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Loss of MMR function impacts the colonic homeostasis

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Abstract
The fine balance between proliferation, differentiation, and apoptosis in the colonic epithelium is tightly controlled by the interplay between WNT, Notch, and bone morphogenetic protein (BMP) signaling. How these complex networks coordinate the colonic homeostasis, especially whether cancer predisposing mutations such as mutations in the DNA mismatch repair (MMR) are present, is unclear. Inactivation of the MMR system has long been linked to colorectal cancer; however, little is known about its role in the regulation of the colonic homeostasis. It has been shown that loss of MMR promotes the proliferation of colon epithelial cells that renders them highly susceptible to transformation. The mechanism through which MMR mediates this effect, yet, remains to be determined. Using a MMR-deficient mouse model, we show that increased methylation of Dickkopf1 (DKKI) impacts its expression, and consequently, the ability to negatively regulate WNT signaling. As a result, excessive levels of active β-catenin promote strong crypt progenitor-like phenotype and abnormal proliferation. Under these settings, the
development and function of the goblet cells are affected. MMR-deficient mice have fewer goblet cells with enlarged mucin-loaded vesicles. We further show that MMR inactivation impacts the WNT–BMP signaling crosstalk.

Introduction
The DNA mismatch repair (MMR) is a highly conserved DNA repair system that plays a central role in maintaining the genomic stability of the cells. The MMR proteins recognize DNA single base mutations and small insertion or deletion loops, generated during DNA replication, and coordinate their repair. Initially, the DNA damage is recognized by the protein MutS homologue 2 (Msh2) that forms a complex with one of its partner proteins Msh6 or Msh3 depending on the type of DNA damage (Jiricny, 2006). Msh2 then recruits other downstream components of the pathway that excise and resynthesize the damaged DNA region. Therefore, Msh2 is a key MMR protein and its inactivation completely abolishes the DNA repair process (Modrich and Lahue, 1996; Jiricny, 2006). Loss of MMR activity is associated with both hereditary and sporadic colorectal cancer (CRC) (Ionov et al., 1993; Marra and Jiricny, 2005; Poulogiannis, 2010). One of the hallmarks of MMR deficiency is development of mutator phenotype seen as elevated frequency of single base substitutions and small insertion/deletion mismatches that are generated at repeated DNA sequences, known also as microsatellite instability (MSI). Indeed, MSI is recognized as the main mechanism through which MMR deficiency mediates CRC initiation, nicely reviewed by Boland and Goel (Boland and Goel, 2010). MMR pathway also plays a critical role in initiation of apoptosis in response to high levels of DNA damage. Therefore, failure to trigger apoptosis was recognized as another mechanism through which MMR deficiency contributes to CRC (Negureanu and Salsbury, 2012; Li et al., 2016). Interestingly, inactivation of MMR pathway increases susceptibility to carcinogenesis of particular types of cells. The most susceptible tissue is the colonic epithelium, followed by endometrium, stomach, kidney, ovary and small intestine (Chao and Lipkin, 2006). Significantly lower risk for cancer incidence is reported in prostate, breast and lung tissue (Watson and Lynch, 2001). Studies with MMR mutant mouse models have further provided significant knowledge about the mechanisms of cancer susceptibility in different types of tissue (Heyer et al., 1999; Lee et al., 2016). It has been suggested that high proliferation rate, as well as rapid acceleration/deceleration of the
proliferation rate, play critical roles in the process of cell transformation (Chao and Lipkin, 2006).

Since its discovery, extensive research has been focused on revealing the molecular mechanisms through which MMR deficiency mediates tumor development. In contrast, very little is known about the role of MMR pathway in the regulation of the colonic homeostasis. The colon epithelial cells (CECs) are organized in colonic crypts. The colonic crypt itself is composed by stem cells that are located at the bottom of the crypt which proliferate and give rise to their daughter cells known as transit amplifying cells (TA cells). The stem cells and TA cells form the so-called proliferative compartment of the crypt. As these cells proliferate they also move upwards toward the intestinal lumen where they differentiate into goblet cells, enterocytes and enteroendocrine cells (Medema and Vermeulen, 2011). Three signaling pathways tightly regulate the proliferation and differentiation of the CECs: WNT/β-catenin, Notch, and bone morphogenetic protein (BMP) pathways. It is generally accepted that WNT regulates the proliferation of the CECs. The myofibroblast cells at the bottom of the colonic crypts produce several WNT ligands, so that the stem cells and TA cells are exposed to higher amounts of WNT molecules. Typically, the WNT ligands interact with their receptors and activate signaling cascades that result in disassembling of the β-catenin destruction complex (involves APC, Axin2, CK1, and GSK-3β). This further leads to stabilization and accumulation of non-phosphorylated (active) β-catenin. Activated β-catenin then enters the nucleus and together with its co-factors mediates the transcription of genes that are required to maintain the proliferation of the cells (Medema and Vermeulen, 2011). The myofibroblast cells also secrete several BMP ligands. However, the signaling molecule Noggin effectively inhibits their activity at the bottom of the colonic crypts. The Noggin concentration gradually decreases towards the intestinal lumen so that BMP ligands gain their activity and interact with BMPR1,2 receptors, resulting in phosphorylation of Smad1,5,8 and its cooperation with Smad4. Subsequently, the formed Smad complex transcribes genes that regulate cell cycle arrest, differentiation or apoptosis of the CECs (Medema and Vermeulen, 2011). On the other hand, Notch regulates the equilibrium between cell proliferation and differentiation by directing the response of the cells to specific environmental signals (Artavanis-Tsakonas et al., 1999). Notch 1,2 receptors, their specific ligands Jagged 1 (Jag1), Delta-like 1 and 4 (Dll1, Dll4), and the Notch target genes Hairy and enhancer of split 1 (Hes1), Hes5 and Hes6 are highly active in the proliferative compartment.
of the colonic crypts and are important for the maintenance of the colonic stem cell niche (Jensen et al., 2000; Schroder and Gossler, 2002; VanDussen et al., 2012). Great amount of research has focused on elucidation of the specific roles of WNT, BMP, and Notch signaling in the regulation of proliferation and differentiation of CECs. However, our knowledge of how these pathways crosstalk with each other to maintain the normal colonic homeostasis is still very limited. Moreover, how this crosstalk is impacted by specific genetic mutations that predispose to cancer is unclear.

It has been shown that inactivation of MMR system in mice results in increased proliferation of CECs that renders these cells highly susceptible to transformation events (Belcheva et al., 2014). Although it has been suggested that this phenomenon is caused by elevated WNT/β-catenin signaling, the mechanistic links were not elucidated.

Here we investigated the causative factors leading to overactivation of WNT signaling pathway in Msh2-deficient CECs and its impact on the colonic homeostasis. We found that WNT functions normally upstream of the β-catenin destruction complex, and the presence of excessive active form of the protein is caused by loss of expression of the WNT inhibitor Dickkopf1 (DKK1). Our data show that increased methylation of DKK1 CpG island in Msh2-/- CECs is a mechanism for its downregulation. As a result, the activated β-catenin promotes strong crypt progenitor-like phenotype and enhanced proliferation. Under these settings, the normal development and function of the goblet cells are affected. We observed significant reduction in the goblet cell numbers which, however, produce substantially more Mucin 2 (Muc2). We also show that failure to regulate β-catenin activity in Msh2-deficient CECs has an impact on the molecular crosstalk between WNT and BMP signaling pathways.

**Results**

**Inactivation of MMR pathway is associated with strong stem cell-like phenotype**

The CECs are organized in colonic crypts where the proliferating cells (stem cells and their daughter progenitor cells) and terminally differentiated cells (enterocytes, enteroendocrine and goblet cells) are maintained in precisely controlled homeostasis (Figure 1A). Mutation in Msh2 gene results in an increase in the proliferating compartment of the intestinal crypts (Belcheva et al., 2014), also observed in this study (Supplementary Figure S1). This suggests that the normal homeostatic balance between proliferating and differentiated cells is likely impacted by the Msh2
mutation. To investigate this hypothesis, we analyzed the expression of several stem cell and differentiated cell markers in colonic crypts derived from Msh2<sup>−/−</sup> mice and their WT controls (Msh2<sup>+/−</sup> mice). Previously reported significant increase in Ephrin type-B receptor 2 (EphB2), Ephrin type-B receptor 3 (EphB3) and CD44 mRNA levels in Msh2<sup>−/−</sup> colonic crypts (Belcheva et al., 2014) were also confirmed in this study (Figure 1B and Supplementary Figure S2). In addition, the stem cell marker Achete scute-like 2 (Ascl2), a gene with pivotal role in defining the stem cell identity (Schuijers et al., 2015), was also significantly elevated (Figure 1B). Although not significant, the expression of the stem cell marker CD24 was increased by 1.5-fold in Msh2<sup>−/−</sup> CECs (Figure 1B). The increased EphB2 mRNA levels in Msh2 mutant CECs also correlated with notably higher protein abundance (Figure 1C). In addition, the typical EphB2 expression gradient pattern was altered in Msh2-deficient crypts. We observed extensive EphB2 expression at the bottom of the colonic crypts and prolonged decreasing gradient compared to WT controls (Figure 1C). EphB2 expression pattern is critical for establishment of balanced distribution of proliferating and differentiated cells within the colonic crypt (Batlle et al., 2002) and together with EphB3, CD44, Ascl2, and CD24 are direct transcriptional targets of WNT/β-catenin signaling (Pinto et al., 2003; Schuijers et al., 2015). To further understand whether these stem cell markers were increased in Msh2 mutant CECs due to alterations in WNT activity, we carried out western blot analysis of β-catenin (Figure 1D). We measured higher expression of the active form of β-catenin (non-phosphorylated β-catenin) in Msh2<sup>−/−</sup> colonic crypts. This was concomitant with marked decrease in the expression of the negative cell cycle regulator p21<sup>(CIP1/WAF1)</sup>. Since the reduced p21 expression in Msh2<sup>−/−</sup> CECs is likely associated with the enhanced proliferation of these cells, we further investigated the mechanism responsible for its negative regulation. p21 transcription is controlled by both p53 (Gartel and Tyner, 1999) and β-catenin (p53-independent mechanism) (Kamei et al., 2003). Specifically, p53<sup>(Ser15)</sup> phosphorylation is required for p53 transcriptional activity at the p53-responsive promoters, including p21 promoter (Loughery et al., 2014). Therefore, we assessed the p53<sup>(Ser15)</sup> levels by western blot. The result showed that phospho-p53<sup>(Ser15)</sup> levels were not different between Msh2<sup>−/−</sup> and Msh2<sup>+/−</sup> cells (Figure 1D), suggesting that the reduced expression of p21 results from the elevated β-catenin activity. Taken together, these results indicate that in Msh2 mutant CECs enhanced β-catenin activity promotes crypt progenitor-like phenotype and enhanced proliferation.
**Msh2 mutation impacts the regulation of WNT signaling via DKK1**

The activity of β-catenin is controlled by its phosphorylation status. Typically, in the absence of WNT signal activated glycogen synthase kinase (GSK-3β) plays a central role in phosphorylation of β-catenin, an event that triggers its degradation and shuts down the transcription of β-catenin-regulated genes (Medema and Vermeulen, 2011). Since we measured excessive levels of active β-catenin and marked increase in several stem cell markers in Msh2 mutant colonic crypts we hypothesized that this may be due to alteration in the GSK-3β activity. However, we found that the active form of the kinase (p-GSK-3β) that is detected by the specific phosphorylation on Tyr 216, was not different between Msh2 mutant and Msh2 WT CECs (Figure 2A). Next, we tested the mRNA levels of adenomatous polyposis coli (APC) and Axis inhibition protein 2 (Axin2), important components of the β-catenin destruction complex (Medema and Vermeulen, 2011) and are frequent mutational targets of MMR deficiency (Castiglia et al., 2008). The results showed that these genes were not differentially expressed between Msh2 WT and Msh2−/− mice (Figure 2B), suggesting that the increased active β-catenin levels in Msh2 mutant CECs are neither caused by discrete external stimuli that would alter the activity of GSK-3β (Figure 2A), nor by loss of APC and Axin2 expression (Figure 2B). To explain the abundance of the active β-catenin in Msh2 mutant CECs we further hypothesized that it may be caused by aberrant negative regulation of WNT signaling, mediated by Dickkopf1 (DKK1) protein. DKK1 is a natural WNT antagonist that plays a critical role in the precise regulation of WNT signal transmissions, and therefore, prevents abnormal signaling output (Niida et al., 2004; Gonzalez-Sancho et al., 2005).

Our analysis revealed that the expression of DKK1 was reduced at both mRNA (Figure 2B) and protein (Figure 2C) levels in Msh2−/− CECs. Indeed, DKK1 expression is often reduced in CRC (Gonzalez-Sancho et al., 2005). This may be due to mutations in DKK1 or transcriptional inactivation of DKK1 via hypermethylation of CpG regions (Aguilera et al., 2006; Sato et al., 2007), a process highly associated with MSI and MMR (Rawson et al., 2011).

To assess whether DKK1 inactivation is caused by specific mutation(s), genomic DNA was isolated from CECs of four Msh2−/− and three Msh2+/− mice, the entire gene was amplified and subjected to sequencing analysis. The results showed no mutations (not shown).
DKK1 has two CpG islands. The first spans around its transcription start site and the second is located ~600 bp downstream. The location of the predicted CpG islands, the DKK1 transcription start site and the location of the primers are shown (Figure 1D). To investigate the methylation state of DKK1, we examined the CpG islands by methylation specific polymerase chain reaction (PCR). We used methBLAST algorithm to design primers that are specific to the bisulfite converted genomic DNA (Pattyn et al., 2006). We were only able to amplify one of the CpG regions. The specificity of the selected primers was evaluated by 1.5 % agarose gels. The purified PCR products were sequenced, and the DNA methylation status was determined by comparing the sequencing results with the original DNA sequence. Only presence of a single C-peak that indicates 5-methylcytosine (5mC) in the sequence was considered (Figure 2E). The results revealed that 32 % of the analyzed cytosines were methylated in Msh2 mutant mice compared to 11 % in their Msh2 WT controls (Figure 2E). The 2.9-fold increase in the methylated cytosines of DKK1 in Msh2 mutant CECs indicates that its reduced expression is dependent on DNA hypermethylation, mediated by loss of MMR function.

MMR deficiency is associated with abnormal differentiation of goblet cells

The marked increase in the stem cell signature (Figure 1B and C) and the reduced p21(CIP1/WAF1) expression in Msh2 mutant colonic crypts (Figure 1D) suggest that the normal cell differentiation may be disturbed. This prompted us to investigate the expression of known markers for specific differentiated cells in the colon. We measured a significant increase in the mRNA level of the goblet cell marker Muc2 (Figure 3A) in Msh2 mutant CECs that was also confirmed in colonic tissue sections stained with Muc2 specific antibody (Figure 3B). In addition, the expression of Klf4, the main transcription factor that regulates the differentiation of intestinal goblet cells (Katz et al., 2002), was 1.8-fold reduced in Msh2−/− CECs (Figure 3A), leading us to further hypothesize that the normal development of the goblet cells might be impacted. To investigate this possibility, we stained colonic tissue with Periodic Acid-Schiff (PAS), a specific dye that stains mucins and enables visualization of goblet cells (Figure 3C). In agreement with the above results, the PAS staining produced more intensive staining pattern in Msh2-deficient colonic tissue compared to their WT controls, indicating an increase in the mucin compounds. In addition, we counted less goblet cells per colonic crypt in Msh2−/− mice, which was more
prominent in the distal part of the colon (Figure 3C) and correlated with the reduced expression levels of Klf4.

Furthermore, we assessed the morphology of the goblet cells using electron microscopy. Our analysis revealed two important features. The first and most striking was the observation that in Msh2⁻/⁻ mice the goblet cells had notably larger mucin-loaded vesicles compared to their WT controls (Figure 3D). Second, some of the goblet cells in Msh2⁻/⁻ mice were smaller in size (Figure 3D), suggesting for lack of maturity of these cells.

To investigate the development of the colonic enterocytes we measured the expression of their main product sucrase-isomaltase (SIM) and two transcription factors Hepatocyte nuclear factor beta 1 (Hnf1β) and Homeobox protein Cdx2 that regulate their terminal differentiation (Benoit et al., 2010; D'Angelo et al., 2010). The results showed no differences in the mRNA levels of these genes (Figure 4A). Other specific characteristics for mature enterocytes such as cell polarization and microvilli development were also not different between the genotypes (Figure 4B). Furthermore, the development of the colonic enteroendocrine cells was also unaffected by the loss of MMR function, as indicated by the expression levels of synaptophysin (SYP) and Nk2 homeobox 2 (Nkx2.2), the transcription factor shown to regulate their differentiation (Desai et al., 2008) (Figure 4C). Taken together, the results indicate that inactivation of MMR system leads not only to changes in the proliferative but also in the differentiated cell compartment, specifically affecting the normal goblet cell development and function.

Regulation of the colonic homeostasis in Msh2⁻/⁻ mice

Here we present evidence that in Msh2 mutant mice deregulated WNT signaling leads to an increased stem cell-like phenotype, enhanced proliferation, and abnormal goblet cell development and function. In general, the proliferation and differentiation in the colonic crypts are precisely regulated by the interplay between WNT, BMP and Notch pathways. WNT signaling maintains proliferation, while the BMP pathway regulates the differentiation of the colonocytes (Medema and Vermeulen, 2011). Notch seems to have dual functions in the colonic crypt by being responsible for directing both proliferation and differentiation (VanDussen et al., 2012). Therefore, we assessed whether the Notch and BMP signaling pathways function normally in Msh2 mutant colonic epithelium. First, we examined the expression of the Notch responsive gene Hes1 that mediates reduction in the members of the secretory lineage (i.e. goblet
and enteroendocrine cells) and expansion of the absorptive enterocytes (Stanger et al., 2005). However, Hes1 expression was normal in Msh2-deficient CECs (Figure 5A). On the other hand, loss in the Notch activity will normally lead to an induction of Math1 expression in the colonic crypts (van Es et al., 2005). Similarly, Math1 mRNA levels were not affected by the Msh2 mutant genotype (Figure 5A), suggesting that Notch activity is not altered under Msh2-deficient background.

Next, we investigated the expression of major BMP signaling components since downregulation of BMP signaling has been shown to impact the goblet cell terminal differentiation (Auclair et al., 2007). Our results showed a significant increase of BMPR2 and BMP2 mRNA levels in Msh2-deficient cells (Figure 5B). We also detected higher protein expression of BMP2,4 in Msh2 mutant colonic crypts by immunofluorescence. BMP2,4 were expressed predominantly by differentiated (Ki-67 negative) cells (Figure 5C), suggesting for elevated BMP activity in Msh2-deficient mice.

Taken together, our results demonstrate that MMR system plays a key role in the maintenance of the colonic homeostasis. Here we show that under MMR-deficient background increased methylation of DKK1 causes elevated WNT/β-catenin activity that results in strong stem cell-like phenotype and increased proliferation. In addition to the elevated WNT the BMP signaling was also increased. These events impact the normal goblet cell development and function and hence the colonic homeostasis.

Discussion
Inactivation of MMR pathway in CECs leads to abnormal proliferation compared to their WT controls, a feature that highly predisposes these cells to transformation (Belcheva et al., 2014). Although it has been suggested that this is caused by overactivation of WNT/β-catenin, the mechanistic details were not elucidated. In the present study, we investigated the causative factors that mediate deregulated WNT signaling in MMR-deficient CECs and its impact on the colonic homeostasis. We first tested whether the enhanced proliferation in Msh2 mutant CECs correlates with specific pattern of stem cell markers and found significant increase in mRNA expression levels of EphB2, EphB3, CD44 (in agreement with our previous study) (Belcheva et al., 2014) and Ascl2. Another stem cell marker CD24, although not significant, was also elevated in Msh2 mutant cells (Figure 1B and Supplementary Figure S2). The change in the expression of EphB2 and EphB3 is particularly interesting. Eph receptors belong to a family of receptor
tyrosine kinases and are highly abundant in proliferating cells, and gradually decrease in differentiated cells (Figure 1A). The Eph receptors and their cognate ligands are important in defining the cell positioning and migration within the intestinal crypts (Guo et al., 2006). Because EphB proteins are directly regulated by β-catenin and are expressed by proliferating cells, they have been implicated in CRC development (Batlle et al., 2002; Jagle et al., 2014). However, the role of EphB proteins in the process of carcinogenesis seems to be very controversial. Reduction of EphB2 and EphB3 expression has been found in advanced stages of colon cancer where the extent of EphB2 expression correlates with the cancer aggressiveness (Batlle et al., 2005; Guo et al., 2006; Jagle et al., 2014). In contrast, Merlos-Suarez and colleagues have reported that EphB2 defines strong proliferating capacity of CECs and has distinct cancer initiating properties (Merlos-Suarez et al., 2011). In this study, we show evidence that EphB2 expression follows the typical gradient pattern, which however, is considerably prolonged in Msh2-deficient colonic crypts (Figure 1C). How the increased EphB2 expression in Msh2 mutant CECs is related to their enhanced proliferative potential and high cancer susceptibility still remains to be investigated.

Furthermore, our results suggest that the enhanced stem cell-like phenotype and increased proliferation result from excessive levels of the active form of β-catenin (Figure 1D). In addition, this was concomitant with loss of expression of the negative cell cycle regulator p21(CIP1/WAF1) (Figure 1D). The expression of p21 can be induced by p53 (Gartel and Tyner, 1999) or negatively regulated by β-catenin (Kamei et al., 2003). Specifically, p53(Thr15) phosphorylation is required for transcriptional activation of p21 promoter (Loughery et al., 2014). Therefore, we investigated whether the loss of p21 in Msh2 mutant CECs is caused by reduced p53(Thr15) levels in Msh2−/− cells (Figure 1D). The result showed that p53(Thr15) expression was similar between Msh2 mutant and Msh2 WT CECs, suggesting that loss of p21 is caused by p53-independent mechanism. It has been shown that overactivation of WNT leads to induction of c-Myc that represses p21 transcription to efficiently maintain proliferation and suppress differentiation (van de Wetering et al., 2002). Therefore, the reduction of p21 in Msh2 mutant mice likely results from the overactive WNT and contributes to the increased proliferation capacity of the CECs.

To further understand the causative factors leading to the excessive levels of the active β-catenin in Msh2-deficient CECs we investigated the activity of WNT pathway upstream of β-catenin. Since GSK-3β plays a key role in the phosphorylation of β-catenin in response to environmental
signals (van de Wetering et al., 2002) it was important to investigate changes in its activity between the genotypes. We found that the active form of the kinase (p-GSK-3β\textsuperscript{Tyr216}) was not different between Msh2 mutant and Msh2 WT CECs (Figure 2A). We also investigated the expression of APC and Axin2, two key components of the β-catenin destruction complex that are also frequently mutated in MMR-deficient cells (Castiglia et al., 2008). However, we found that their expression was not altered in Msh2-deficient CECs (Figure 2B). These results, therefore, suggest that the excessive levels of active β-catenin in Msh2 mutant CECs result neither from discrete external stimuli that modulate the activity of GSK-3β, nor by loss of expression of APC or Axin2. Instead, we detected that both the mRNA and protein expression levels of the negative WNT regulator DKK1 were dramatically lost in Msh2 mutant cells (Figure 2B and C). DKK1 is a secreted antagonist of WNT signaling. It has been suggested that the protein forms a ternary complex with LRP5/6 and Kremen receptors and mediates the endocytosis of this complex resulting in depletion of LRP5/6 from the cell surface (Niida et al., 2004). Indeed, DKK1 expression is also regulated by β-catenin/TCF and has been shown to function as an important negative feedback loop in WNT pathway. In this way, DKK1 plays a critical role in the precise control of the transmission of WNT signals and avoid abnormal signaling output. It is not surprising that DKK1 expression is often reduced in CRC (Gonzalez-Sancho et al., 2005). Since inactivation of the MMR system leads to about 11-fold increase in the mutation frequency in intestinal epithelium (Andrew et al., 1997) we hypothesized that mutational inactivation of the DKK1 gene may be the causative reason for the loss of DKK1 expression. However, no mutations were present in the DKK1 sequences from Msh2 mutant CECs. Another mechanism that can lead to loss of DKK1 is via hypermethylation of CpG regions (Aguilera et al., 2006; Sato et al., 2007). Since this process has been previously associated with inactivation of MMR (Rawson et al., 2011) we investigated the methylation status of DKK1. The gene has two regions containing high frequency of CpG sites that are potential targets for methylation. To assess the methylation status of the gene we used methylation specific PCR sequencing (Li and Tollefsbol, 2011), however, we were able to analyze only the CpG island located about 600 bp downstream of the transcription start site (Figure 2D and E). The data revealed 2.9-fold increase in methylated cytosines in Msh2\textsuperscript{−/−} CECs and indicates that loss of DKK1 expression is likely caused by enhanced methylation of CpG regions in Msh2\textsuperscript{−/−} CECs. Taken together, our results suggest that in Msh2 mutant CECs loss of DKK1 expression causes deregulation of WNT
signaling resulting in excessive levels of active β-catenin that promotes strong stem cell-like properties and enhanced proliferation.

The abnormally high expression of stem cell markers, the prolonged gradient of EphB2 and the loss of p21(CIP1/WAF1) suggest that in Msh2 mutant colonic crypts the normal balance between proliferating and differentiated cells is disturbed. This prompted us to investigate the expression of several markers specific to the three types of differentiated cells in the colon: goblet, enterocytes and enteroendocrine cells. We found that the goblet cell marker Muc2 was elevated at both mRNA and protein levels in Msh2 mutant CECs (Figure 3A and B). Muc2 is an important component of the intestinal barrier and its expression is frequently reduced in CRC tumors compared to adjusted normal tissue. Also, loss of Muc2 expression correlates with poor prognosis in CRC (Kang et al., 2011; Wang et al., 2017). However, a small fraction of CRCs (10%–20%), known as mucinous colorectal adenocarcinomas, heavily express Muc2 (Perez et al., 2008). It has been suggested that the expression differences could arise from hypermethylation (Hanski et al., 1997) or hypomethylation (Gratchev et al., 2001) of the Muc2 promoter. Several studies demonstrate that the regulation of Muc2 is quite more complex and responds to different stimuli. The promoter region of the Muc2 contains binding sites for p53 (Ookawa et al., 2002), AP1 elements and consensus binding site for c-Myc (Velcich et al., 1997), suggesting that Muc2 overexpression could be associated by the enhanced WNT/β-catenin signaling. However, Muc2 overexpression can be induced also via activation of BMP pathway (Prakash et al., 2011). Because Muc2 is exclusively secreted by the intestinal goblet cells we hypothesized that the increased expression of the protein in Msh2-deficient mice will correlate with an increase in the goblet cell numbers. To our surprise, we observed reduction in the goblet cells per colonic crypt (Figure 3C) that, however, contained larger mucin-loaded vesicles (Figure 3D). Also, we found that some of the goblet cells in Msh2-deficient mice were smaller in size compared to their WT controls (Figure 3D), suggesting for a delay in their maturation. We also investigated the expression of Klf4, a goblet cell differentiation marker, with key role in regulation of the proliferation/differentiation in the gut epithelium (Katz et al., 2002; Yu et al., 2016). The protein is almost undetectable in exponentially proliferating cells due to the suppressive action of β-catenin/TCF signaling (Shields et al., 1996; Flandez et al., 2008; Zhang et al., 2012) and its overexpression in colon cancer cells was shown to cause G1/S arrest (Chen et al., 2001). Deletion of Klf4 in mice abrogated specifically the differentiation of the intestinal
goblet cells and was associated with increased WNT/β-catenin signaling and proliferation (Ghaleb et al., 2011). These studies demonstrate that Klf4 is a critical factor that directs the differentiation of the intestinal goblet cells. We found that Klf4 was downregulated by 1.8-fold in Msh2 mutant cells (Figure 3A). This result, therefore, correlates with the reduced goblet cell number and their delayed maturation (Figure 3C and D). The reduced Klf4 can also lead to an increase in the Muc2 production, as previously reported (Yu et al., 2016). Interestingly, the other specialized cell types, namely enterocytes and enteroendocrine cells, were not altered under MMR-deficient background (Figure 4).

Taken together, these results indicate that inactivation of the MMR system leads to dramatic alterations in the stem cell and differentiated cell compartments and hence, disturbed colonic homeostasis.

The differentiation of the intestinal proliferating cells is regulated by the complex interplay between WNT, Notch and BMP pathways. Specifically, activation of Notch signaling leads to induction of one of its responsive genes Hes1 that further mediates reduction in the members of the secretory lineage and expansion of the absorptive cells (Stanger et al., 2005). On the other hand, loss in the Notch activity will lead to Math1 expression in the colonic crypts (van Es et al., 2005). However, the expression of both genes (Hes1 and Math1) was not altered between Msh2-deficient and Msh2 WT CECs, suggesting that Notch functions normally under Msh2-deficient background (Figure 5A). In addition, BMP pathway controls the terminal differentiation of the secretory cells. Since we observed alterations in the goblet cell differentiation and function it was important to investigate the BMP signaling components and we measured an increase in BMPR2 and BMP2 mRNA levels in Msh2-deficient CECs (Figure 5B). Inactivation of BMP signaling in Bmpr1a mutant mice led to no differences in the number of goblet cells and Muc2 expression, although they were smaller in size and contained smaller mucin granules (Auclair et al., 2007). Our results, therefore, suggest for unique changes in the goblet cell function that likely result from alterations in both WNT and BMP signaling. These results are intriguing, especially in the context of the cancer susceptibility of MMR-deficient CECs. While the tumor promoting properties of WNT signaling are well documented, the role of BMP in carcinogenesis is very controversial. Because BMP is a natural antagonist of WNT it was suggested that the pathway acts as a tumor suppressor (Hardwick et al., 2008). Yet, it has been shown that BMP4 overexpression promotes invasiveness of colon cancer cells (Deng et al., 2007), while BMP2 induces
epithelial-mesenchymal transition (EMT), and hence, strengthening the metastatic potential of colon cancer cells (Kang et al., 2009; Kim et al., 2015). It was also shown that BMP pathway promotes growth of primary colon tumors in vivo (Lorente-Trigos et al., 2010). On the basis of this and other studies, the current hypothesis is that BMP initially may act as tumor suppressor and later as a tumor promoter. What are the specific conditions and factors that affect the biological effects of BMP pathway is poorly understood. Our results indicate that in Msh2-deficient CECs overactivation of WNT is concomitant with enhanced BMP activity. The physiological meaning of this crosstalk likely plays a central role in maintenance of the colonic homeostasis. Our work showed that in Msh2 mutant colonic crypts improper regulation of β-catenin activity promotes a crypt progenitor-like phenotype and increased proliferation that impact the normal differentiation and function of the goblet cells. To what extent the impaired homeostasis in MMR-deficient CECs is important in their susceptibility to transformation remains to be investigated.

**Materials and methods**

*Mice*

The Msh2−/− mice have been previously described (Reitmair et al., 1995). Briefly, 68 base pairs in the Msh2 gene were deleted by neomycin resistance cassette that was inserted in an antisense orientation in exon 11. This has resulted in a loss of both copies of the Msh2 allele (Msh2−/−) and complete loss of Msh2 protein expression. All mice were on the C57BL/6J background and raised under specific pathogen-free conditions. Msh2 heterozygous (Msh2+/−) mice characterized by normal DNA repair were used as controls in this study. The mice were genotyped as previously described (Peters et al., 2003). All experiments and analysis were conducted with 6-week-old mice.

*Isolation of colon epithelial cells*

Mouse colons were removed, flushed with PBS, cut into 0.5 cm pieces, and transferred in Ca2+/Mg2+ free PBS supplemented with 5 mM EDTA. The tissue was incubated for 20 min at 37°C. The solution was replaced with pure PBS, and CECs were released by vigorous shaking. Cells were collected by centrifugation at 1300 rpm for 5 min at 4°C. The procedure was repeated 3 times. The CECs were collected and used for mRNA or DNA isolation or cell lysates.
**mRNA isolation and quantitative polymerase chain reaction**

CECs were homogenized prior to RNA isolation using PureLink™ RNA Mini Kit (Ambion, Life Technologies) and reverse transcribed to cDNA using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fischer Scientific). Relative quantification of gene expression was performed using PowerUp™ SYBR Green Master Mix (Applied Biosystems). QPCR was carried out with initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 sec and extension at 60°C for 1 min. Relative gene expression levels were calculated by ΔΔCt method and normalized to the reference gene β-actin. The primer sequences are shown in Supplementary Table S1.

**Immunofluorescence**

Frozen Swiss-rolled colons were cut into 5-μm-thick sections and fixed with ice-cold acetone. The tissue was blocked with 3% BSA in PBST for 1 h at room temperature and incubated with specific antibodies detecting Ki67 (Abcam), EphB2 (Cell Signaling Technology), Mucin 2 (F-2), BMP-2/4 (H-1), and total β-catenin (H-102) obtained from Santa Cruz Biotechnology. Typically, the primary antibodies were used at 1:100 dilution at 4°C for overnight incubation. Subsequently, tissue sections were washed 3 times with PBST and incubated with secondary antibodies Goat Anti-Rabbit IgG Alexa-488 or Alexa-647 (Abcam) and Goat Anti-Mouse IgG Alexa-488 (Santa Cruz Biotechnology) in 1:250 for 1 h at room temperature. Nuclei were counterstained with 4’,6-diamino-2-phenylindole (DAPI) obtained from Sigma. The tissue was then covered with Fluoromount™ Aqueous Mounting Medium (Sigma) and imaging was performed with a Zeiss LSM510 confocal microscope at the Danish Molecular Biomedical Imaging Center (DaMBIC), University of Southern Denmark.

**Periodic Acid-Schiff staining**

The 5-μm-thick sections were fixed with 10% formalin followed by hydration in water. The tissue was stained with Periodic Acid Solution (Sigma) for 5 min, Schiff’s Reagent for 15 min, and Hematoxylin solution for 90 sec. Samples were dehydrated in increasing concentrations of ethanol (70%, 90%, 95%, and 100%) followed by incubation in Xylene and finally sealed with a
coverslip using Fisher Chemical™ Permount™ Mounting Medium (Fisher Scientific). Goblet cells were observed and counted in individual well-preserved colonic crypts using a Leica DMRBE microscope.

**Transmission electron microscopy**

A fragment of the distal part of the mouse colon was obtained and the tissue was fixed and processed for transmission electron microscopy (TEM) imaging according to standard methods (Almeida Junior et al., 2017). Goblet cells and enterocytes were observed using a JEOL 1400 plus transmission electron microscope at the Department of Pathology, Odense University Hospital.

**Western blotting**

Isolated CECs were lysed with RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease inhibitor cocktail (Sigma). The proteins were separated by 10%–15% SDS-PAGE and blotted onto a PVDF membrane (Bio-Rad). Membranes were blocked in TBS containing 0.1% Tween® 20 (Fisher BioReagents) and 5% skim milk powder (Oxoid) for 1 h at room temperature. Membranes were incubated with total β-catenin (H-102) (Santa Cruz Biotechnology), non-phospho (active) β-catenin (Ser33/37/Thr41); p-53 (Ser15), and EphB2 (D2X2I) obtained from Cell Signaling, p-GSK-3α/β (Tyr 279/216), p21 (F-5), and DKK-1 (B-7) from Santa Cruz Biotechnology. β-actin (Cell Signaling), GAPDH (Sigma), or β-tubulin (Thermo Fisher Scientific) were used as loading control. Typically, the primary antibodies were used at 1:1000 at 4°C overnight incubation. After washing 3 times with TBST, the membranes were incubated with Goat Anti-Rabbit IgG-AP (Santa Cruz Biotechnology) or Goat Anti-Mouse IgG-AP (Cell Signaling) at 1:20000 for 1 h at room temperature. Protein–antibody complexes were visualized using a chemiluminescent substrate Tropix® CDP-Star® (Applied Biosystems) and detected on CL-X Posure™ Film (Thermo Scientific). Each western blot was repeated at least three times and the protein expression levels were determined by densitometry analysis and ImageJ.

**DKK1 gene sequencing and CpG island methylation analysis**
Genomic DNA from CECs derived from four Msh2+/- and four Msh2-/- mice was isolated using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma). The DKK1 gene was amplified using primer sequences listed in Supplementary Table S2 and the Phusion High-Fidelity PCR Kit (Thermo Fisher Scientific). PCR conditions consisted of initial denaturation at 98ºC for 3 min followed by 35 cycles of denaturation at 98ºC for 10 sec, annealing at 63ºC for 30 sec and extension at 72ºC for 2 min completed with final extension at 72ºC for 10 min. The PCR products were analyzed on 1% agarose gel and bands corresponding to 3570 bp PCR product were cut out and DNA was purified using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific).

For analyses of the methylation status of DKK1, genomic DNA was bisulfite converted using the EpiTect® Bisulfite Kit (Qiagen). A region encompassing a predicted CpG island was amplified with specific primers for bisulfite conversion-based PCR. The primers were designed using MethPrimer Version 2.0 (primer sequences are listed in Supplementary Table S2). The JumpStart™ Taq DNA Polymerase (Sigma) was applied and PCR conditions consisted of initial denaturation at 94ºC for 3 min followed by 40 cycles of denaturation at 94ºC for 30 sec, annealing at 59ºC for 30 sec and extension at 72ºC for 1 min completed with final extension at 72ºC for 10 min. PCR products were analyzed by 1.5% agarose gel electrophoresis and bands corresponding to 388 bp were gel extracted and submitted to sequencing. All samples, i.e. the entire DKK1 gene and the methylation-specific PCR-purified products, were sequenced by Eurofins Genomics using the same primers as used for PCR amplifications. Bisulfite sequencing DNA methylation data were analyzed with the BDPC DNA methylation analysis platform (BISMA).

**Statistical analysis**

Data are presented as mean±standard deviation. All graphically presented data were analyzed using GraphPad Prism version 7.0 and two-tailed t-tests. A value of p<0.05 was considered statistically significant. Asterisks are defined as *p<0.05, **p<0.01, and ***p<0.001.

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**Conflict of interest:**

none declared.

**Author contributions**

K.N. and A.B. designed the study. K.N. planned the experiments, conducted data collection and analysis, and wrote the manuscript. C.M., N.C., M.L.C., C.L.M., and S.S.N. contributed to data collection and preparation of the manuscript. T.E.T. provided important reagents and helped with analysis of the results and preparation of the manuscript. A.B. designed and supervised the study, performed data collection and analysis, and wrote the manuscript.

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**Figure 1** MMR deficiency mediates strong stem cell-like phenotype that supports enhanced proliferation in colon epithelial cells. (A) A typical colonic crypt that comprises stem cells, progenitor cells (transit amplifying cells), and differentiated cells (goblet, enterocytes, and enteroendocrine cells). The gradient expression of WNT, Notch, and BMP pathways as well as EphB2 is denoted. (B) mRNA expression levels of the stem cell markers *EphB2*, *Ascl2*, and *CD24* relative to β-actin in Msh2+/− and Msh2−/− CECs. Each dot represents an individual mouse. (C) Western blot analysis of EphB2 expression in CECs from Msh2+/− and Msh2−/− mice and representative images showing its expression in Msh2+/− and Msh2−/− colonic crypts. The intensity of western blot bands relative to their respective GAPDH bands was measured by densitometry analysis and ImageJ. This analysis was carried out with three individual mice per genotype from 3 individual western blots. (D) Protein expression of active and total β-catenin, the negative cell cycle regulator, p21(CIP1/WAF1) and p-p53(Ser15) in Msh2+/− and Msh2−/− CECs. The intensity of western blot bands relative to their respective β-actin or β-tubulin bands was determined by densitometry analysis and ImageJ in 3 individual mice per genotype.
Fig. 1
**Figure 2** Analysis of the main factors involved in the regulation of WNT signaling. (A) Representative western blot of active p-GSK3-α/β (Tyr 279/216) in Msh2+/− and Msh2−/− CECs. (B) mRNA expression levels of the components of the β-catenin destruction complex, APC and Axin2, and the WNT signaling pathway inhibitor DKK1 in Msh2+/− and Msh2−/− CECs. (C) Expression of DKK1 in Msh2+/− and Msh2−/− CECs detected by western blot. The numbers indicate individual mice. The intensity of western blot bands relative to their respective β-tubulin bands was measured by densitometry analysis using ImageJ in 3 individual mice per genotype. (D) Schematic representation of the DKK1 region containing two CpG islands. The position of the transcription start site, the CpG islands, and the primers used in the methylation-specific PCR are shown. (E) Bisulfite sequencing analysis of the DKK1 CpG island (+380 to +720). Genomic DNA isolated from purified CECs, matching the cell lysates used for western blot in C, was used for bisulfite sequencing analysis. The numbers on the left correspond to the individual mice also used in C. Open circles indicate unmethylated cytosines and black circles show methylated cytosines. Percentages indicate the fraction of methylated cytosines.
Figure 3 MMR deficiency impacts the normal differentiation and function of the intestinal goblet cells. (A) mRNA expression levels of the goblet cell-specific markers *Muc2* and *Klf4*. (B) Expression of Muc2 protein (green) and β-catenin (red) in Msh2+/− and Msh2−/− colonic crypts. (C) Goblet cells visualized by staining of colonic tissue with PAS reagent. Mucins that are produced by the goblet cells are stained in magenta. The number of goblet cells per colonic crypt is determined by averaging the number of goblet cells counted in at least 7 morphologically well-preserved crypts of each mouse (n=6). (D) Transmission electron microscopy analysis of goblet cells (indicated by arrows) showing differences in their cell size and mucin-loaded vesicles.
Fig. 3
**Figure 4** Analysis of the enterocytes and enteroendocrine cells in Msh2 mutant mice. (A) Analysis of the mRNA levels of *SIM*, a specific marker of intestinal enterocytes, and the transcription factors *Hnf1β* and *Cdx2* that regulate their differentiation. (B) Representative transmission electron microscope pictures showing normal enterocyte morphology, cell polarity, and microvilli formation. (C) Analysis of mRNA expression of *SYP* and *Nkx2.2* markers for intestinal enteroendocrine cells.
Fig. 4
Figure 5 Analysis of Notch and BMP signaling pathways in colonic tissue derived from Msh2 mutant mice. (A) The expression levels of the Notch responsive genes Hes1 and Math1 are shown relative to β-actin. (B) mRNA expression levels of BMP receptors BMPR1 and BMPR2, BMP ligands BMP2 and BMP4, and the signal transducer Smad4. (C) Expression of BMP2,4 ligands (green) and the proliferation marker Ki-67 (magenta) in colonic crypts from Msh2+/− and Msh2−/− mice.
Fig. 5
**Supplementary Figure S1.** Visualization of the proliferating compartment of colonic crypts from Msh2<sup>+</sup>- and Msh2<sup>-/-</sup> mice. Proliferating cells (Ki67-positive) visualized by immunofluorescence staining of colonic crypts derived from Msh2 WT and Msh2 deficient mice.

**Supplementary Figure S2.** mRNA expression levels of the stem cell markers EphB3 and CD44.

**Supplementary Table S1.** DNA oligonucleotide primers used for qPCR analysis

**Supplementary Table S2.** DNA oligonucleotide primers used for DKK1 gene mutation and CpG island methylation analysis and sequencing