig. S1 *F*, quantification on the *Right*). The morphology of intestinal villi of *Shp2* mutants was slightly aberrant; some villi appeared to be shortened or thickened (Fig. 1*A*, *Upper Right* and Fig. S1 *A–D*, *Right*). *Shp2* mutants also developed diarrhea and anal bleeding, which are symptoms of severe colitis, and were growth retarded (Fig. S1 *G*). We observed a corresponding reduction in mRNA of *Aquaporins 7* and *8* in the intestines of *Shp2* mutants, as assessed by qRT-PCR (Fig. S1 *H*). These genes are known to be down-regulated in human and mouse colitis (24). The data thus show that the *Shp2* mutation in the intestine results in goblet cell deficits and colitis.

Ablation of Shp2 Induces Premature Differentiation of Paneth Cells and Activates Wnt/ β -Catenin Signaling. Differentiated paneth cells that produce lysozyme and matrilysin (MMP7) appear only after

birth (25) and were thus rare in intestines of control mice at P6 (Fig. 2*A*, *Left*). *Shp2* mutants, however, exhibited a massive increase in paneth cells at this stage (Fig. 2*A*, *Right*, quantification in Fig. 2*B*), which indeed appear only in *Shp2*-deficient crypts (Fig. S2*A*, yellow arrow points to *Shp2*-deficient crypt with paneth cells, blue arrowhead points to a nonrecombined crypt without paneth cells). The noted expansion of paneth cells potentially mimics an increase in canonical Wnt signals. Wnt signaling has been implicated in paneth cell differentiation (8), and accordingly, administration of Wnt3a increases paneth cell numbers in intestinal organoid cultures (26). Increases of paneth cells were also noted after tamoxifen-induced ablation of *Shp2* in intestinal organoids from *villinCreERT2* mice, as assessed by IHC or qRT-PCR for lysozyme (Fig. 2*C* and Fig. S2*B*). This led us to examine whether the effects of the *Shp2* mutation and the enhanced paneth cell differentiation are mediated by activation of canonical Wnt signaling. We performed genome-wide expression analyses on mutant and control intestines using Illumina microarrays (GSE50785), and then used gene set enrichment analysis to compare these with data previously obtained on *Wnt/ β -catenin*-dependent genes in the intestine (27, 28). *Shp2* mutant intestines exhibited a marked up-regulation of *Nol5a*, *Myc*, and *Cd44* and several other Wnt/ β -catenin-dependent genes (Fig. 2*D* and Fig. S2*C*). Increases in the expression of *Wnt3*, the Wnt target gene *Cd44*, and genes encoding *Lgr4* and the closely related *Lgr5*, were also noted in *Shp2* mutant intestines, as confirmed by qRT-PCR (Fig. 2*E*). Thus, Wnt/ β -catenin signaling is indeed enhanced after *Shp2* ablation in the murine small intestine.

Also up-regulated in *Shp2* mutants was the gene *Lgr5*, which is normally uniquely expressed in stem cells (1). We used the *Lgr5*-IRES-GFP-CreERT allele to identify *Lgr5*⁺ stem cells by IHC for GFP (29) and observed an increase in their numbers in *Shp2* mutant mice (Fig. 2*F*). This was confirmed by in situ hybridization for *Olfm4*, another marker of *Lgr5*⁺ stem cells (Fig. 2*G* and ref. 30). Next, we compared genes that are deregulated in *Shp2* mutant intestines to those of *Lgr5*⁺ stem cells (31). Remarkably, many stem cell genes were increased in the small intestine of *Shp2* mutants (Fig. 2*H* and Fig. S2*D*). Further work using qRT-PCR demonstrated an up-regulation of stem cell genes, including *Ascl2*, *Adora1*, *Claa4*, *Aqp1*, and *Smoc2* in the mutants (Fig. 2*I*, marked in bold in Fig. S2*D*). Thus, ablation of *Shp2* in the intestine leads not only to enhanced paneth cell differentiation, but also increases a presumptive *Lgr5*⁺/*Olfm4*⁺ stem cell population.

Shp2 Controls Erk1/2-MAPK Signaling in the Intestinal Crypts. In many cell types, Shp2 activates Mek1, which in turn phosphorylates Erk1/2. Immunohistological analysis of control intestines demonstrated high nuclear phospho-Erk1/2 levels in goblet cells as well in cells located in the lowest part of the villi and in the upper parts of the crypts; phospho-Erk1/2 levels were strongly reduced in the *Shp2* mutant (Fig. 3*A*, *Insets*), reinforcing the notion that Shp2 might control goblet and paneth cell differentiation through the regulation of Mek1/MAPK signaling. To pursue this, we took advantage of a transgenic mouse strain that expresses a gain-of-function variant of *Mek1* (*Mek1DD*) upon removal of a translation stop cassette through Cre-mediated recombination (Fig. S3*A*) (32, 33). *Mek1DD* expression could be activated by *villinCre* in the *Shp2* mutant background. In the intestine of *Mek1DD*; *Shp2* double mutants at P6, nuclear p-Erk1/2 levels were strongly elevated (Fig. S3*B*, *Right*), but overall proliferation was not significantly changed, as assessed by Ki67 staining (Fig. S3 *C* and *D*). Remarkably, the Shp2-dependent reduction of goblet cells both in the small intestine and the colon was rescued in *Mek1DD*; *Shp2* double mutants (Fig. 3*B*, *Right* and Fig. S3*E*). The numbers of rescued goblet cells in the intestine were comparable to goblet cell numbers in control mice (Fig. S3*F*). Further, paneth cells were strongly reduced in *Mek1DD*; *Shp2*

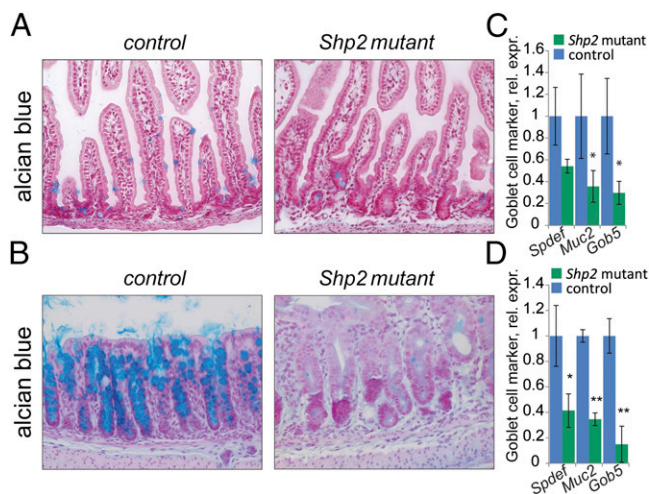


Fig. 1. (A and B) Reduction of goblet cells in the small intestine (A) and colon (B) of villinCre-induced *Shp2* loss-of-function mutant mice at P9, as seen on paraffin sections stained with alcian blue. (C and D) Reduction of goblet-cell-specific mRNA expression in the small intestine (C) and the colon (D) in *Shp2* mutant mice at P9, assessed by qRT-PCR ($n = 5$, significance calculated with *t* test; * $P < 0.05$, ** $P < 0.01$). Counterstaining, nuclear fast red. (Scale bars, 100 μ m.)

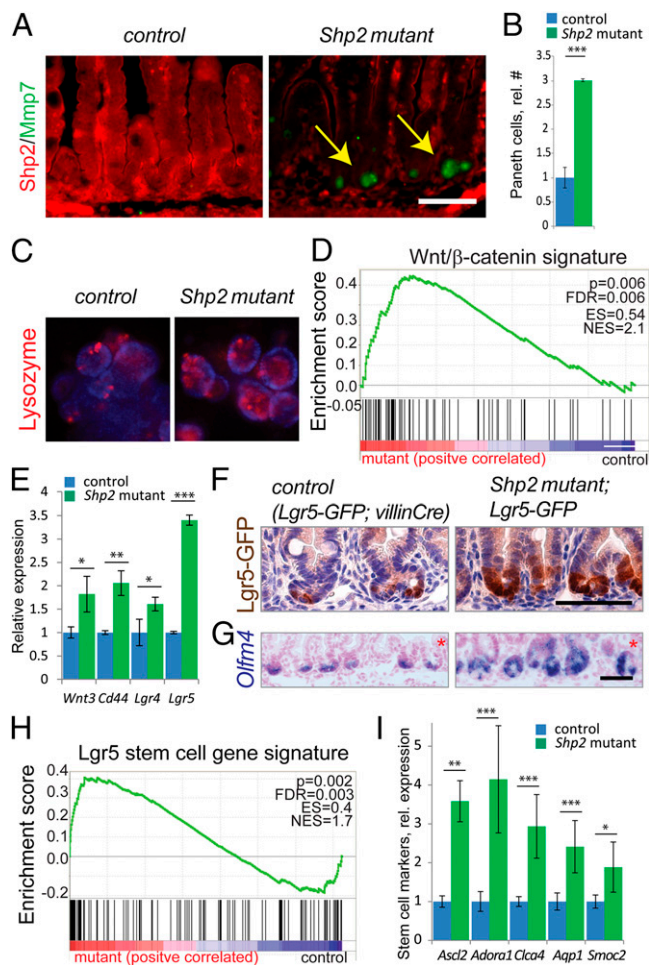


Fig. 2. (A) Increase of paneth cells in *Shp2* mutants at P6, in comparison with controls, as seen on paraffin sections stained by immunofluorescence for Shp2 and Mmp7. (B) Quantification of lysozyme-positive paneth cells from the experiment in Fig. S2A ($n = 4$). (C) Increase of paneth cells in organoid cultures of tamoxifen-inducible *Shp2* mutants and controls, stained for lysozyme by immunofluorescence; counterstain was with DAPI. (D) Gene set enrichment analysis (GSEA): plot of enrichment score of control versus *Shp2* mutant intestinal tissue at P6 for the *Wnt/β-catenin* gene signature. (E) qRT-PCR for mRNAs of Wnt signaling components in the small intestine of *Shp2* mutant and control mice at P6 ($n = 5$). (F) Identification of Lgr5+ cells by immunohistochemistry for GFP in *Shp2* mutants and controls crossed with *Lgr5-IRES-GFP-CreERT*. (G) In situ hybridization for *Olfm4* in *Shp2* mutants and controls; red asterisks mark crypts. (H) GSEA: plot of enrichment score of control versus *Shp2* mutant intestinal tissue at P6 for the Lgr5 stem cell signature. (I) Confirmation of stem-cell-associated genes by qRT-PCR at P6 ($n = 5$). (Scale bars, 100 μm .) Significance calculated with t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

double mutants at P6 and P16, as shown by lysozyme staining (Fig. 3C and Fig. S3G). Loss of paneth cells was previously observed in mice that express an oncogenic variant of *K-Ras* and suggested to be caused by increased *Hes1* expression (12). However, *Hes1* expression was not significantly changed in *Shp2* single and *Mek1DD*; *Shp2* double mutants (Fig. S3H). Thus, in *Mek1DD*; *Shp2* double mutant mice, goblet cells are rescued, and this appears to occur at the expense of paneth cells. This is reminiscent of the phenotype observed after overexpression of the Ets transcription factor *Spdef*; *Spdef* overexpression promoted goblet cell differentiation at the expense of paneth cells (22). In control mice, goblet and paneth cells produce the Ets transcription factor *Spdef*, as assessed by IHC (Fig. 3B, marked by arrows, *Left*). In *Shp2* mutants, *Spdef* production was observed in the few residual

goblet cells (Fig. 3B, located in villi) as well as in paneth cells (located in crypts, *Center*). In *Mek1DD*; *Shp2* double mutants, the production of *Spdef* was limited to the rescued goblet cells in the villi (Fig. 3B, *Right*). Apparently, *Mek1* activity in intestinal epithelial cells does not change the cell-type-specific expression pattern of *Spdef*. Overall, we conclude that *Mek1*/MAPK signaling regulates the choice between goblet and paneth cell differentiation.

The *Shp2* mutation does not only affect goblet and paneth cell fates, but also increases the presumptive Lgr5+ stem cells (see above and Fig. 2F). We therefore analyzed stem cells in *Mek1DD*; *Shp2* double mutants and observed down-regulated expression of *Lgr5*, *Olfm4*, and *Ascl2* by in situ hybridization and qRT-PCR (Fig. 3D and Fig. S3J). Thus, *Shp2*/MAPK signaling also controls the number of stem cells.

Shp2/Mek1 Signaling Regulates the Secretory Cell Fate Switch by Interfering with β -Catenin/Tcf4 Signaling. The transcription factor Tcf4 activates *Wnt/β-catenin* target genes and is critical for the maintenance and differentiation of intestinal epithelia (34, 35). Because mutation of the *Tcf4* gene in the adult small intestine induced a paneth to goblet cell transition (35), we analyzed Tcf4 production in the small intestine by immunohistochemistry. Nuclear Tcf4 protein was observed throughout the entire crypt–villus axis, with the strongest levels in the crypts (Fig. S4A). Goblet cells in controls also showed strong nuclear Tcf4 (Fig. S4A, a). However, no difference in Tcf4 production was observed between control and *Shp2* mutants (Fig. S4A). However, Tcf4 splice variants with different transcriptional capacities might be differentially produced (36) and regulate cell fate decisions in the intestinal crypts. To analyze this, we took advantage of organoid cultures of the small intestine, which finally consist mainly of crypt structures. Organoids with ablated *Shp2* or activated *Mek1DD* confirmed the inverse correlation of paneth cells and pErk

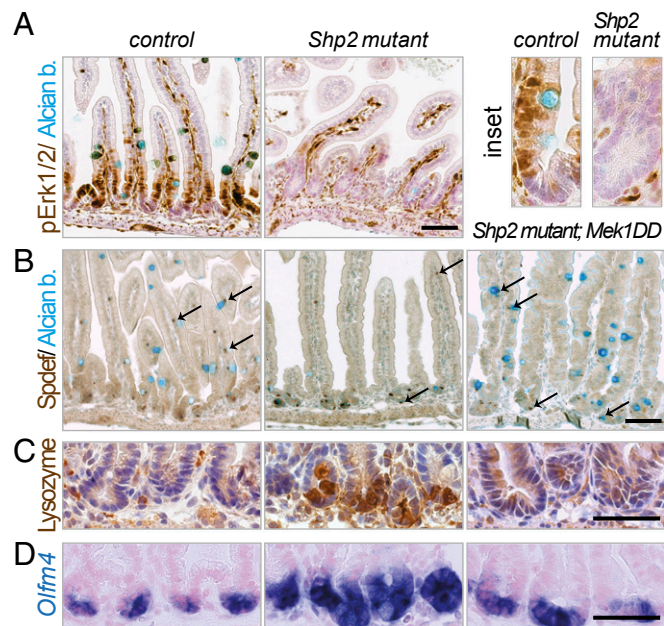


Fig. 3. (A) Immunohistochemistry of phospho-Erk1/2 (for activated *Mek1/2* signaling) at P6 on sections of control and *Shp2* mutant small intestines. (Inset) Magnifications. (B and C) Rescue of goblet cell and paneth cell switches in *Shp2* mutants by the *MekDD1* allele, as shown by staining for goblet cells with alcian blue and *Spdef* (B) and for paneth cells with lysozyme (C). Arrows in B point to *Spdef*-stained nuclei. *Spdef* signals are in part overlapping with strong alcian blue staining in *Mek1DD* mutants. (D) Rescue of *Olfm4* mRNA expression in *Shp2* mutants by *MekDD1*, as shown by in situ hybridization. (Scale bars, 100 μm .)

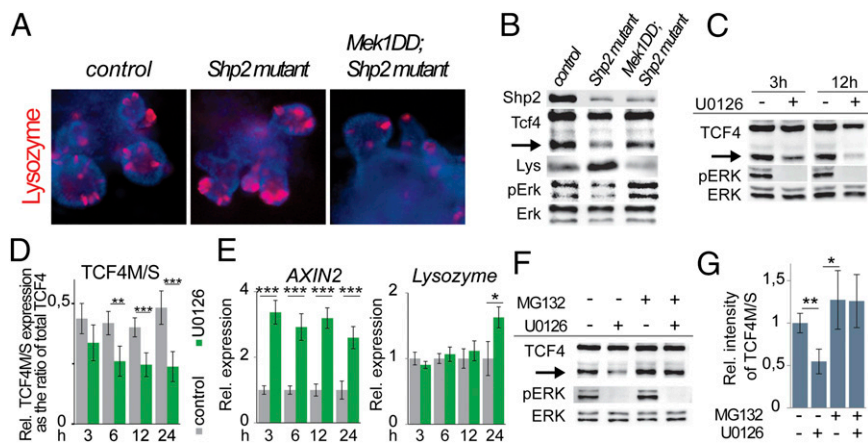


Fig. 4. (A) Paneth cell switch in organoid cultures of tamoxifen-inducible *Shp2* mutants, *Mek1DD*; *Shp2* double mutants, and controls stained for lysozyme; counterstain was with DAPI. (B) MAPK-dependent regulation of Tcf4M/S (marked by arrow) production in organoid cultures of tamoxifen-induced *Shp2* and *Mek1DD* mutants, as analyzed by Western blotting for Shp2, lysozyme, Tcf4, and pErk. (C) MEK1/2 inhibition in HT29 cells with U0126 for 3 h and 12 h particularly decreased the shorter TCF4 (TCF4M/S) splice variant (arrow). (D) Quantification of the time-dependent decrease of the TCF4M/S isoforms in U0126 treated (green bars) and control (gray bars) HT29 cells. (E) Up-regulation of the Wnt target gene *AXIN2* (Left) and of *Lysozyme* (Right) in HT29 cells through MEK1/2 inhibition (green bars) in a time-dependent manner ($n = 3$). (F) Prevention of degradation of the TCF4M/S isoforms in U0126-treated HT29 cells by inhibition of proteasomes with MG132 for 6 h. (G) Quantification of the alterations in TCF4M/S proteins following MG132 treatment ($n = 3$). Significance was calculated with t test; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

activities, as assessed by immunofluorescence and Western blotting (Fig. 4A and B). Two major Tcf4 protein isoforms were produced in control organoids but remarkably, *Shp2* mutant organoids showed reduced amounts of the shorter isoform, which was rescued by the *Mek1DD* allele (Fig. 4B, the shorter Tcf4 isoform is marked by an arrow). The longer isoform at 70 kDa is Tcf4E, which harbors a binding site for the transcription factor carboxyl-terminal binding protein (CtBP) and an extended C-terminal domain including a C clamp, which is a further DNA binding domain (37). In contrast, the shorter Tcf4 band at 50 kDa (arrow in Fig. 4B) corresponds to the Tcf4M and -S isoforms, which lack the binding sites for CtBP and carry no or an incomplete C clamp (37). The Tcf4M and -S isoforms can therefore bind to Wnt response elements on the DNA but lack the capacity of promoter activation, for instance of the Wnt target gene *Axin2* (37). The different amounts of the Tcf4 isoforms by changed MAPK signaling is regulated on the translational level, because the mRNAs for the *Tcf4* isoforms were not altered between control and mutant organoids (Fig. S4B). Moreover, in human HT29 colon cancer cells, pharmacological inhibition of MEK1/2 by the small molecule U0126 also produced reduced amounts of the shorter TCF4M/S isoforms in concentration and time-dependent manners (Fig. 4C and Fig. S4C and D). Reduction of the TCF4M/S isoforms was rapid, starting already at 3 h, and was significantly reduced at 6 h of MEK1/2 inhibition (Fig. 4D and Fig. S4E), at time points when the large TCF4 isoform was not significantly altered. Importantly, pharmacological inhibition of MEK1/2 by U0126 also rapidly increased the expression of the canonical Wnt target gene *AXIN2*, already at 3 h (Fig. 4E, Left). However, up-regulation of the paneth cell differentiation marker lysozyme occurred only after 24 h of MEK1/2 inhibition (Fig. 4E, Right). shRNA-mediated knockdown of *SHP2* in HT29 cells did not affect MEK1/2 activity (Fig. S4F), which is in line with the fact that HT29 cells harbor an oncogenic *B-RAF* mutation (38) that activates MAPK signaling downstream of SHP2. Furthermore, *shSHP2* interference did not alter the expression of the TCF4 isoforms (Fig. S4F), indicating that the alterations of the TCF4M/S isoforms are regulated by MEK1/2 signaling and not via other SHP2-mediated events. Coimmunoprecipitation of TCF4 with β -catenin from nuclear fractionations showed that the interaction of β -catenin with TCF4E was persistent upon MEK1/2

inhibition (Fig. S4G). To pursue further the mechanism of the MEK1/2-dependent reduction of TCF4M/S proteins, we inhibited proteasome protein degradation with MG132. Remarkably, this inhibition prevented the MEK1/2-dependent reduction of the shorter TCF4M/S isoforms (Fig. 4F, quantification in G), indicating that MEK1/2 activity protects the shorter isoforms from proteasomal degradation. In conclusion, MEK1/2 inhibition appears to affect canonical Wnt signaling by rapidly changing the relative abundance of the TCF4 isoforms, i.e., suppressing the transcriptionally inactive/inhibitory isoforms, while leaving the activating form unchanged. These data thus suggest that *Shp2*/MEK1/MAPK signaling regulates the choice between goblet and paneth cell fate by regulating Wnt/ β -catenin signaling through interfering with the protein stability of particular Tcf4 isoforms.

Discussion

Goblet and paneth cells represent two secretory cell types in the intestinal epithelium. Here we show that in mouse intestinal development, the tyrosine phosphatase *Shp2* promotes the generation of goblet cells at the expense of paneth cells. Conditional *Shp2* ablation in the intestine, which decreases MAPK signaling, reduces the numbers of goblet cells and increases the numbers of paneth cells. In contrast, sustained activation of MAPK signaling by the *Mek1DD* allele has the converse effects. Paneth and goblet cells express overlapping sets of genes (22, 39), and cells with initially intermediary phenotypes have indeed been described (35, 40). Paneth and goblet cells are thus closely related and originate from a common precursor (Fig. S5, Left). The third secretory lineage, enteroendocrine cells, is generated independently of *Shp2* and MAPK activity and is believed to diverge from secretory precursors at an earlier stage of development (Fig. S5, Left) (41). Overall, our data thus show that the level of *Shp2*/MAPK signaling determines the choice between goblet and paneth cell fates.

We also found that *Shp2*/MAPK attenuates canonical Wnt signaling, which may mediate the effect of *Shp2*/MAPK on the goblet/paneth cell fate changes: goblet cells differentiate when MAPK and Wnt activity are high and low, respectively, whereas paneth cells differentiate when MAPK and Wnt signals are low and high, respectively (Fig. S5, Left). Our analysis of mutant intestinal

organoid cultures and of HT29 colon cancer cells using pharmacological interference is supporting such a model and provides mechanistic insights: MAPK inhibition increases Wnt/ β -catenin signaling and promotes paneth cell characteristics, whereas high MAPK activity or inhibition of Wnt signaling favors goblet cell properties (Fig. S5, Right).

The *Shp2* mutation also triggers colitis and reduced body weight, which are fully rescued by MAPK activation. *Shp2/Mek1DD* double mutant mice are viable and develop no diarrhea or anal bleeding. For function, Shp2 is recruited to several tyrosine kinase receptors and other cell surface receptors (13). Compound *Egfr^{wa-2}*; *Shp2^{+/-}* mutants exhibited intestinal defects, like desquamated epithelia and shortened villi (18), which are also observed in mild form in conditional *Shp2* mutants. Furthermore, *Egfr* activity is known to control the maturation of goblet cells (42), whereas ErbB2, ErbB3, and ErbB4 receptors promote the recovery from chemically induced colitis (43, 44). Thus, Shp2 might act downstream of tyrosine kinase receptors like the *Egfr* in intestinal development and prevention of disease. Moreover, we show that MAPK acts downstream of Shp2 in intestinal development, because activation of MAPK signaling fully rescued the *Shp2* phenotypes, i.e., the deficits in goblet/paneth cell fate determination and the colitis. Loss of paneth cells and an increase of goblet cell numbers were also observed in intestines after conditional overexpression of oncogenic *K-rasG12D* (12). In addition, *K-rasG12D* produced hyperplastic polyps and severely distorted crypt architecture, which we do not observe in the *Mek1DD* mutants. The loss of paneth cells was attributed to Ras-mediated expression of the transcription factor *Hes1* (12). In contrast, we did not observe changes in *Hes1* expression in *Mek1DD* or *Shp2* mutants. In *Ras* mutant intestines, no effect on Wnt signaling has been reported (12). In contrast, oncogenic *Ras* acted synergistically with canonical Wnt signaling in *APC* mutant intestines (45). Apparently, oncogenic K-Ras controls larger sets of downstream signaling events than Shp2 and *Mek1*, which may explain the additional phenotypes.

We observed that Shp2/MAPK signaling affected canonical Wnt activity in the intestinal epithelium: high Shp2/MAPK activity decreased Wnt/ β -catenin signals and promoted goblet cell differentiation, whereas *Shp2* ablation increased Wnt/ β -catenin activity and promoted paneth cell differentiation (Fig. S5, Right). Canonical Wnt signals downstream of MAPK thus appear to control the choice between goblet and paneth cell differentiation, as is also indicated by genetic analysis of *Tcf4* (35). A common secretory precursor has recently been located at the +5 position in small intestines (3). Cells at the +5 position are believed to receive intermediary levels of Wnt/ β -catenin, and our data show that these cells also display intermediary MAPK activity. In contrast, the highest canonical Wnt activity is observed at the base of the crypts (46), whereas MAPK signaling (as assessed by nuclear pErk1/2 distribution) is high in the upper part of the crypts. We therefore suggest that the balance between Wnt/ β -catenin and Shp2/MAPK activities controls the choice between goblet/paneth cell differentiation in these precursors.

To define mechanisms by which Shp2/MAPK exert a negative effect on Wnt/ β -catenin activity, we analyzed intestinal organoids genetically and colon cancer cells using pharmacological inhibition. We observed that Shp2 and MAPK signaling interfere with the production of Tcf4 isoforms. Tcf4 transcription factors are critical for the differentiation and maintenance of intestinal epithelia (34, 35). Different variants of Tcf4 proteins exist that are generated from differentially spliced mRNAs and exhibit different transcriptional activation capacities (36, 37). The shorter isoform corresponds to Tcf4M/S, which lack the CtBP-binding and transactivation motifs (37). In contrast, the longer Tcf4E isoform exhibits the unique capacity of promoter recognition and activation, based on the extended C-terminal domain. Tcf4E and Tcf4M/S proteins are produced in intestinal organoids and colon

cancer cells, and reduction of MAPK markedly decreased the levels of the short Tcf4M/S isoforms, i.e., reduced the production of the Tcf4 isoforms with the lower/inhibitory potential of Wnt target gene activation. Inhibition of proteasomes prevented the degradation of the TCF4M/S isoform, indicating that active MAPK signaling stabilizes this TCF4 isoform, presumably by affecting an E3 ligase that targets TCFM/S for degradation. Thus, MAPK signaling can influence Wnt target gene activation by regulating the protein stability of isoforms of TCF4 transcription factors. It has been shown that changes in the relative abundance of Tcf4 variants can indeed fine tune target gene expression in a cell-context-dependent manner (47). Because the Tcf4M/S proteins can bind β -catenin, we assume that by reducing these isoforms by MAPK inhibition, more β -catenin becomes accessible for canonical Wnt target gene activation through the large Tcf4E transcription factor. Thus, we provide here evidence for a unique mechanistic link between MAPK and canonical Wnt signaling in intestinal secretory progenitors.

Ablation of *Shp2* in the intestine triggers both an increase in paneth cells and an increase in the number of epithelial stem cells, as assessed by gene enrichment profiling and histological analyses. This connection is interesting because the control of the small intestinal stem cell niches has generally been assigned to paneth cells, which provide Wnt signals known to be essential for the maintenance of stem cells (4). Paneth cells do not seem to be solely responsible for this, however; sustained activation of MAPK resulted in the complete loss of paneth cells, decreasing but not completely eliminating the presumptive Lgr5+ stem cells. Thus, the absence of paneth cells did not completely disrupt the stem cell niches. This confirms recent work that noted a decrease but not complete loss of Lgr5+ stem cells after genetic ablation of paneth cells (5, 6). By contrast, in organoid cultures the interaction between the two cell types is crucial: Lgr5+ stem cells are only able to generate organoids in the presence of paneth cells (4, 26). This difference suggests that in vivo, additional cell types such as stromal components might produce factors that allow stem cell maintenance in the absence of paneth cells (5, 6). Ablation of *Shp2* could increase the numbers of Lgr5+ cells indirectly, i.e., by increasing the numbers of paneth cells that in turn produce factors that stimulate stem cells. This view is supported by experiments that stimulation of Wnt signaling produced supernumerary paneth and Lgr5+ cells (26). Alternatively, increased Wnt signaling might allow the maintenance of supernumerary Lgr5+ stem cells or impair their differentiation. Further work is required to identify the factors that paneth cells and other cell populations provide to stem cells in the small intestine.

Methods

Breeding conditions and genotyping of the mouse strains, *Shp2^{lox}*, *villinCre*, *villinCreERT2*, and *Lgr5-EGFP-IRES-creERT2*, have been described (16, 20, 29). All animal experiments were conducted according to regulations established by the Max Delbrück Center together with the Landesamt für Gesundheit und Soziales (LAGe So) and the European Union. Mouse intestines were fixed in 4% (wt/vol) formaldehyde and immunohistochemistry, immunofluorescence, and in situ hybridization were performed on 5- μ m paraffin sections. Primary antibodies were anti-pErk1/2, pStat3 (Cell Signaling), Shp2, Mmp7 (Santa Cruz), lysozyme (Dako), Gob5, ChroA, and GFP (Abcam), Ki67 (Neolabs), Cre (Novagen), and SPDEF (kindly provided by J. A. Whitsett (Cincinnati Children's Hospital Medical Center, Cincinnati, OH)). For immunofluorescence, cyanine-labeled secondary antibodies (Jackson ImmunoResearch) and for immunohistochemistry, HRP-conjugated polymer and DAB reagent (DAKO) were used. In situ hybridization was performed using digoxigenin-labeled (DIG) RNA probes (Roche). Western blotting was performed as described (16) on organoid and cell culture lysates, and blots were probed with anti-Shp2 (Santa Cruz), pErk1/2 (Sigma), lysozyme (Dako), β -catenin (BD Biosciences), and Erk1/2 and TCF4 (Cell Signaling) antibodies.

Nuclei from HT29 cells were enriched as described before (48) followed by brief sonification, and coimmunoprecipitations were performed at 4 °C with anti- β -catenin antibody (BD Biosciences) and Protein-G-Sepharose (GE Healthcare). Beads were washed four times with PBS/Nonidet P-40

(0.1%), and immunoprecipitated proteins were analyzed by Western blotting. RNA was isolated from pieces of proximal small intestine, which was processed for gene profiling (Illumina) according to the manufacturer's protocol (Illumina total prep, Ambion, Life Technologies). Gene set enrichment analysis was performed using GSEA software from the Broad Institute (27).

Cells were cultured in DMEM supplemented with 10% FCS (Gibco) and treated with 10 μ M U0126 (Calbiochem) for 3 h–24 h or as indicated. Proteasomes were inhibited for 6 h by 25 μ M of MG132 (Sigma) with or without 10 μ M U0126. Organoid culture (49) was performed with supplement from HEK293 cells, which overexpressed recombinant R-spondin1. The R-spondin1 plasmid was a gift of Christof Niehrs (Institute of Molecular Biology, Mainz,

Germany). Tamoxifen-inducible *Shp2* mutant organoids were split 2 d before treatment with 400 nM 4-hydroxitamoxifen (4-OHT) (Sigma) for 2 consecutive days and cultured for an additional 4 d.

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