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Morfologická a funkční charakterizace střevního epitelu z hlediska exprese protein LGR4

Morphological and functional characterization of intestinal epithelium in the context of LGR4 expression

Diplomová práce

Vedoucí závěrečné práce: Mgr. Vítězslav Kříž, Ph.D.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedl všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

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Abstrakt

Leucine-rich repeat containing G-protein-coupled receptor 4 a příbuzné LGR5 a LGR6 proteiny představují B podskupinu transmembránových proteinů patřícím k rodině G-protein spřaženým receptorům (GPCRs). LGR4 je exprimován v širokém spektru embryonálních a dospělých tkání. Na určitém genetickém pozadí bývá absence LGR4 spojována s embryonální/perinatální letalitou. Funkce LGR4 je nejvíce svázána se stimulací Wnt dráhy po interakci LGR4 se svými ligandy, R-spondiny. Pro získání nástroje pro klasifikaci LGR4 specifických populací a pro charakterizaci LGR4 interakčních partnerů, jsme vytvořili epitopově značenou LGR4 myš s tripleinfluenza hemagglutininovou značkou (3HA) vloženou do N koncové části proteinu LGR4 ($Lgr4^{3HA/3HA}$). $Lgr4^{3HA/3HA}$ myš je životaschopná a plodná. Imunohistochemie založená na použití anti-HA protilátky odhalila podobný expresní vzor v tenkém a tlustém střevě, jaký byl již dříve detekován pomocí anti-LGR4 protilátek. V tenkém střevě byl zpozorován silný signál v Panethových buňkách, v rychle se dělících buňkách označovaných jako transit amplifying cells a v buňkách kmenových. Naopak, v tlustém střevě byl zaznamenám nejsilnější signál na vrchní části krypt, který slábnul směrem k bázi krypt. Kromě toho jsme sledovali expresi *Lgr4* i na úrovni mRNA. Zatímco v tenkém střevě byla *Lgr4* mRNA přítomna převážně na dně krypt, v tlustém střevě byl signál více rozptýlený ve střední části krypt. Použitím průtokové cytometrie jsme mohli charakterizovat genovou expresi LGR4 pozitivních buněk tenkého střeva. Dále jsme pomocí anti-HA magnetických kuliček byli schopni imunoprecipitovat LGR4 protein pro hmotnostní spektrometrii, která může být využita pro identifikaci jeho vazebných partnerů.

Klíčová slova:

Leucine-rich repeat containing G-protein-coupled receptor 4/5 (*Lgr4/5*), tenké střevo, tlusté střevo, immunohistochemie (IHC), western blot (WB), immunoprecipitace (IP), Fluorescenčně aktivovaná průtoková cytometrie (FACS), quantitativní real-time polymerázová řetězová reakce (qRT-PCR), geneticky modifikovaný myší model, hemagglutininová (HA) značka, TALEN

Abstract

Leucine-rich repeat containing G-protein-couple receptor 4 and related LGR5 and LGR6 proteins represents a B subgroup of transmembrane proteins belonging to the G-protein-coupled receptors (GPCRs) family. LGR4 is expressed in the broad spectrum of embryonic and adult tissue and at certain backgrounds its deficiency is connected with embryonal/perinatal lethality. The function of LGR4 is mainly characterised in relation with promotion of Wnt signalling upon binding its ligands R-spondins. To obtain a tool for classification of LGR4 specific populations and for characterization LGR4 interaction partners, we have generated epitope-tagged LGR4 mouse with tripleinfluenza hemagglutinin tag (3HA) inserted into N terminal part of LGR4 protein ($Lgr4^{3HA/3HA}$). $Lgr4^{3HA/3HA}$ mouse is viable and fertile. Anti-HA antibody based immunohistochemistry revealed similar expression pattern in the small intestine and in the colon, which was previously detected with anti-LGR4 antibodies.In the small intestine, a strong signal was observed in Paneth cells, transit amplifying cells and in stem cells. Conversely, in the colon the strongest signal was noticed at the upper part of colonic crypts and it diminished towards crypt base. Besides that, we have followed *Lgr4* expression at the mRNA level. While in the small intestine, *Lgr4* mRNA was presented mostly at the crypt bottom; in the colon, the signal was more dispersed in the central part of the colonic crypt. Using flow cytometry, we could characterize gene expression profile LGR4 positive cells from the small intestine. Finally, by anti-HA magnetic beads, we were able to immunoprecipitate LGR4 protein for mass spectrometry, which can be employed for identifying its binding partners.

Key words:

Leucine-rich repeat containing G-protein-coupled receptor 4 (*Lgr4*), small intestine, colon, immunohistochemistry (IHC), western blot (WB), immunoprecipitation (IP), Fluorescence-activated cell sorting (FACS), quantitative real-time polymerase chain reaction (qRT-PCR), gene modified mouse model, hemagglutinin (HA) tag, TALEN

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List of abbreviations:

ALPI	Intestinal alkaline phosphatase
APC	Adenomatous polyposis coli
APC	Streptavidin-allophycocyanin
3HA	triple influenza hemagglutinin
7TM	seven transmembrane configuration
APC	Adenomatous polyposis coli
Bmi1	Polycomb protein lymphoma Mo-MLV insertion region 1 homolog
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CaMKII	Calcium/calmodulin-dependent protein kinase II
Cas	Clustered regularly interspaced short palindromic repeats -associated
CBC	Crypt base columnar cells
CK1α	Casein kinase 1 α
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	Clustered regularly interspaced short palindromic repeats ribonucleotide acid
Crypt	Cryptidin
Def5	Alfa-defensin 5
DEPC	Diethylpyrocarbonate
Dhh	Desert hedgehog
Dl1/3/4	Delta 1/3/4
DMEM	Dulbecco´s modified eagle medium
DR	Direct repeat
DSB	Double strand break
Dvl	Dishevelled
ESC	Embryonic stem cells
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
Fzd	Frizzled
Gli 1-3	Glioma-associated oncogene 1-3
gRNA	Guide-ribonucleotide acid
GSK-3β	Glycogen synthase kinase 3 β

HES	Hairy/Enhance of split
Hhip	Hh interacting protein
HR	Homologous recombination
IHC	Immunohistochemistry
Ihh	Indian hedgehog
InDel	Insertion or deletion of nucleotide
INSL3	Insulin-like peptide 3
IP	Immunoprecipitate
IQGAP1/3	IQ motif containing GTPase-activating protein 1 and 3
Jag 1/2	Jagged 1/2
JNK	c-Jun-N-terminal kinase
JP	Juvenile Polyposis
LacZ	β-galactosidase
LEF	Lymphoid enhancer-binding factor
LGR	Leucine-rich repeat containing G protein-coupled receptor
Lrp5/6	Low-density lipoprotein receptor-related protein 5/6
LRR	Leucine repeats
Mmp7	Matrix metallopeptidase 7
Muc2	Mucine 2
mRNA	messenger RNA
NHEJ	Non-homologous recombination
NICD	Intracellular domain of Notch receptor
Olf4	Olfactomedin 4
PAM	Protospacer adjacent motif
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PKC	Protein kinase
PORCN	O-acetyltransferase Porcupine
pre-crRNA	Precursor of clustered regularly interspaced short palindromic repeats ribonucleotide acid
Ptch	Patched receptor
qRT-PCR	Quantitative real-time polymerase chain reaction
Rac1	GTPases ras-related C3 botulinum toxin substrate 1

RBPJ	Recombination signal binding protein for immunoglobulin Kappa J region
RhoA	Ras homolog hebe family member A
Rho-kinase	Rho-associated kinase
RNF43	Ring finger ligase 43
RSPO	R-spondins
RT	Room temperature
RVD	The repeat-variable diresidue
Shh	Sonic hedgehog
Smo	Smoothened receptor
TA cells	Transit amplifying cells
TALE	Transcription activator-like effector
TALEN	Transcription activator-like effector nuclease
TCF	T-cell factor
TCL	Total cell lysate
TGF-β	Transforming growth factor-β
tracrRNA	Trans-activating clustered regularly interspaced short palindromic repeats ribonucleotide acid
TSR-1	Thrombospondin type 1 repeat
Ubb	Ubiquitin B
WB	Western blot
Wls	Wntless transmembrane protein
WT	Wild type
ZF	Zinc finger
ZFN	Zinc finger nuclease
ZNFR3	Zinc and ring finger ligase 3
β-TrCP	F-box-containing β-transducin repeat containing

1 Introduction

Wnt signalling is one of the most important signalling pathways in the maintenance of intestine homeostasis. Several studies have revealed that mutations in members of this pathway could cause intestinal crypts demission and excessive cell proliferation. The leucine-rich repeat-containing G protein-coupled receptor 4 (*Lgr4*) represents an additional receptor of Wnt signalling. As ligands for this receptor, R-spondins were identified. It was confirmed that creation of R-spondin-*Lgr4* complex boosts Wnt signalling in many ways. The expression of *Lgr4* was, except for other organs, also detected in the small intestine and colon. During investigation of *Lgr4* functions, mice from several genetic backgrounds were developed. The results concluded that in some knock-out mice with homozygous deletions of *Lgr4*, there were a lot of structural changes in the small intestine, such as lower depth of crypts together with reduction of epithelial proliferation, mostly associated with Paneth cells. Deletion of *Lgr4* use to be also connected with perinatal mortality. A homologue partner of *Lgr4*, *Lgr5* was identified among other as a marker of intestinal stem cells. The phenotype of mice with homozygous deletion in *Lgr5* gene also caused mortality in very young mice, like in some cases of homozygous deletions of gene *Lgr4*. For better morphological and functional analyses, our laboratory generated epitope (triple hemagglutinin; 3HA)-tagged Lgr4 mouse ($Lgr4^{3HA/3HA}$). The 3HA tag provides better possibilities to investigate of *Lgr4* gene with specific anti-HA antibody.

2 Aims of the thesis

The aims of my thesis are to clarify Lgr4 expression profile in the small intestine and colon at different expression levels, to detect co-expression of Lgr4 and Lgr5 in various intestine cell types.

3 Literature review

3.1 Intestine

The intestine is created by two major parts, the small intestine and the colon. In the small intestine, digestive processes are finished and arised nutrients are absorbed. The most important function of the colon is the absorption of soils and water that helps the creation of mucus (mucine) and causes the thickening of the intestine content which comes from the small intestine. The small intestine is divided into three parts: the duodenum, jejunum and ileum. With the naked eye we can notice bordering on the wall of the intestine which is formed by permanent transverse circular folds (plicae circulares, Kerckringi), in which the mucosa (created by epithelial cells, lamina propria and muscularis mucosae) and submucosa extend. These folds reach their greatest abundance in the duodenum and at the beginning of the jejunum, but towards the ileum they gradually decrease. Adjacent to the submucosa, there are two layers of muscularis mucosae (inner circular and outer longitudinal) created by smooth muscle cells, which are responsible for peristaltic movements. The last layer covering the outer surface of the intestine is the serosa. The serosa consists of connective tissue binding the intestine to other organs. The mucosa in the small intestine constitutes finger-like structures called villi that significantly increase the absorbent surface of the mucosa. The villi are longest in the duodenum. Sags between the villi create short tubular glands, the crypts (Lieberkühn crypts) (Fig. 1A). In contrast to the small intestine, Lieberkühn crypts in the colon do not project to the villi (Fig. 1B) (van der Flier and Clevers 2009, Shepers and Clevers 2012, Barker 2014, Bloemendaal 2016, Miyoshi *et al.* 2016).

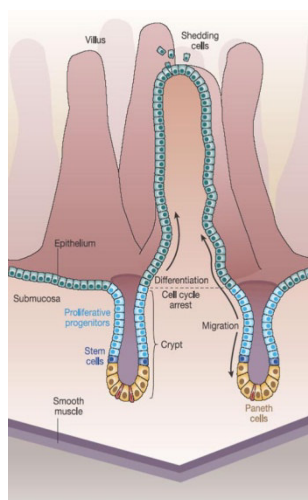

Fig. 1.A

Small intestine (Reya and Clevers 2005)

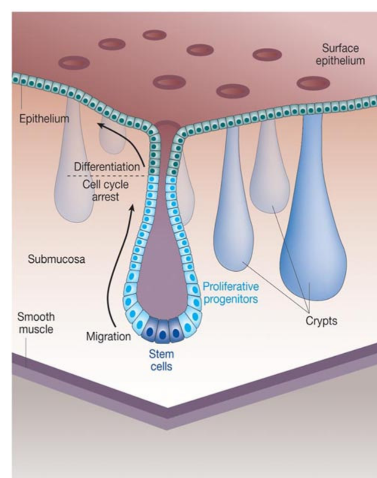

Fig. 1.B

Colon (Reya and Clevers 2005)

The uppermost surface of the villi and the crypts are covered by a simple columnar epithelium. The cells of the epithelium occur in various types. The most numerous cell types of the villi are enterocytes. The individual enterocytes are part of the absorption epithelium and in relation of their content of enzymes, they are responsible for several functions. Among the enterocytes, there are incorporated goblet cells which are responsible for producing mucine that is important for creating a protective mucus barrier which protects the mucosa against pancreatic enzymes and bacteria. The antigen presenting cells of the small intestine represent M cells. These cells populate in the Peyer´s patches and are typical for their immune function. The enteroendocrine cells colonize severe parts of the villi as well as the crypts and their main function is to produce hormones and release them into the lumen. The base of the crypts is covered by Paneth cells. With their production of antimicrobial protein complexes (e.g. defensin, fosfolipase A2 and lysosyme) and polysaccharides, they are important to immune resistance (Sato *et al.* 2011). Intestinal stem cells are located at the basal part of the crypts. In contrary to the small intestine, in the colon there are no Paneth cells (Fig. 2) (reviewed in Potten *et al.* 2009, Clevers and Bevins 2013, Du *et al.* 2015, Miyoshi 2016).

The epithelium is a very dynamic tissue and has to be frequently renewed because of exposure to bacteria toxins, digested substances and mechanical stress. During 4 – 7 days, the old or damaged epithelial cells undergo apoptosis and are shed into the lumen and replaced by new cells migrating from the crypts up to the top of the villi. During this migration, new cells undergo differentiation into specific epithelial cell types. The sources of this self-renewal processes are the stem cells interleaved with Paneth cells, also called the crypt base columnar cells (CBCs), in the intestinal crypt bases. Stemness of the CBC is closely dependent on interactions between CBC and its local microenvironment is named niche. Recent studies noticed that CBCs are responsible for continual renewal processes in the crypts with their marker gene Leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5; Barker *et al.* 2007), Troy (Fafílek *et al.* 2013) and Olfactomedin 4 (Olfm4; van der Flier 2009). CBCs give rise to their progeny, transit amplifying cells (TA cells) and Paneth cells. Whereas after about two days TA cells migrate up the crypts, undergo 4-5 divisions and differentiate into the specialized epithelial cells, Paneth cells migrate to the opposite site (down the crypts, van der Flier and Clevers 2009).

Besides CBCs, there is another type of the cell which has self-renewal ability, slowly cycling +4 cells (Potten *et al.* 1997, Potten *et al.* 2009, Bloemendaal 2016).

This cell is responsible for replenishment of the epithelium after injury. Its typical marker is polycomb protein lymphoma Mo-MLV insertion region 1 homolog (Bmi1; Sangiorgi and Capecchi 2008; Potten *et al.* 2009, Du *et al.* 2015).

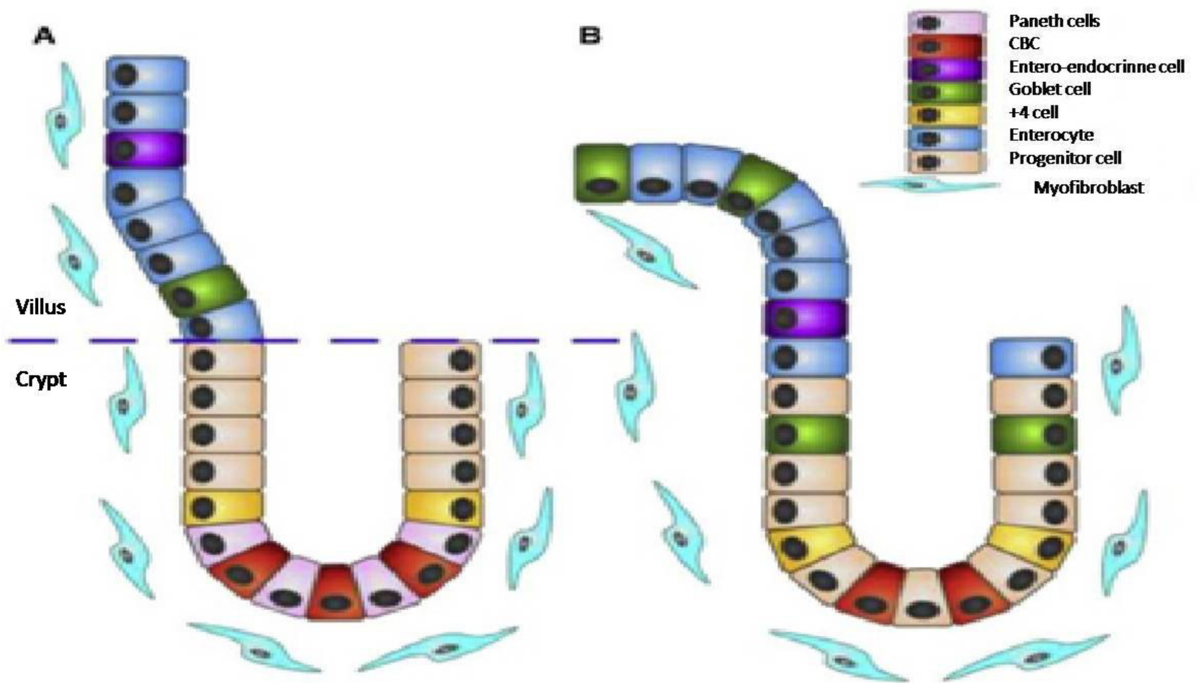

Fig. 2.

Architecture of A) small intestine, B) colon. (reviewed in Bloemendaal *et al.* 2016)

3.2 Regulation of intestinal homeostasis

The homeostasis of the constant renewal intestinal epithelium still has to be sustained and controlled (Lander *et al.* 2012). The influences of various signalling pathways are involved in maintenance of the intestine homeostasis and in its regulation. Bone morphogenetic protein (BMP), Notch signalling, Hedgehog signalling and Wnt signalling are the main pathways keeping balance between cell renewal, apoptosis, cell differentiation and cell migration (Fig. 3).

3.2.1 BMP signalling

Bone morphogenetic proteins are soluble factors belonging to the Transforming growth factor-β (TGF – β) superfamily and play a critical role during embryonic growth and differentiation (Mishina 2003).

BMP signal is transduced through heterocomplex of Ser/Thr kinase transmembrane receptors type I and II (Bmpr1, Bmpr2) that facilitate receptor-mediated phosphorylation of BMP-specific receptor-regulated Smad1/5/8 transcription factors. These already phosphorylated transcription factors create a complex with common mediator Smad4. The complex enters the nucleus and regulates expression of BMR signalling target genes, e.g. Msx – 1, Msx – 2, c- fos, Egr – 1, c- jun (Hollnagel *et al.* 1999, Hardwick *et al.* 2008;).

In some patients suffering from familial Juvenile Polyposis (JP), mutations were found in these proteins resulting in inhibition of BMP signalling pathway. JP is a rare autosomal dominant polyposis syndrome of the gastrointestinal tract (Harned *et al.* 1999, Woodford-Richens *et al.* 2000, Waite and Eng 2003). In the intestine, BMP signalling is activated both in mesenchymal and epithelial cells (Li *et al.* 2006, Kosinski *et al.* 2007). The BMP pathway creates a gradient with the lowest activity at the crypt bottom and the highest at the top of the villi (He *et al.* 2004, Kosinski 2007). By generating transgenic mice overexpressing the BMP inhibitor Noggin, Haramis and colleagues observed that this inhibition leads to the formation of excessive amount of crypt-like structures created by proliferating epithelial cells migrating from intervillus to villus regions. The extension of these structures resulted into dilated cysts, typical for JP. Because BMP signalling has an important role during gastrointestinal development (Haramis *et al.* 2004), following experiments revealed that BMP signalling has an influence on crypt fission via inhibition of suppression of stem cell proliferation (He *et al.* 2004).

3.2.2 NOTCH signalling

Notch signalling is a very important signalling pathway using interactions between neighbouring cells. Developing individuals of the animal kingdom exert this pathway to regulate intercellular relations of stem cells (in the stomach and also in the small intestine), also to influence differentiation and proliferation and to cell survival. Indeed, Notch signalling is dependent on factors which are specifically produced by certain cells and thus this signalling is capable to influence many particular processes that are important for cell differentiation and development (Artavanis-Tsakonas *et al.* 1999, Mum and Kopan 2000, Demitrack and Samuelson 2016).

The Notch signalling pathway is mediated by four types of transmembrane receptors Notch 1-4 and by five types of ligands – Jagged 1 (Jag 1), Jagged 2 (Jag 2), Delta-like 1 (Dl1), Delta-like 3 (Dl3) and Delta-like 4 (Dl4), collectively referred to as DSL (Mumm and Kopan 2000, Chiba, 2006, Kopan and Ilagan 2009). After creation of a complex ligand-

receptor, the receptor undergoes intramembrane proteolysis that causes the release of intracellular domain of Notch receptor (NICD). NICD translocates into the nucleus and regulates the activation of Recombination Signal Binding protein For Immunoglobulin Kappa J Region (RBPJ) protein from its repressor form to activator form. The activator enables transcription of Hairy/Enhance of Split (HES) genes (acting like transcription repressors). In the non-active state, the RBPJ protein stays in repressor form and blocks the transcription (Artavanis-Tsakonas *et al.* 1999, Mumm and Kopan 2000, Baron 2003).

The inhibition of Notch signalling is related to inhibition proliferation and activity of the stem cells, whereas the activation of this pathway is characterised by increased proliferation and activation of these cells. It is known that Notch signalling has a key role as a regulator of self-renewal of stem cells in the gastrointestinal tract (Demitrack and Samuelson 2016) and also controls the fate of small intestinal cells in differentiation either to secretion or absorption lineages (Vanuytsel *et al.* 2013).

3.2.3 HEDGEHOG signalling

Hedgehog signalling pathway influences the development of several organ systems (including organs of the gastrointestinal tract) (Bitgood and McMahon 1995, Ramalho-Santos *et al.* 2000, van den Brink, 2007).

In vertebrates, there are three paralogues of the Hh gene, Sonic hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh) (Ingham *et al.* 2011). Patched (Ptch) transmembrane receptor was identified as the first receptor for Hh proteins (Nakano *et al.* 1989, Hooper and Scott 1989). In "offstate" (no Hh ligand binding), Ptch receptor itself affects a transmembrane receptor Smoothened (Smo) by inhibiting its positive role in activation of relevant transcription factors from the protein family Glioma-associated oncogene (Gli 1 – 3), major effectors specific for Hh signalling pathway. The "onstate" of Hh signalling is mediated by binding of Hh ligands on Ptch receptors. When Hh ligands are present and create complex Hh–Ptch, Smo receptor is released, enabling activation of Gli transcription factors and thus, transcription of target genes, such as p53, Gli1, Bcl-2, Myc, N-myc, Cyclin D, Hh interacting protein (Hhip) and Bmi1 (Ferreti *et al.* 2005, Kolterud and Tofgard 2007).

In adults, active the Hh pathway manifests its influence on regulation of tissue homeostasis and its repair and self-renewal, and also on stem cell maintenance (Hooper and Scott 2005). Kolterud *et al.* (2009) revealed that in embryonic intestine, this signalling

pathway is always paracrine, mediated by interaction between ligands produced by an epithelium and receptors that are located in mesenchyme cells. This paracrine signalling continues till adult life. After inhibition of Hh signalling (by pan-Hh inhibitor or by knock-out of Hh ligand secreted by epithelium), there are visible histological changes. In the case of mesenchyme, there was obvious loss of smooth muscle differentiation. Whereas in epithelium, a huge epithelial proliferation was observed accompanied with deep crypts and blunted villi (Zacharias *et al.* 2010, van Dop *et al.* 2010). In the case of relationships to other signalling, this constitutive active epithelial/mesenchyme cross-talk induces Bmp signalling (Madison *et al.* 2005, Mao *et al.* 2010) but, on the other hand, it was discovered that inhibition of Hedgehog signalling pathway causes elevation of Wnt signalling pathway (van den Brink *et al.* 2004, Madison *et al.* 2005, Zacharias *et al.* 2010).

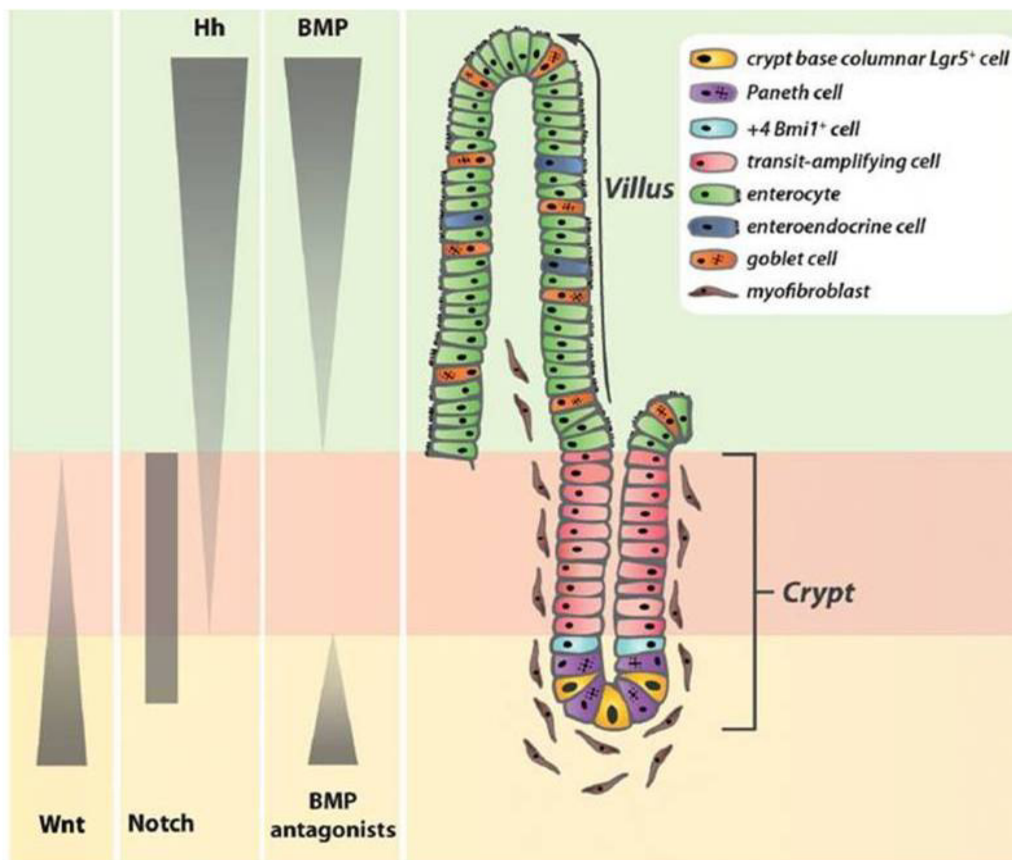

Fig. 3.

Illustration of cell populations creating epithelium of the small intestine (modified from Krausova and Korinek 2012).

3.2.4 WNT signalling

Wnt genes represent a part of a large secretory polypeptides family which are expressed locally and tissue-specifically (Wodarz and Nusse 1998). Secretory proteins from the Wnt family are crucial members in many biologic processes such as development, differentiation and proliferation (reviewed in Logan and Nusse 2004, Gregorieff and Clevers 2005, Krausova and Korinek 2014).

Wnt ligands perform 19 secreted glycoprotein morphogens (Papkoff *et al.* 1987) which play a key role in Wnt signalling pathways. Like many mature proteins, Wnt proteins undergo a lot of modifications that are necessary for ligand maturation.The first of these modifications is palmitoylation which is provided by O-acetyltransferase Porcupine (PORCN) and is situated in the endoplasmic reticulum (Willert *et al.,* 2003, Takada *et al.* 2006). After palmitoyl modification, Wnt proteins are bonded to a transmembrane protein Wntless (Wls) which is responsible for the secretion of lipid-modified Wnts (Bänziger *et al.* 2006). In dependence on binding Wnt ligands on their receptors, Wnt ligands stimulate β-catenin dependent (‘‘canonical’’) or β-catenin independent (‘‘non-canonical’’) pathways (Valenta *et al.* 2011, Cruciat and Niehrs 2012, Anastas and Moon 2013).

Integration of Wnt ligands to the canonical or non-canonical signalling groups is not specified exactly. Some of the Wnt ligands, namely Wnt1, Wnt3 and Wnt8, were noticed as the most important members of canonical pathway due to their regulation of Wnt target genes (in the case of Wnt3a - Bmp2, Lef1 or Fgf8) (Shimizu *et al.* 1997, Kengaku *et al. 1998,* Miller *et al.* 2001, Willert *et al.*, 2003). Others Wnts are connected mostly with non-canonical signalling like Wnt 4, Wnt5a and Wnt11 (Davis *et al.* 2008, Komiya and Habas 2008, Nishita *et al.* 2010). But some later studies observed that Wnt5A and Wnt11 could have an influence on canonical pathways based on receptor context (Tao *et al.* 2005, Mikels and Nusse 2006, Ying *et al.* 2008).

Canonical pathway

The canonical Wnt signalling pathway regulates β-catenin stabilization and its subsequent translocation into nucleus. In the presence of Wnt, Frizzled (Fzd) and its co-receptor Low-density lipoprotein receptor-related protein 5/6 (Lrp5/6) forms a complex with Wnt ligand. Upon activation Frizzled recruits Dishevelled (Dvl) from cytoplasm to the cell membrane, Lrp5/6 is phosphorylated by casein kinase 1 α (CK1α) and Glycogen synthase kinase 3 β (GSK-3β). Phosphorylated Lrp5/6 aggregates of several proteins as axis inhibition

protein Axin, Amer1 and Adenomatous polyposis coli (APC).Recruitment of this protein complex to the cell membrane enables accumulation of β-catenin in the cytoplasm and its transfer to nucleus. In the nucleus, β-catenin binds by its transactivation domain of the lymphoid enhancer-binding factor/T-cell factor (LEF/ TCF) family transcription factors (TCFs) (Bienz and Clevers 2003, Bejsovec 2005, Gordon and Nusse 2006, Tanneberger *et al.* 2011). TCF/β-catenin promotes transcription of several genes as c-myc (He *et al.* 1998), cyclin D1 (Tetsu and McCormick 1999) and Axin2 (Lustig *et al.* 2002).

In the absence of Wnt, β-catenin does not accumulate in the cytoplasm. It is bound by destruction complex consisting of APC, Axin1, Amer1 and two serine-threonine kinases CK1α and GSK-3β. Recruited β-catenin is phosphorylated (He *et al.* 2004). By this phosphorylation, the β-catenin is identified and ubiquitinated by F-box-containing beta-transducin repeat containing (β-TrCP), subunit E3 of ubiquitin ligase, and intended for degradation through a proteasome (Aberle *et al.* 1997). When β-catenin is degraded, there is no activation of the transcription factor TCF and so TCF is associated with the transcription repressor Groucho. Hereby, the expression of Wnt-responsive genes is blocked (Roose and Clevers 1999).

The β-catenin Wnt signalling pathway is critical in regulation of the stem cells in the process of differentiation and in the maintenance of intestinal crypts (Fevr 2007). There are two sources of Wnt signalling; the first source is represented by mesenchyme that produces several Wnt ligands such as Wnt2b, Wnt4 and Wnt5a and the other source is Paneth cells (review by Clevers 2014). Paneth cells are an important part of the niche, especially because of their production of Wnt3a (Farin 2012). Wnt3a gradient production correlates with proliferation and differentiation of different cell types along the crypt and villi. The highest Wnt activity is at the bottom of the crypts, where stem cells are present.Wnt activity gradually decreases toward the intestinal lumen which is connected to cell differentiation (van der Flier *et al.* 2009, Clevers 2013). Several studies confirmed the essential role of the Wnt pathway in the preservation of stem cell proliferation. Genetic disruption of Wnt effectors Tcf4 (Korinek 1998) and β-catenin (Ireland *et al.* 2004, Fevr *et al.* 2007) leads to intestinal crypts demission. Contrarily, Wnt pathway over-activations by Wnt agonist R-spondin causes an increased stem cell number (Kim *et al.* 2005).

Noncanonical pathways

Non-canonical Wnt pathways are autonomous on β-catenin signalling. There are two main branches of β-catenin independent Wnt signalling: Fzd/Planar Cell Polarity (PCP) and Wnt/Ca^{2+} pathway (reviewed in Schulte 2010, Najdi *et al.* 2012).

The Fzd/PCP signalling pathway was first identified in Drosophila (Gubb and García-Bellido 1982). This signalling is mediated by Fzd and Dvl, leading, in this case, to activation of specific GTPases ras-related C3 botulinum toxin substrate 1 (Rac1) and ras homolog gene family member A (RhoA), resulting in activation of kinases, such as c-Jun-N-terminal kinase (JNK) and Rho-associated kinase (Rho-kinase). Active PCP pathway influences cells behaviour, such as their motility, division and rearrangement (Adle, 2002, Wang and Nathans 2007). Even though Fzd/PCP does not play thus crucial role in intestine homeostasis as β-catenin pathway, it is important for oriented cell divisions in developing gut epithelium (Matsuyama *et al.* 2009).

The Wnt/Ca^{2+} pathway is also one of the major Wnt non-canonical signalling pathways where Wnt5a is needed (Kohn 2005). In this case, calcium cations are released from intracellular stores (Slusarski *et al.* 1997) which afterwards stimulate the next on calcium dependent cellular processes (Kühl *et al.* 2000, Klaus and Birchmeier 2008) and proteins, calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) (Sheldahl *et al.* 1999, Kühl *et al.* 2000) influencing inhibition of the canonical Wnt signalling pathway (Kühl *et al.* 2000).

3.3 R-spondins (RSPO)

R-spondins (RSPOs) are Wnt pathway activators. There are four R-spondin (RSPO 1-4) members of a large group of thrombospondin type 1 repeat (TSR-1)-containing proteins. The increase of phosphorylation of Wnt/Fzd coreceptor LRP6 and nuclear accumulation of β-catenin upon RSPO-mediated Wnt signalling suggested that RSPOs participate in enhancing Wnt signalling (reviewed in de Lau *et al.* 2012) (Kazanskaya *et al.* 2004, Kim *et al.* 2005). It was recently disclosed that Leucine-rich repeat-containing G protein-coupled receptors 4 and 5 (Lgr4/5) are receptors for RSPOs and that RSPOs physically interact with extracellular domains and create complexes with them (Kazanskaya *et al.* 2004, Carmon *et al* 2011, de Lau *et al.* 2011, Ruffner *et al.* 2012,).

Hao and colleagues demonstrated that LGR4 and cell-surface transmembrane E3 ubiquitin ligase zinc and ring finger 3 (ZNRF3) together with its homologue ring finger (RNF43) creates a complex of receptors for RSPOs (Hao *et al.* 2012). ZNRF3 and RNF43 ligases represent negative regulators of Wnt signalling. If there are no RSPOs, these ligases bind and ubiquitylate frizzled receptors, which leads to frizzled and LRP6 degradation, and to attenuation of Wnt signalling. However, in RSPOs presence, Lgr4/5 and RSPO make a complex with ZNRF3 and RNF43 which is internalized from the membrane. It leads to an increase of frizzled and LRP6 levels and enhancement of Wnt signalling (Fig. 4) (Hao *et al.* 2012, Koo *et al.* 2012).

As stated above, the interaction of RSPO ligands with an LGR4 receptor participates in activation of the Wnt/β-catenin signalling pathway. However, according to recent data, the Wnt activation in the present of RSPO is connected particularly with E3 ubiquitin ligase internalization, but even stronger Wnt activation comes from the RSPO-LGR4 complex itself. How the interaction between RSPO-LGR4 complex and Wnt signalosome proceeds was investigated. By co-immunoprecipitation, two potential candidate genes were identified. These are IQ motif containing GTPase-activating protein 1 and 3 (IQGAP1 and IQGAP3) which play roles in intracellular signalling mediation. The interaction of RSPO with LGR4 enhances the affinity of IQGAP1 to the cytoplasmic mediator DVL. This stronger affinity causes the RSPO-Lgr4 to create a supercomplex with the Wnt receptor system resulting in augmentation of canonical Wnt signalling. The complex RSPO-Lgr4-IQGAP1 also plays an important role in non-canonical Wnt signalling by regulation of F-actin assembly, which is responsible for coordination of the cytoskeletal organization (Carmon *et al.* 2014).

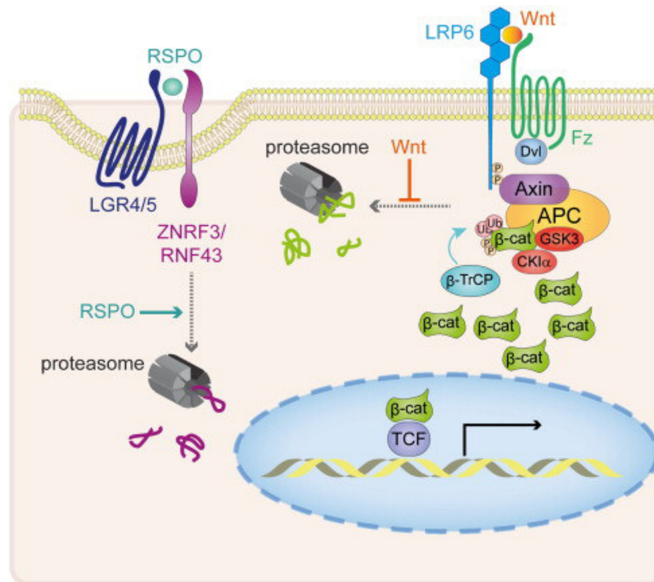

Fig. 4.

Canonical Wnt signalling. RSPOs enhance Wnt signalling via binding on LGR4/5 and recruiting of ZNRF3 and RNF43 ligases (Krausova and Korinek, 2014).

3.4 LGR proteins

The leucine-rich repeat-containing G protein-coupled receptors (LGRs) represent a group of receptors characterized by the presence of seven transmembrane configuration (7TM) large outer domain (ectodomain) with multiple leucine repeats (LRR) and unique linking region connecting those features together (Hsu *et al.*, 2003). LRR form horseshoe-like structures and offer themselves as a region for the binding of ligands (Hsu *et al.* 1998). The 7TM region plays a role in G protein activation and subsequent downstream signalling (Luo *et al.* 2005).

Groups of LGR proteins could be divided into three subgroups (groups A, B and C) (Luo *et al.* 2005, Barker *et al.* 2013).

Higher similarity occurs among proteins from group A and C in which the extracellular domains contain 7 – 9 LRR (group C is typical by its additional presence of on cystein-rich motif low-density lipoprotein receptor class A). Group A includes receptors binding glycoprotein hormones – follicle-stimulating hormone receptor (*Lgr1*), luteinizing hormone receptor (*Lgr2*) and thyroid-stimulating hormone receptor (*Lgr3*). Members of the C group are *Lgr7* and *Lgr8* with their ligands – relaxin and insulin – like peptide 3 (INSL3). (Hsu *et al.* 2000, Luo *et al.* 2005).

In contrast to receptors from A and C groups, receptors from B group contain ectodomains that are characterised by the presence of 13 – 18 LRR. This group is represented by three receptors: *Lgr4*, *Lgr5* and *Lgr6*.

3.4.1 LGR6

The ectodomain of the *Lgr6* gene contains only 13 leucine-rich repeats (Hsu *et al.* 2000).

The receptor Lgr6 was specified like a Wnt signalling independent marker of stem cells in various organs such as skin (sweat glands and interfollicular epidermis), nails, lungs, and taste buds (Snippert *et al.* 2010, Oueztuerk-Wider *et al.* 2012, Ren *et al.* 2014, Lehoczky and Tabin 2015). Stem cell marker LGR6, it plays an important role during wound repair, development of hair follicle and nail regeneration. But a knock-out mouse of the *Lgr6* gene did not display any defects and those animals were healthy and fertile (Snippert *et al.* 2010, Lehoczky and Tabin 2015). Zhang *et al.* (2015) generated knock-in mice to investigate the expression of the *Lgr6* gene. They noticed that *Lgr6* positive cells give rise to hair cells *in vitro* and that they are also supposed to be marks of hair progenitors in the Corti organ.

3.4.2 LGR5

The *Lgr5* gene represents one of the Wnt target genes. Its expression was noticed in intestine stem cells which are characterized by active proliferation in the case of an active Wnt signalling pathway (Barker *et al.* 2007). LGR5 as the stem cell marker was later identified in other various mice tissues such as stomach, hair follicles, kidney, taste buds, ovary and mammary glands (Jaks *et al.* 2008, Barker *et al.* 2010b, Barker *et al.* 2012; Plaks *et al.* 2013, Yee *et al.* 2013, Ng *et al.* 2014). It was confirmed that LGR5 positive intestinal stem cells are multipotent and actively proliferating, giving rise to all differentiated intestinal cell types (Barker *et al.* 2007, Sato *et al.* 2011).

As a member of Wnt/Tcf4 target genes, expression of *Lgr5* was detected in colorectal cancers (van de Wetering *et al.* 2002), basal cell carcinomas (Tanese *et al.* 2008) and its overexpression was noticed in ovary and liver tumours (McClanahan *et al.* 2006, Zucman-Rossi *et al.* 2007). In mice with homozygous mutation in this gene, neonatal mortality was discovered. This is caused by ankyloglossia, characterised by fusion of the tongue with the floor of the oral cavity characterised (Morita *et al.* 2004). This abnormality is associated with

Lgr5 gene expression in the tongue epithelium and lower jaw in developing embryos. Conditional deletion of *Lgr5* gene in the small intestine in adult mice did not result in a clear crypt phenotype (de Lau *et al.* 2011) which is in contrast to a study where Paneth cells prematurely differentiated in *Lgr5* null neonatal mice (Garcia *et al.* 2009).

The deletion of the *APC* gene in LGR5 positive intestinal stem cells resulted in transformation of these cells and subsequent growing of adenomas at the lumen of the small intestine and colon (Barker *et al.* 2009). Recently, studies concerned with colorectal cancers in human reported that high *Lgr5* expression occurs mostly in the basal layer of adenoma cells and that this expression extends in many cell lines of colon cancer in the metastatic stage (Uchida *et al.* 2010, Takahashi *et al.* 2011). LGR5 positive tumour cell populations are in relation with poor prognosis in patients with colorectal carcinomas. It all suggests that LGR5 stem cells represent a predominant cell-of-origin of colorectal cancers (Merlos-Suárez *et al.* 2011).

3.4.3 LGR4

In mice with a marked *Lgr4* gene, the expression was manifested in some locations. Strong expression was detected in cartilage, heart, kidneys, adrenals, salivary glands, reproductive system, neural system and also in the digestive tract (Van Schoore *et al.* 2005). High levels of expression were also noticed in basal and granular cells of the skin, in the epithelial cells of breast ducts, island cells of the spleen, and also in most cells of a colon tumour (Yi *et al.* 2013). The consequence of phenotype is related to the genetic background of mice models. One of the models was generated by genetic modification resulting in *Lgr4* knock-out mice with characteristic fusion protein formed by extracellular domain of the *Lgr4* and intracellular domain of β-galactosidase enzyme. This *Lgr4* knock-out mouse is associated with intrauterine growth retardation. This retardation is associated with high perinatal mortality. Most of the mice with homologous deletion of the *Lgr4* gene and some heterozygous mice died on the first day after birth (Mazerbourg *et al.* 2004). In other genetic background, the homozygous deletion of the *Lgr4* gene was not connected with lethality, however it was associated with abnormal development of the male reproductive tract (Mendive *et al.* 2006). Other abnormal phenotypes were noticed like female infertility (Mazerbourg *et al.* 2004), impaired prostate development (Luo *et al.* 2013), insufficient uterine development (Sone *et al.* 2013), noneffective erythropoiesis (Song *et al.* 2008),

slowdown of differentiation osteoblasts (Luo *et al.* 2009), kidney hypoplasia (Kato *et al.* 2006), and insufficient gallbladder development (Yamashita *et al.* 2009).

Activation of the Wnt/β-catenin signalling pathway via the interaction of RSPO1 with LGR4 is critical for small intestinal organoid growth (long-life and self-organizing crypt-villus and *in vitro* created structure that grow without non-epithelial niche cells and with the presence of all differentiated cell types (Sato *et al.* 2009, Ruffner *et al.* 2012).

Whereas *Lgr5* was identified as the marker of stem cells in the small and large intestine, stomach and hair follicle (Barker *et al.* 2010), *Lgr4* was rather detected in the proliferating cells (Van Shoore *et al.* 2005).

Positivity of *Lgr4* at the mRNA and protein level was observed in the small intestinal crypts, especially up the zone of the Paneth cells, in so-called transit-amplifying cells, next in crypt basal columnar cells, and as vesicle structures in Paneth cells. On a lesser scale, it was also noticed that stem cells in the small intestinal crypts show positive staining (Mustata *et al.* 2011, Yi *et al*. 2013). Beside the epithelium, *Lgr4* is present in a mesenchyme and smooth muscle region, in myofibroblasts and nerve cells. In other parts of the small intestine the protein expression was very similar (Mustata *et al.* 2011).

When the influence of *Lgr4* on the development of epithelial cells in the small intestine was investigated, there was a comparison between mice with homozygous deletion of the *Lgr4* gene (knock-out mice) and mice that had the *Lgr4* gene fully functional (wild type mice). The results showed that knock-out mice (despite the common development of crypts in time) even had 35% lower depth (on 15thday of postnatal period) and, at the same time, they had 50% reduction of epithelial proliferation. These changes were not related to the differentiation stages of cells, such as absorptive cells, enteroendocrine and goblet cells, but the difference was in Paneth cell differentiation where they showed up to 85% reduction of their abundance (postnatal day 21) (Mustata *et al.* 2011).

In contrast, in mice large intestine the positivity in the cytoplasm of all cells was remote but on the epithelial surface the positivity was stronger. However, the positivity was not detected in stem cells of the large intestine. So, it was confirmed that weak reactivity in stem cells of the large intestine is related to the absence of Paneth cells. In humans, the expression of *Lgr4* in the small intestine was weakly detected in the epithelial cells. In comparison with Paneth cells, stronger immunoreactivity manifested more in stem cells. In contrast to mice, no vesicles in the Paneth cells were observed in humans. The immunoreactivity of the human large intestinal epithelium was not noticed (Yi *et al.* 2013).

3.5 Gene editing technologies

For several reasons, the mouse is the most popular animal model for human diseases. The whole mice genome is known and it was also found that approximately 99 % of human genes correspond to genes in mice. The anatomy of a mouse body is very similar to a human, in consideration of organs, physiology and tissues. Moreover, mice have a short lifespan, high reproduction rate with short generation time and they are not difficult to breed and house them together. Later, with the discovery of homologous recombination (HR), the mouse embryonic stem cells (ESC) proved to be an excellent tool for gene editing (reviewed in (Wijshake *et al.* 2014)) (Lin *et al.* 1985, Doetschman *et al.* 1987, Mansour *et al.* 1998).

Origins of studies dealing with gene functions *in vivo* reach to the second half of the 20th century, when non-targeted genetic modifications in somatic cells were executed by integration of exogenous DNA with the use of microinjection into fertilized eggs (Brinster *et al.*, 1981, 1982, Palmiter *et al.*, 1982).Later studies focused on genomic manipulations using gene targeting in germ line cells (reviewed in Capecchi 2005). The basic of gene editing manipulations is DNA repair via two systems that initialize repairing after identification of double strand breaks (DSBs). One of the systems is non-homologous end-joining (NHEJ) when two broken ends are ligated together. HNEJ is connected with insertions or deletions of nucleotides (InDels) (Barnes 2001). The second repairing system is homologous recombination which use the homologous template to renew the DSBs without involuntary integrated nucleotides (van den Bosch *et al.* 2002).

HR together with the ability of isolation ESCs facilitated generation of knock-out mice. In 2007, Marion Capecchi, Martin Evans and Oliver Smithies were awarded by Nobel Prize in Physiology or Medicine for establishing a first knock-out model of mouse from isolated embryo-derived stem cells with the use of genetic engineering, *in vitro* fertilization and breeding (in 1989) (Vogel 2007). Before this cooperation, these three researchers had worked in different lines of investigations. Evans was concerned with isolation of embryonic cell lines which could *in vitro* differentiate and *in vivo* create teratocarcinoma (Evans 1972, 1975). Capecchci and Smithies participated in research focused on insertion of specific DNA into mammalian cells with the use of homologous recombination (Capecchi 1989, Smithies *et al.* 1985). Though all these discoveries considerably improved insights into understanding of gene functions *in vivo*, the processes of generation transgenic animals were very time-consuming and work-intensive.

In recent years, nuclease engineering has brought new advantages to gene editing technologies. In contrast to earlier technologies based on long template homologous

recombination, genome editing using nucleases enables gene modification in the broad spectrum of organisms (such as plants (Jiang *et al.* 2013, Romay and Bragards 2017), *Caenorhabditis elegans* (Sugi *et al.* 2016), *Drosophila melanogaster* (Bibikova *et al.* 2002, Liu *et al.* 2012, Gratz *et al.* 2013), *zebrafish* (Doyon *et al.* 2008, Sandler *et al.* 2011, Xiao *et al.* 2013), mouse (Wang *et al.* 2013, Li *et al.* 2013, Shen *et al.* 2013, Zhang *et al.* 2014) and rats (Tesson *et al.* 2011, Li *et al.* 2013) and cell lines like blood cells (reviewed in (Weiss and Mullighan 2016)). The next great improvement is a high specificity in gene-target modifications. In the presence of short homologous template it enables to make delicate changes as point mutations. Moreover, nuclease engineering reduces time-consuming work, enables easier design and construction of targeting constructs (reviewed in (Wijshake *et al.* 2014, Rocha-Martins *et al.* 2015).

Here I want to report three methods using nucleases that cause DNA double-stranded breaks at a specific sequence of the gene:

3.5.1 ZFN

Zinc finger nucleases represent the technology of genome editing. This tool consists of two domains – Zinc finger (ZF) domain and nuclease *FokI*. Zinc finger proteins are common transcription factors in eukaryotes, which are responsible for highly specific recognition and DNA binding (Wolfe 2000). It was noticed that ZFN with three ZF domains can recognise 9-18 bp on the target DNA (Miller 1985). The *FokI* nuclease creates catalytic domain with cleavage activity. As noted above, the *FokI* nuclease needs to be in dimer status for cleavage activity. Thus, ZFN activity requires two monomers and each will bind the target sequence on the DNA at the complementary strands and generate a DSB. The recognition DNA binding sequence in the *FokI* nuclease was replaced by ZFs, which give the binding specificity of these proteins.

3.5.2 TALEN

Transcription activator-like effector nucleases (TALENs) introduce the next method which uses targeted DSBs for genome editing. This system comprises a DNA binding domain and nonspecific nuclease which come from diverse organisms. The DNA binding domain is derived from ‘‘Transcription activator-like effectors’’ (TALEs) which are proteins from bacteria *Xanthomonas* binding on promotor regions in host plants and altering host gene

transcription (Boch *et al.* 2009, Bogdanove *et al* 2010). DNA recognition domain *FokI* with cleavage activity was derived from a restriction enzyme in *Flavobacterium okeanokoites* (Wah *et al.* 1998).

TALE DNA binding domain consists of tandem repeats. Each of these repeats include 33-35 amino acid residues that define the specificity of binding between amino acids of TALE domain and nucleotides of the target DNA. The binding specificity of these tandem repeats on the TALE domain is given by an amino acid sequence at positions 12 and 13 (Deng *et al.* 2012, Mak 2012). The region of polymorphisms at positions 12 and 13 is called ‘‘the repeat-variable diresidue’’ (RVD) (Boch *et al.* 2009, Moscou *et al.* 2009). In TALENs the nuclease *FokI* represents the catalytic domain. Because *FokI* nucleases can cleave DNA molecules only after their dimerisation (Bitinaite *et al.* 1998), it is important that TALENs be in dimer status. After dimerisation, a whole complex of TALENs is able to generate DSBs on the target DNA sequences (Christian *et al.* 2010, Miller *et al.* 2011). Each monomer from this dimer complex binds one strand of DNA so that the whole dimer generates cleavages on the complementary strands. Because between binding domains of each monomer inside the TALEN dimer is a distance of 12-16 nucleotides, the cleavage creates overlaps of four nucleotides (Urnov *et al.* 2010).

3.5.3 CRISPR/Cas9 system

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) is a bacterial and archeal adaptive immune system which helps these organisms with protection against foreign genetic elements by using RNA-guided nucleases (Horvath and Barrangou 2010, Bhaya *et al.* 2011, Terns and Terns 2011, Wiedenheft *et al.* 2012). There are three types of CRISPR system (I-III) (Makarova *et al.* 2006). The CRISPR/Cas9 system belongs to the type II and is predominantly used in genome engineering.

Guide-RNA (gRNA) is a complex of CRISPR RNA (crRNA) with trans-activating crRNA (tracrRNA). crRNA contains ‘‘spacer’’ which is a unique target sequence consisting of 20 base pairs (bp). Primary crRNA arises from its precursor pre-crRNA that contains nuclease guide sequences (spacers) interspaced by palindromic direct repeats (DRs) and is coded in the bacterial genome (Deltcheva *et al.* 2011). Complex gRNA directs Cas9 nuclease protein to the target DNA via Watson-Crick base pairing between the spacer on the crRNA and the ‘‘protospacer’’ that is a complementary sequence to the target DNA. To successful

match Cas9 to the protospacer on the target DNA, a ''protospacer adjacent motif'' (PAM) is needed. The PAM sequence varies in different CRISPR systems, but in Streptococcus pyogenes the PAM sequence was identified as 5´-NGG. After recognition and connection Cas9 to the target DNA, Cas9 cleaves the target sequence three bp upstream of the PAM (Jinek *et al.* 2012).

Even though ZFNs represented a big success in genome editing, later development of TALENs quickly substituted ZFN technology. In contrast to ZFNs, TALENs facilitate more sequence specific cleavage and are also easier to construct (Chen *et al.* 2013). But in comparison with TALENs, the newest CRISPR/Cas9 technology is easier to design, suitable for simultaneous modifying of genes, and is not dependent on DNA methylation (advantage in using in targeting to CG-rich regions). But with respect to off-target activity, TALENs are preferred because of containing 30nt long target sequence which is identical to the genome of mice. However, CRISPR/Cas9 could cause a high off-target activity, because its guide RNA tolerates multiple mismatches (Pattanayak *et al.* 2013, Wefers *et al.* 2013).

The presented work analysed knocking mouse created by TALEN based technology.

4 Materials and methods

4.1 Animal model

The genetically modified mice, housed in the animal facility of the Institute of Molecular Genetics, were recently generated in Dr. Korinek's lab by microinjection of mRNAs of transcription activated effector nucleases (TALENs) in C57BL/6J mouse egg together with template DNA. The template composed of right and left homology arm spanning 93 nucleotides (nt) coding triple influenza hemagglutinin (3HA) tag. The tag is localized in N-terminal region next to the signal peptide of Lgr4 receptor. Correct insertion into the genome was precisely verified by PCRs spanning left and right arm of the homologous template.

Lgr5-EGFP-IRES-CreERT2 mouse strain was purchased from Jackson laboratory (Bar Harbor Main, USA).

4.2 DNA isolation, genotyping

The tip of the tails from mice were cut, placed into tubes with lysis buffer (1 M Tris pH 8, 0.5 M EDTA, 10% SDS, 5.0 M NaCl, deionized water) supplemented with Proteinase K (20 mg/ml; Thermo Scientific) and incubated overnight at 55°C. The tubes were then spun for 5 minutes (14000g), 400 µl of solute was pipetted out and 400 µl of isopropanol (Penta) was added, mixed by inverting and spun (5 min, 14000g). After, we added 400 µl of 70% ethanol (Penta) and spun (5 min, 14000g), pipetted out and repeated. DNA was dried on a heat block (Thermocell Cooling and Heating Block CHB-202; BIOER) at 55°C, dissolved in 200 µl of tissue water and kept on the heat block at 55°C for 3 hours.

PCR based genotyping was performed from DNA of adult mice, newborns or embryos. Each reaction tube contained 50% Master mix (Dream Taq Green, PCR Master Mix; Thermos Scientific), 0.75mM betaine (Sigma-Aldrich), 0.5µM of each primer (Table 1; Sigma) and DNA. The reaction tubes were placed into cycler T100 Thermo Cycler (BioRad) and run under conditions listed in Table 2. Then, the samples and ladder were loaded onto 1.5% agarose gel dyed by DNA G strain (Serva). Electrophoresis (Electrophoresis Power Supply-EPS301) was set up on 120 V and 30 minutes and after, the samples were detected on UV transilluminator (Major Science).

Table 1.

Primers for genotyping and qRT-PCR (Primer were designated by Primer3 web tool (http://bioinfo.ut.ee/primer3-0.4.0))

Type of reaction	Name of primer	Sequence
Genotyping	P1 Lgr4-HA	GGAGGCGAGTCGAGCGAGAGGAG
	P2 Lgr4-HA	GCACTCACAGTGCTTGGGTGAAGGC
qRT-PCR	Lgr4	AACCTGGAAACCCTGGACTT
		CTCCATCCGGGATAACAGAA
	Lgr5	CCTGTCCAGGCTTTCAGAAG
		CTGTGGAGTCCATCAAAGCA
	Mmp7	GGCCTAGGCGGAGATGCTCACT
		AACAGGAAGTTCACTCCTGCGTCC
	Def5	TTCTCCAGGTGACCCCCAGCC
		GCAGACCCTTCTTGGCCTCCAAAG
	Crypt	AGGAGCAGCCAGGAGAAG
		ATGTTCAGCGACAGCAGAG
	Axin2	TAGGCGGAATGAAGATGGAC
		CTGGTCACCCAACAAGGAGT
	Muc2	GGCCTCACCACCAAGCGTCC
		CGAAGGCGTGGCACTGGGAG
	Ubiquitin B	ATGTGAAGGCCAAGATCCAG
		TAATAGCCACCCCTCAGACG

Table 2.

Steps in PCR reaction

Step	Temperature	Duration	
Initial denaturation	95°C	30 s	
Denaturation	95°C	30 s	34x
Annealing	68°C	30 s	
Amplification	72°C	60 s	
Infinitive hold	72°C	300 s	

4.3 Immunohistochemistry (IHC)

Separated organs (colon and small intestine) were fixed in 4% formaldehyde (Penta) overnight, store in 70% ethanol (Penta) and then continued with the dehydratation over ascending alcohols, xylene and paraffin in a tissue processor (Leica ASP200S) overnight. Saturated tissues were embedded into paraffin blocks (Leica Paraffin Embedding Station EG1150H) and kept in 4°C. Next, the blocks were cut (Leica Microtome RM2255) to 7 µm thick slides. Slides with samples were deparaffinized in Xylene (Lachema) 2x for 8 minutes, incubated with isopropanol (Lachema) for 5 minutes, 100% ethanol and proceed with rehydratation to 70% ethanol and deionized water for 3 minutes. Heat induced antigen retrieval was performed in Tris-EDTA buffer (pH 9). Endogenous peroxidases were blocked 0.3% H_2O_2 (Sigma) in methanol and unspecific immunoglobulins were eliminated by incubation in blocking solution; 10% BSA (bovine serum albumin) and 5% goat serum in TBS (50mM Tris Cl, 150mM NaCl, pH 7.6) for 1 hour. Immunostaining was performed by primary antibodies anti-HA (goat anti-rabbit, diluted 1:100, Cell Signaling) and in blocking solution at 4°C overnight. The slides were rinsed in TBS + 0.01% TritonX-100 (Fluka) 1x 5 minutes, in TBS 4x 5 minutes, immunolabelled by biotin conjugated goat anti rabbit secondary antibody (Life Technologies) and again washed just like before. Subsequently, the detection was provided by treatment with Vecastain ABC kit (Vector) for 30 minutes, staining with 3, 3′-diaminobenzidine (DAB; 30 mg/100 ml 50 mM Tris pH 7.6, 0.018% H_2O_2). Hematoxylin was used for counterstaining. Stained sections underwent dehydration over ascending alcohols and xylene. Samples were mounted in Solacryl medium. The visualization of stained tissues was captured by a Leica DM6000B microscope.

4.4 In situ hybridization

To avoid RNAse contamination, all equipment used during in situ hybridization was baked at 200°C or washed by diethylpyrocarbonate (DEPC)-treated H_2O. All solutions were also prepared from DEPC-treated water. Intestinal tissue underwent the same deparaffinization process as in immunohistochemistry. Samples were denaturized with 0.2M HCl for 15 minutes and washed in PBS. For better probe penetration, the samples were incubated with Proteinase K in PBS (30 µg/ml, room temperature (RT), 15 min). To block the protease, the tissue was rinsed in 0.2% glycine in PBS. The post-fixation was done with 4% paraformaldehyde (PFA) for 10 min and rinsed 2x in PBS. To reduce background, the slides were incubated in acetic solution pH 8 with Triethanolamine for twice 5 min, rinsed 2x in

PBS and subsequently in 5xSSC pH 4.5. Next, the slides were placed in prehybridization mixture (2% block solution (Roche), 50% formamide, 5x SSC pH 4.5, 5mM EDTA, 0.05% Chaps, 50 µg/ml heparin and 1 µg/ml yeast total RNA and DEPC-treated water) and incubated at 70°C for 1 hour. After, the prehybridization solution was removed and the slides were covered with the same mixture containing probe and incubated at 55°C for 36-48 hours. Probes (Lgr4 sense, Lgr4 anti-sense and anti-HA rabbit antibody) were available in our laboratory. Post-hybridization wash was first in 1x SSC pH 4.5, 50% formamide, 0.1% Tween20 (Sigma) (50°C, 30 min); then, 3x in 0.5x SSC pH 4.5 and 0.1% Tween 20 (50°C, 20 min) and the last two washes were in MATB solution (100 mM Maleic acid pH 7.5, 150 mM NaCl, 0.1% Tween20) (RT, 20 min). To avoid unspecific binding during immunological detection, samples were incubated in blocking solution (0.5% Blocking powder in MATB, RT, 30 min). After the blocking step, samples were treated with sheep anti-dioxigenin Fab-Ab antibody (dilution 1:1000; Roche) in blocking solution (4°C, overnight). Next day, the slides were washed 5x in MATB at RT for 20 min, 2x in NTM (0.1M Tris pH 9.5, 0.05M MgCl, 0.1M NaCl) and incubated in nitro blue tetrazolium (NTB)/bromo-chloro-indolyl-phosphate (BCIP) diluted in NTM buffer in the dark. After observation violet staining, samples were mounted in Mowiol (Calbiochem) and captured using a Leica DM 6000B microscope.

4.5 Fluorescence-activated cell sorting (FACS)

Small intestine and colon were taken out from mouse and washed in PBS. Then they were longitudinally cut and, in the case of small intestine, the villi were removed using coverslip. The tissues were vigorously agitated and washed in PBS several times to remove villi and waste. To release cells of crypts from connective tissue, samples were incubated with 5mM EDTA (4°C, 30 min). Then, the mixture was filtrated with 70µm strainers (Fisher Scientific), spun down and the pellet was processed for immunostaining or for immunoblotting/immunoprecipitation.

To obtain single cell suspension for immunostaining, the pellet was shaken with dispaze (Corning, 18U) in serum free media (37°C, 2x 10 minutes, 800 rpm). Collected supernatant was spun down and cells were incubated with an anti-HA biotin-conjugated antibody (monoclonal rabbit antibody, 1:25, Cell Signaling Technology) at 4°C for 15 minutes, washed in 3% foetal bovine serum (FBS; Sigma-Aldrich) in DMEM (Dulbecco´s Modified Eagle Medium; Thermo Fisher Scientific) and incubated with streptavidin-allophycocyanin (APC; 1:100; BD Biosciences) secondary antibody at 4°C for 15 minutes. Washed cells were analyzed by flow cytometry using influx high speed cell sorter (BD

Sciences) and sorted to RNA lysis buffer (Qiagen). Gated areas were evaluated by FlowJo software (Tree Star).

4.6 Immunoprecipitation

Isolated cryptic cells were lysed in lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% NP40) supplemented by protease inhibitor cocktail (Roche, dilution 1:500). The cell lysate was homogenized and spun down (4°C, 20 min, 20 000g). Supernatant was removed into a new tube and mixed with Laemmi sample buffer (5x Laemmli: 0.5M Tris-HCl pH 6.8, 45% glycerol, 5% SDS, 0.25% Bromophenom blue, 1.78M β-mercaptoethanol) and used as total cell lysate (TCL) in immuniprecipitation. The TCL samples were boiled (15 min), spun down and used for western blotting.

In the case of immunoprecipitation, supernatant was incubated with magnetic beads (anti-HA tag) (clone 2-2.2.14, Thermo Fisher Scientific) on a carousel at 4°C for 1 hour. After incubation, the magnetic beads were washed by inverting the tubes and collected by magnetic separation rack (3x in lysis buffer, 2x in lysis buffer without detergent), resuspended in 50 µl of lysis buffer, mixed with Laemmli sample buffer and used for western blotting as an IP sample.

4.7 Western blot (WB)

The IP and TCL samples were loaded onto 10% denaturation acrylamide gel and separated by vertical electrophoresis (Mini-Protean Tetra System, BioRad) (150 V, 90 min). The gel was blotted by semidry blotting to nitrocellulose membrane (Trans-blot SD, Semi-dry transfer cell, BioRad) (20 V, 30 min). Subsequently, the membrane was blocked in 5% low fat milk and 0.025% Tween20 in PBS for 1 hour and incubated with primary antibodies (rabbit anti-HA monoclonal antibody, dilution 1:1000 (Cell Signalling)) or rabbit anti-actin (whole rabbit serum cat. no. A2668, Sigma-Aldrich) at 4°C overnight. The next day, the membrane was washed with Tween20 in PBS and incubated with secondary antibodies (goat anti-rabbit conjugated to peroxidase, dilution 1:1000; Sigmal-Aldrich) in 5% low fat milk and 0.025% Tween20 in PBS at RT for 1 hour, washed 3x 10 min in 0.025% Tween20 in PBS, placed into the cassette, incubated for 5 minutes with chemiluminescent substrate for detecting horseradish peroxidase (Femto or Pico; Thermo Scientific) and immediately exposed to film. The developing was carried out developing machine Fomei Optimax.

4.8 RNA isolation and reverse transcription

RNA was isolated with RNeasy Micro Kit according to manufacturer´s instruction. RNA was eluted in 14 µl nuclease free water. Reverse transcription was performed by Thermo Scientific Maxima Reverse Transcriptase RNA according to manufacturer´s instructions. Briefly, RNA was mixed with random primer hexamer (final concentration 7 µM) and dNTP (final concentration 0.5 mM) and incubated at 60°C for 5 min. Then, the sample was completed with Reverse Transcriptase buffer, RiboLock RNase Inhibitor and Maxima Reverse Transcriptase and incubated in thermocycler (25°C for 10 min, 50°C for 30 min, 85°C for 5 min).

4.9 Quantitative real-time polymerase chain reaction (qRT-PCR)

cDNA was diluted according to the number of analyzed genes with RNase free water. Each reaction mixture contained 2.5 µl 2x LightCycler 480 SYBR Green I Master mix (Roche), 2 µl cDNA and 0.5 µl primer mix (final concentration 0.5 µM each; Table 1). Each sample was run in duplicate in at least three independent experiments. The mixture was pipetted in 384-well plate (Roche). The plate was spun down and placed into the LightCycler 480 facility (Roche) where qRT-PCR reaction proceeded (Table 3).

Table 3. Programme of qRT-PCR

Step		Temperature	Duration
Initial denaturation		95°C	7 min
Cycling	Denaturation	95°C	14 s
	Annealing	61°C	14 s
	Amplification	72°C	14 s
Melting curve		95°C	15 s
		55°C	61 s
		37°C	61 s
		95°C	until end

5 RESULTS

5.1 DNA genotyping

The successful 3HA tag insertion was previously verified by other sets of primers, priming upstream and inside of the target region and inside and downstream the targeted region (data not shown). For regular genotyping, the confirmation of epitope-tagged $Lgr4^{3HA/3HA}$ allele was performed by left and right arm PCR with P1 Lgr4-HA and P2 Lgr4-HA primers (Table 1; Fig. 5). The difference between size of $Lgr4^{WT/WT}$ allele (252 bp) and $Lgr4^{3HA/3HA}$ allele (345 bp) is 93 bp which corresponds with the size of inserted 3HA tag. Importantly, when heterozygotes $Lgr4^{3HA/WT}$ were mated, viable and fertile homozygotes were obtained in expected Mendelian ratio (data from 77 pups; 12 litters: 24.6% $Lgr4^{3HA/3HA}$; 49.4% $Lgr4^{3HA/WT}$; 26.0% $Lgr4^{WT/WT}$). As a "by product" of targetting, Lgr4 knockout (Lgr4-) alleles were generated on C57BL/6 backgroud. Interestingly, when heterozygotes for knockout allele ($Lgr4^{-/WT}$) were mated, none Lgr4 knockout ($Lgr4^{-/-}$) was born (data from 64 pups; 12 litters: 68.75% $Lgr4^{-/WT}$; 31.25% $Lgr4^{WT/WT}$) (Fig. 6). Absence of $Lgr4^{-/-}$ animals and normal viability of $Lgr4^{3HA/3HA}$ mice made us believe, that presence of 3HA tag should not affected LGR4 function.

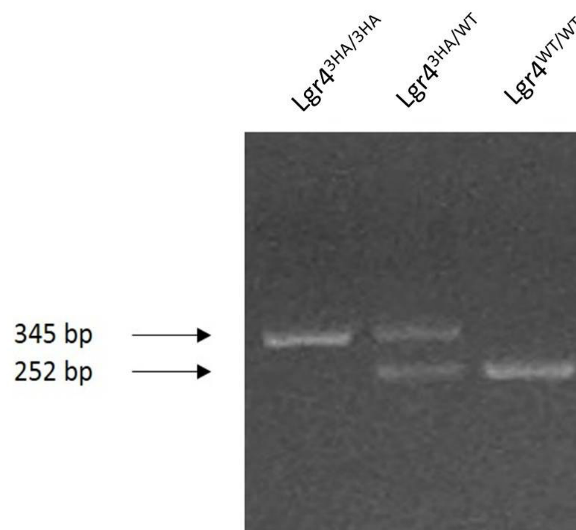

Fig. 5. Genotyping of tail biopsies
After DNA isolation, PCR was performed and genotypes were visualized by exposing 1.5% agarose gel with UV transilluminator.

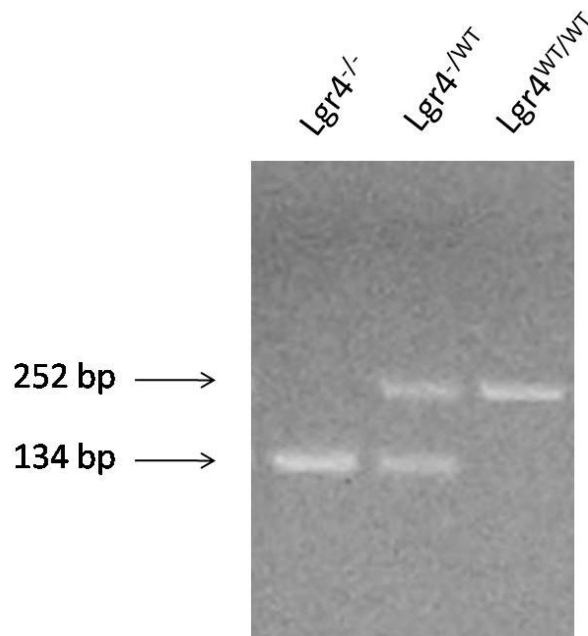

Fig. 6. Genotyping for presence of knockout allele
WT allele is presented as 252 bp band, Lgr4- allele is indicated as 134 bp band. Note that homozygote for the knockout allele (Lgr4$^{-/-}$) was observed only in embryonic stages, but not in adult mice.

5.2 Immunohistochemistry, in situ hybridization

The presence of HA epitope in Lgr4$^{3HA/3HA}$ mouse was observed over the all parts of the small intestine (duodenum, jejunum, ileum) and the results were consistent with previous data screening *Lgr4* expression in this organ (Mustata *et al.* 2011, Yi *et al.* 2013). Very strong positivity was detected at the crypt bottom, up and at the zone of transit amplifying cells. Then the strength of signal gradually became weaker along the crypt-villus axis (Fig. 7A, 8A). In contrary to the small intestine, in the colon we noticed a strong positivity rather on the epithelial cells closest to the colonic lumen and weakest intensity at the bottom of the crypts (Fig. 7B, 8B), which is in contrast with previous studies based on the mRNA level, where the signal of *Lgr4* was strongest at the bottom of the crypts (Mustata *et al.* 201, Liu *et al.* 2013).

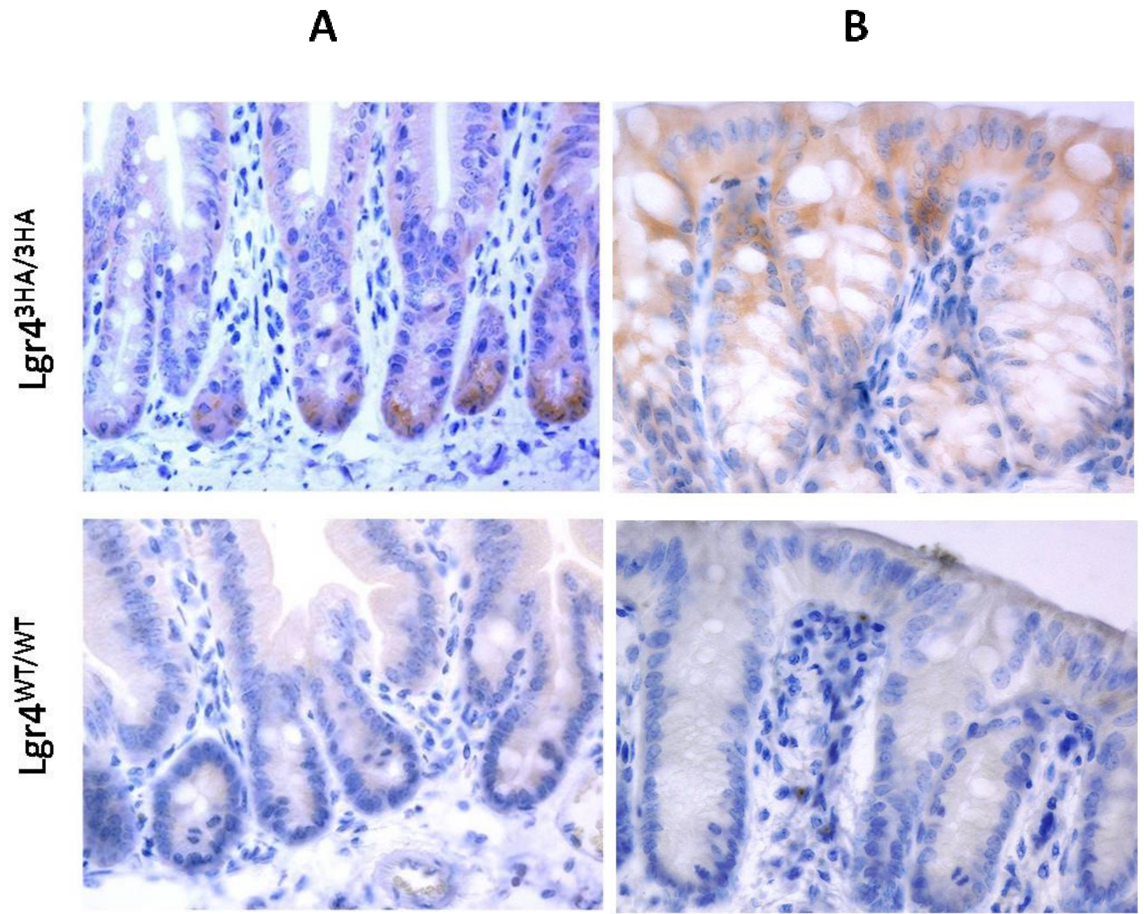

Fig. 7. HA antigen has the strongest expression at the bottom of the small intestinal crypts and at the surface of the colon (400x).

A: Immunohistochemical staining in the jejunum. **B:** Immunohistochemical staining in the colon. Anti-HA rabbit antibody detects the pattern of Lgr4$^{3HA/3HA}$. As a negative control we used wild type mouse.

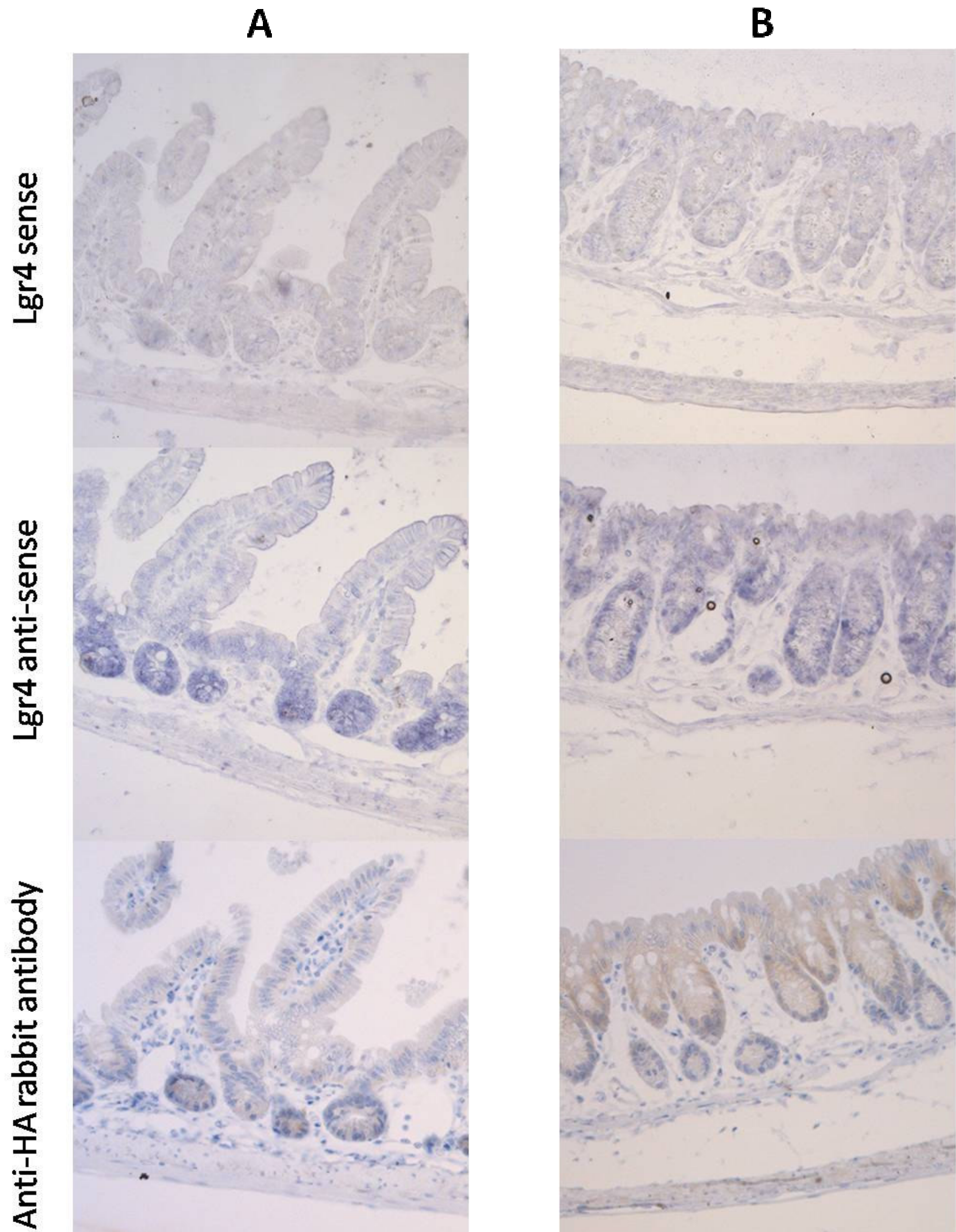

Fig. 8. The expression of *Lgr4* is strongest in the central part of the colon and at the crypts in the jejunum (200x).

A: In situ hybridization in the jejunum. **B:** In situ hybridization in the colon. Lgr4 sense probe detects *Lgr4* expression and anti-HA rabbit antibody detects 3HA-LGR4 in $Lgr4^{3HA/3HA}$ mouse. As a negative control we used Lgr4 anti-sense probe.

5.3 Fluorescence-activated cell sorting (FACS)

LGR4 receptor in Lgr4$^{3HA/3HA}$ mouse carries a 3HA tag in its extracellular part and thus the receptor should be detectable by anti-HA antibody on the cell surface. We took the opportunity that EGFP-IRES-CreERT2 mouse is available in our laboratory. In EGFP-IRES-CreERT2 mouse, GFP protein expression is driven by Lgr5 promoter (Lgr5^{GFP}) and makes it possible to distinguish intestinal Lgr5+ stem cells as GFP positive. Lgr4$^{3HA/3HA}$ strain was crossed with Lgr5^{GFP} and we have obtained Lgr4$^{3HA/3HA}$ / Lgr5^{GFP} hybrids. We isolated crypts from the small intestine Lgr4$^{3HA/3HA}$ / Lgr5^{GFP} and Lgr5^{GFP} mice. Subsequently, we visualized HA epitope by biotin conjugated anti-HA antibody together with streptavidin conjugated APC antibody. The sorted cells were distributed according to the presence of GFP and APC markers into five populations gated as S1 – S5 (Fig. 9) and collected into separate tubes. Interestingly, population S1 and S2, which contain Lgr4 positive cells, contained much less cells in the sample originated from Lgr5^{GFP} than in the sample originated from Lgr4$^{3HA/3HA}$ / Lgr5^{GFP}. This result gives evidence about the specificity of anti-HA/APC staining.

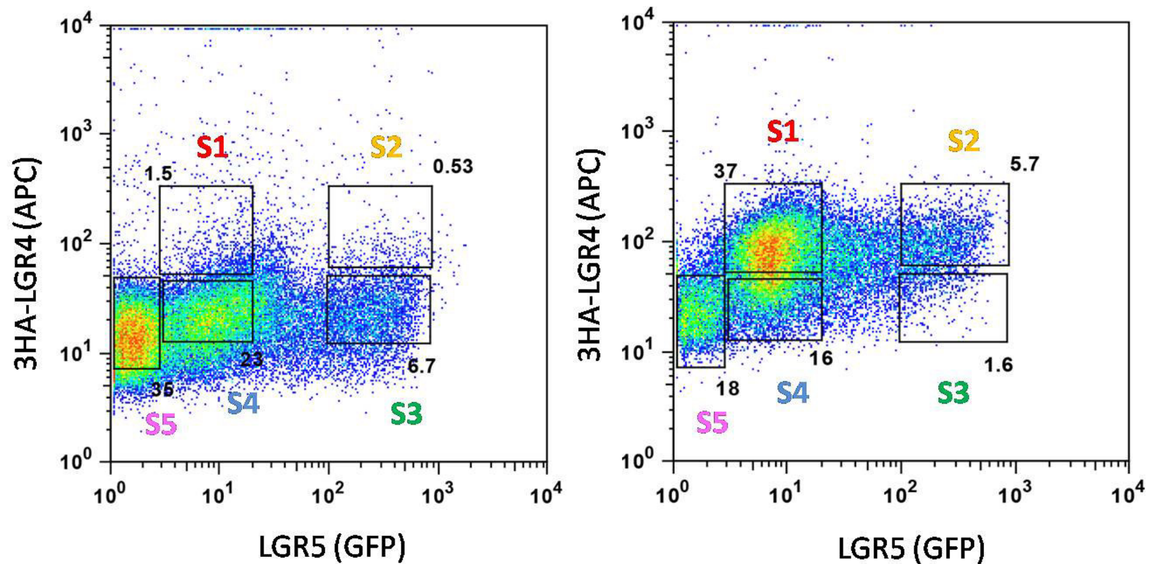

Fig. 9. Fluorescence-activated cell sorting separated five (S1-S5) populations according to the positivity of LGR4 and LGR5 proteins.

On the left: The intestine cells only from Lgr5^{GFP} mouse. **On the right:** The intestine cells from Lgr4$^{3HA/3HA}$/Lgr5^{GFP} mouse strain. The numbers next to the frames show percentage of cells in specific population.

5.4 Quantitative real-time polymerase chain reaction (qRT-PCR)

In the cells that were sorted into separated tubes during FACS analyses, we next performed qRT-PCR to measure the RNA expression profile with the use of predominant markers which are specific for individual small intestine cells (Fig. 10). Single positive Lgr4^{+} cells defined as S1 population display a high expression of *Lgr4* mRNA and Paneth cells markers like *alfa-defensin 5* (*Def5*), *cryptidin* (*Crypt*) and *matrix metallopeptidase*7 (*Mmp7*). Elevation of *mucin 2* (*Muc2*) expression is caused by the presence of goblet cells precursors (Van Dussen and Samuelson 2010). The S2 population of *Lgr4* and *Lgr5* double positive cells represent lineage of cells where the high co-expression of these two homologue genes occurs. This also indicates high levels of gene *Axin2* which is a target gene of Wnt signalling. S3 population is characteristic for its low levels of *Lgr4*, *Lgr5* and also *Axin2*, but increased levels of *Crypt* what could suggested occurrence of secretory precursors above the stem cell population (Buczacki *et al.* 2013). Double negative *Lgr4* and *Lgr5* cells with high levels of *Muc2* are typical for goblet cells (Van Dussen and Samuelson 2010) but with respect to the presence of *Mmp7* and *Crypt*, we cannot exactly confirm which population it is. Also, S5 population is *Lgr4* and *Lgr5* double negative and because of very low levels of all markers it was decided that this population could represent enterocytes. The relative expression was normalized to *ubiquitin B* (*Ubb*) expression.

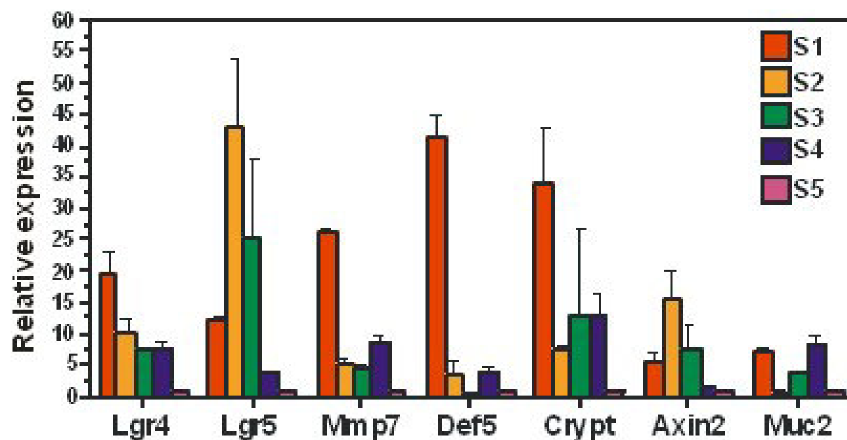

Fig. 10. qRT-PCR analysis of specific markers of small intestinal cells
S1-S5 populations are illustrated in Fig. 9 performed by FACS analysis

5.5 Immunoprecipitation and western blot

With the use of an anti-HA tag-specific antibody, western blotting and immunoprecipitation analysis was performed on samples from Lgr4$^{WT/WT}$ and Lgr4$^{3HA/3HA}$ mice. The signal was revealed in TCL only from Lgr4$^{3HA/3HA}$ mice but not from samples of Lgr4$^{WT/WT}$ mice. We choose actin as a loading control. In the case of IP samples, there was a visible arising band in the size around 110 kDa only in samples from Lgr4$^{3HA/3HA}$ mice but not insamples from Lgr4$^{WT/WT}$ origin. This result gives evidence that we are able to differentiate and detect LGR4 protein from Lgr4$^{3HA/3HA}$ mouse using anti-HA antibody also by immunoblotting and immunoprecipitation (Fig. 11).

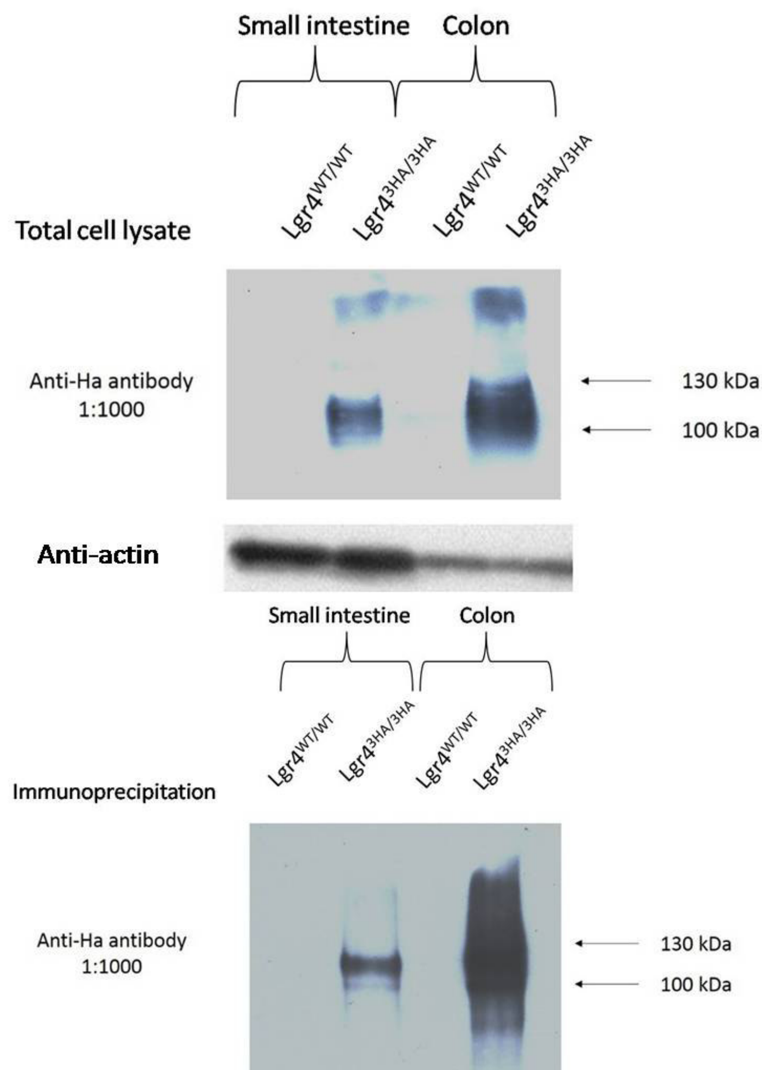

Fig. 11. Western blot if TCL and IP samples.

The use antibody rabbit anti-Ha monoclonal primary antibody and rabbit anti-actin antibody

6 Discussion

The major purpose of the study was characterization of the Lgr4$^{3HA/3HA}$ mouse as a tool for analysis LGR4 expression pattern and determination LGR4 binding partners. The first aim of our work was analyze if presence of 3HA does not affect normal function of LGR4 protein. We have investigated offsprings of twelve Lgr4$^{WT/3HA}$ breeding pairs and as expected the pups were born in Mendelian ratio. Moreover breading twelve Lgr4$^{-/WT}$ heterozygotes we were not able to get any Lgr4$^{-/-}$ animals. These results illustrate that Lgr4$^{-/-}$ are not viable on C57BL/6 background at the same time 3HA tag does not affect viability or fertility of Lgr4$^{3HA/3HA}$ mice.

Our next intention was to determine *Lgr4* expression in the small intestine. The expression outline of *Lgr4* in the small intestine is well considered at the RNA level and protein level. The previous studies performed by on in situ hybridization (Mustata *et al.* 2011), by the β-galactosidase (LacZ) reporter expressed from the *Lgr4* locus (de Lau *et al.* 2011, Mustata *et al.* 2011) and by immunohistochemistry (Yi *et al.* 2013) revealed *Lgr4* expression all along the small intestine crypts, especially in Paneth cells, CBC cells and above the Paneth cell zone – in TA cells. Outside the epithelial region, *Lgr4* expression was detected in smooth muscle layers, myofibroblasts and neurons. Similarly, we have disclosed corresponding expression signs of HA antigen in Lgr4$^{3HA/3HA}$ mouse during IHC analysis, the strongest signal was detected at crypt bottom and middle part but no specific staining was recorded at the intestine villi. Correspondingly, we have disclosed similar expression pattern on RNA level using in situ hybridization. Conversely, *Lgr4* expression pattern in the colon is not clear. The studies exploring *Lgr4* expression at the mRNA level as Lgr4-LacZ reporter expression (Liu et a, 2013) and in situ hybridization (Mustata *et al.* 2011) show the strongest *Lgr4* signal at the bottom of the crypts. By contrast, LGR4 protein was shown to be mostly present at the apical part of the crypts and diminishing to the crypt bottom (Yi *et al.* 2013). Our data based on IHC show the strongest outline at the crypt part facing to the lumen of the colon and signal vanishing toward the crypt bottom; those data support expression outline published by Yi and colleagues. Moreover, our in-situ data shows strongest signal in the middle part of the crypts. Thus, our results are in the contradiction with results published by others (Mustata *et al.* 201, Liu *et al.* 2013). The next goal was to confirm, if HA tag enables specific marking and sorting of *Lgr4* expressing cells by Fluorescence-activated cell sorting analysis and subsequently characterize those cells by quantitative real-time PCR. For the FACS analysis, we were employing crypts from small intestine of Lgr4$^{3HA/3HA}$/Lgr5^{GFP}

mouse, where promoter for stem cell marker *Lgr5* drives GFP protein. We were gated five cell population. Population S1 (3HA-LGR4 single positive cells) was approved with the highest *Lgr4* expression, because of high abundance of mRNA for Paneth cell markers (*Mmp7*, *Def5* and *Crypt)* we confirm *Lgr4* expression in Paneth cells. Moreover, increased expression marker for Goblet cells (*Muc-2*)*,* suggests certain subpopulation of *Lgr4* positive cells as precursors for goblet cells. The next population S2 ($LGR4/5^+$ cells) was disclosed by qRT-PCR disclosed as stem cell population when stem cell markers as *Lgr5* and *Axin2* are highly abundant. Interestingly, S3 population is very small. It displayed low *Lgr4* expression and somewhat reduced levels of *Lgr5* and *Axin2*. Since these cells produce *Crypt,* it might represent the secretory precursors localized above the stem cell zone. S4 (LGR4/5 double-negative) cell population needs to be father characterized. It shows increase expression of goblet cell marker *Muc-2* and positivity for Paneth cell markers *Mmp7* and *Crypt*, but quite low expression for another Paneth cell marker *Def5*. S5 population is double negative (LGR4/5 double-negative). Because of low expression of all investigated markers we suggest the population represent enterocytes. Similarly, S5 population needs additional analysis for some specific enterocyte market as intestinal alkaline phosphatase (ALPI).

Our final goal was to analyze if we could reveal LGR4 protein in $Lgr4^{3HA/3HA}$ mouse in immunoblotted and immunoprecipitated samples using anti-HA antibody. Western blot analysis noticed specific signal only in the tissue (the small intestine and the colon) originated from total cell lysate $Lgr4^{3HA/3HA}$ mouse but not from the WT animal. Nevertheless, the signal was not present as a single band but rather as a smear; its presence only in $Lgr4^{3HA/3HA}$ animals confirm its specificity. Moreover, this result is in the line with our IHC data, where we have noticed HA positive cells both in the small intestine and the colon. $Lgr4^{3HA/3HA}$ samples immunoprecipitation and their subsequent analysis by western blot determined specific band around 110kDa only in tissue from $Lgr4^{3HA/3HA}$ mouse but not from WT tissue. The size of the band corresponds to LGR4 protein (104kDa) with an increased size of triple hemagglutinin tag (3KDa). We believe that 3HA-LGR4 protein can be used for mass spectrometry analysis, which can release new LGR4 binding partners.

7 Conclusion

To conclude, we were able to generate an epitope tagged Lgr4$^{3HA/3HA}$ mouse. The animals were fertile and viable and LGR4 receptor function was not affected by presence of triple hemagglutinin tag on its N terminus. We have characterized expression pattern of HA tag in the small intestine and colon, which corresponded to previously published data. In addition, we have compared expression outline of HA tagged LGR4 protein with expression *Lgr4* mRNA in the small intestine and the colon. Moreover, by using of fluorescence-activated cell sorting and quantitative real-time polymerase chain reaction, we measured mRNA expression in separate small intestine cell populations and we could categorize some of those cell groups. Finally, we detected 3HA-LGR4 protein by western blot in the total cell lysate and after immunoprecipitation with anti-HA magnetic beads. In summary, Lgr4$^{3HA/3HA}$ mouse represent a new tool for characterization cell population the small intestine and colon and open the gate for searching for novel LGR4 binding partners.

8 References

Aberle, H., Bauer, A., Stappert, J., Kispert, A., & Kemler, R. (1997). Beta-Catenin Is a Target for the Ubiquitin-Proteasome Pathway. *The EMBO journal*, *16*(13), 3797–804.

Adler, P. N. (2002). Planar signaling and morphogenesis in Drosophila. *Developmental Cell*, *2*(5), 525–535.

Anastas, J. N., & Moon, R. T. (2013). WNT signalling pathways as therapeutic targets in cancer. *Nat Rev Cancer*, *13*(1), 11–26.

Artavanis-Tsakonas, S., Rand, M. D., Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science (New York, N.Y.)*, *284*(5415), 770–776.

Bänziger, C., Soldini, D., Schütt, C., Zipperlen, P., Hausmann, G., Basler, K. (2006). Wntless, a Conserved Membrane Protein Dedicated to the Secretion of Wnt Proteins from Signaling Cells. *Cell*, *125*(3), 509–522.

Barker, N. (2014). Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nature reviews. Molecular cell biology*, *15*(1), 19–33.

Barker, N., Huch, M., Kujala, P., van de Wetering, M., Snippert, H. J., van Es, J. H., Sato, T., Stange, D. E., Begthel, H., van den Born, M. *et al.* (2010). Lgr5+ve Stem Cells Drive Self-Renewal in the Stomach and Build Long-Lived Gastric Units In Vitro. *Cell Stem Cell*, *6*(1), 25–36.

Barker, N., Ridgway, R. A., van Es, J. H., van de Wetering, M., Begthel, H., van den Born, M., Danenberg, E., Clarke, A. R., Sansom, O. J., Clevers, H. (2009). Crypt stem cells as the cells-of-origin of intestinal cancer. *Nature*, *457*(7229), 608–11.

Barker, N., Rookmaaker, M. B., Kujala, P., Ng, A., Leushacke, M., Snippert, H., van de Wetering, M., Tan, S., Van Es, J. H., Huch, M. *et al.* (2012). Lgr5+ve Stem/Progenitor Cells Contribute to Nephron Formation during Kidney Development. *Cell Reports*, *2*(3), 540–552.

Barker, N., Tan, S., & Clevers, H. (2013). Lgr proteins in epithelial stem cell biology. *Development (Cambridge, England)*, *140*(12), 2484–94.

Barker, N., van Es, J. H., Kuipers, J., Kujala, P., van den Born, M., Cozijnsen, M., Haegebarth, A., Korving, J., Begthel, H. Peters, P. J., Clevers, H. (2007). Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature*, *449*(7165), 1003–1007.

Barnes, D. E. (2001). Non-homologous end joining as a mechanism of DNA repair. *Current Biology*, *11*(12), 455–457.

Baron, M. (2003). An overview of the Notch signalling pathway. *Seminars in Cell & Developmental Biology*, *14*(2), 113–119.

Bejsovec, A. (2005). Wnt pathway activation: New relations and locations. *Cell*, *120*(1), 11–14.

Bhaya, D., Davison, M., Barrangou, R. (2011). CRISPR-Cas Systems in Bacteria and Archaea: Versatile Small RNAs for Adaptive Defense and Regulation. *Annual Review of Genetics*, *45*(1), 273–297.

Bibikova, M., Golic, M., Golic, K. G., Carroll, D. (2002). Targeted chromosomal cleavage and mutagenesis in Drosophila using zinc-finger nucleases. *Genetics*, *161*(3), 1169–1175.

Bienz, M., & Clevers, H. (2003). Armadillo/beta-catenin signals in the nucleus- proof beyond a reasonable doubt? *Nat Cell Biol*, *5*(March), 179–182.

Bitgood, M. J., & McMahon, A. P. (1995). HedgehogandBmpGenes Are Coexpressed at Many Diverse Sites of Cell–Cell Interaction in the Mouse Embryo. *Developmental Biology*, *172*(1), 126–138.

Bitinaite, J., Wah, D. A., Aggarwal, A. K., Schildkraut, I. (1998). FokI dimerization is required for DNA cleavage. *Proceedings of the National Academy of Sciences*, *95*(18), 10570–10575.

Bloemendaal, A. L. A., Buchs, N. C., George, B. D., Guy, R. J. (2016). Intestinal stem cells and intestinal homeostasis in health and in inflammation: A review. *Surgery (United States)*, *159*(5), 1237–1248.

Bogdanove, A. J., Schornack, S., Lahaye, T. (2010). TAL effectors: Finding plant genes for disease and defense. *Current Opinion in Plant Biology*, *13*(4), 394–401.

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., Lahaye, T., Nickstadt, A., Bonas, U. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. *Science (New York, N.Y.)*, *326*(5959), 1509–12.

Brinster, R. L., Chen, H. Y., Trumbauer, M., Senear, A. W., Warren, R., Palmiter, R. D. (1981). Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell*, *27*(1 PART 2), 223–231.

Brinster, R. L., Chen, H. Y., Warren, R., Sarthy, A., Palmiter, R. D. (1982). Regulation of metallothionein-thymidine kinase fusion plasmids injected into mouse eggs. *Nature*, *296*(5852), 39–42.

Buczacki, S. J. a, Zecchini, H. I., Nicholson, A. M., Russell, R., Vermeulen, L., Kemp, R., Winton, D. J. (2013). Intestinal label-retaining cells are secretory precursors expressing Lgr5. *Nature*, *495*(7439), 65–9.

Capecchi, M. R. (1989). Altering the Genome Homologous Recombination by From ES Cells to Germ Line Chimera. *Science*, *244*(4910), 1288–1292.

Capecchi, M. R. (2005). Gene targeting in mice: functional analysis of the mammalian genome for the twenty-first century. *Nature reviews. Genetics*, *6*(6), 507–512.

Carmon, K. S., Gong, X., Lin, Q., Thomas, A., Liu, Q. (2011). R-spondins function as ligands of the orphan receptors LGR4 and LGR5 to regulate Wnt/beta-catenin signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(28), 11452–7.

Carmon, K. S., Gong, X., Yi, J., Thomas, A., Liu, Q. (2014). RSPO-LGR4 functions via IQGAP1 to potentiate Wnt signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(13), E1221-9.

Cerpa, W., Godoy, J. A., Alfaro, I., Farías, G. G., Metcalfe, M. J., Fuentealba, R., Bonansco, Ch., Inestrosa, N. C. (2008). Wnt-7a modulates the synaptic vesicle cycle and synaptic transmission in hippocampal neurons. *Journal of Biological Chemistry*, *283*(9), 5918–5927.

Clevers, H. (2013). The intestinal crypt, a prototype stem cell compartment. *Cell*, *154*(2), 274–284.

Clevers, H. C., & Bevins, C. L. (2013). Paneth cells: maestros of the small intestinal crypts. *Annual review of physiology*, *75*, 289–311.

Clevers, H., Loh, K. M., Nusse, R. (2014). Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. *Science (New York, N.Y.)*, *346*(6205), 1248012.

Cruciat, C. M., & Niehrs, C. (2013). Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harbor perspectives in biology*.

Davis, E. K., Zou, Y., Ghosh, A. (2008). Wnts acting through canonical and noncanonical signaling pathways exert opposite effects on hippocampal synapse formation. *Neural development*, *3*, 32.

de Lau, W. B., Snel, B., Clevers, H. C. (2012). The R-spondin protein family. *Genome Biology*, *13*(3), 242.

de Lau, W., Barker, N., Low, T. Y., Koo, B.-K., Li, V. S. W., Teunissen, H., Kujala, P., Haegebarth, A., Peters, P. J., van de Wetering, M. *et al.* (2011). Lgr5 homologues associate with Wnt receptors and mediate R-spondin signalling. *Nature*, *476*(7360), 293–297.

Deltcheva, E., Chylinski, K., Sharma, C. M., Gonzales, K. (2011). CRISPR RNA maturation by trans -encoded small RNA and host factor RNase III. *Nature*, *471*(7340), 602–607.

Demitrack, E. S., & Samuelson, L. C. (2016). Notch regulation of gastrointestinal stem cells. *The Journal of Physiology*, *53*(9), n/a-n/a.

Deng, D., Yan, C., Pan, X., Mahfouz, M., Wang, J., Zhu, J.-K., Shi, Y., Yan, N. (2012). Structural basis for sequence-specific recognition of DNA by TAL effectors. *Science*, *335*(6069), 720–723.

Doetschman, T., Gregg, R. G., Maeda, N., Hooper, M. L., Melton, D. W., Thompson, S., Smithies, O. (1987). Targetted correction of a mutant HPRT gene in mouse embryonic stem cells. *Nature*, *330*, 576–578.

Doyon, Y., McCammon, J. M., Miller, J. C., Faraji, F., Ngo, C., Katibah, G. E., Amora, R., Hocking, T. D., Zhang, L., Rebar, E. J., Gregory, P. D., Urnov, F. D., Amacher, S. L. (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. *Nature biotechnology*, *26*(6), 702–708.

Du, H., Nie, Q., Holmes, W. R. (2015). The Interplay between Wnt Mediated Expansion and Negative Regulation of Growth Promotes Robust Intestinal Crypt Structure and Homeostasis. *PLoS Computational Biology*, *11*(8), 1–24.

Fafilek, B., Krausova, M., Vojtechova, M., Pospichalova, V., Tumova, L., Sloncova, E., Huranova, M., Stacikova, J., Hlavata, A., Svec, J., *et al.* (2013). Troy, a tumour necrosis factor receptor family member, interacts with Lgr5 to inhibit Wnt signaling in intestinal stem cells. *Gastroenterology*, *144*(2), 381–391.

Farin, H. F., Van Es, J. H., Clevers, H. (2012). Redundant sources of Wnt regulate intestinal stem cells and promote formation of paneth cells. *Gastroenterology*, *143*(6), 1518–1529.e7.

Ferretti, E., Smaele, E. De, Marcotullio, L. Di, Screpanti, I., & Gulino, A. (2005). Hedgehog checkpoints in medulloblastoma: The chromosome 17p deletion paradigm. *Trends in Molecular Medicine*, *11*(12), 537–545.

Fevr, T., Robine, S., Louvard, D., Huelsken, J. (2007). Wnt / β -Catenin Is Essential for Intestinal Homeostasis and Maintenance of Intestinal Stem Cells. *Mol Cell Biol*, *27*(21), 7551–7559.

Garcia, M. I., Ghiani, M., Lefort, A., Libert, F., Strollo, S., Vassart, G. (2009). LGR5 deficiency deregulates Wnt signaling and leads to precocious Paneth cell differentiation in the fetal intestine. *Developmental Biology*, *331*(1), 58–67.

Gordon, M. D., & Nusse, R. (2006). Wnt signaling: Multiple pathways, multiple receptors, and multiple transcription factors. *Journal of Biological Chemistry*, *281*(32), 22429–22433.

Gratz, S. J., Cummings, A. M., Nguyen, J. N., Hamm, D. C., Donohue, L. K., Harrison, M. M., Wildonger, J., O'connor-Giles, K. M. (2013). Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. *Genetics*, *194*(4), 1029–1035.

Gregorieff, A., & Clevers, H. (2005). Wnt signaling in the intestinal epithelium : from endoderm to cancer Wnt signaling in the intestinal epithelium : from endoderm to cancer, 877–890.

Gubb, D., & Garcia-Bellido, A. (1982). A genetic analysis of the determination of cuticular polarity during development in Drosophila melanogaster. *J Embryol Exp Morphol, Apr*, *68*, 67–68.

Hao, H.-X., Xie, Y., Zhang, Y., Charlat, O., Oster, E., Avello, M., Lei, H., Mickanin, C., Liu, D., Ruffner, H., *et al.* (2012). ZNRF3 promotes Wnt receptor turnover in an R-spondin-sensitive manner. *Nature*, *485*(7397), 195–200.

Haramis, A.-P. G., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G. J. a, Clevers, H. (2004). De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. *Science (New York, N.Y.)*, *303*(5664), 1684–1686.

Hardwick, J. C., Kodach, L. L., Offerhaus, G. J., van den Brink, G. R. (2008). Bone morphogenetic protein signalling in colorectal cancer. *Nature reviews.Cancer*, *8*(10), 806–812.

Harned, K., Buck, L., Sobin, H. (1995). Review Article The Hamartomatous Radiologic Features Syndromes : Clinical and. *American Journal of Medical Genetics*, 565–571.

He, T., Sparks, A. B., Rago, C., Hermeking, H., Zawel, L., Costa, L. T., Morin, P. J., Vogelsteing, B., Kinzler, K. W. (1998). Identification of c- MYC as a Target of the APC Pathway. *Science*, *281*(September), 1509–1512.

He, X. C., Zhang, J., Tong, W.-G., Tawfik, O., Ross, J., Scoville, D. H., Tian, Q., Zeng, X., He, X., Wiedemann, L. M., *et al.* (2004). BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nature genetics*, *36*(10), 1117–21.

He, X., Semenov, M., Tamai, K., Zeng, X. (2004). LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development (Cambridge, England)*, *131*(8), 1663–77.

Hollnagel, A., Oehlmann, V., Heymer, J., Rüther, U., Nordheim, A. (1999). Id genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. *Journal of Biological Chemistry*, *274*(28), 19838–19845.

Hooper, J. E., & Scott, M. P. (1989). The Drosophila patched gene encodes a putative membrane protein required for segmental patterning. *Cell*, *59*(4), 751–765.

Horvath, P., & Barrangou, R. (2010). CRISPR/Cas, the immune system of bacteria and archaea. *Science (New York, N.Y.)*, *327*(5962), 167–170.

Hsu, S. Y., Kudo, M., Chen, T., Nakabayashi, K. (2000). The three subfamilies of leucine-rich repeat-containing G protein-coupled receptors {(LGR):} identification of {LGR6} and {LGR7} and the signaling mechanism for {LGR7}, (March), 1257–1271.

Hsu, S. Y., Liang, S.-G., Hsueh, A. J. W. (1998). Characterization of Two LGR Genes Homologous to Gonadotropin and Thyrotropin Receptors with Extracellular Leucine-Rich Repeats and a G Protein-Coupled, Seven-Transmembrane Region. *Molecular Endocrinology*, *12*(12), 1830–1845.

Hsu, S. Y. T. (2003). New insights into the evolution of the relaxin - LGR signaling system. *Trends in Endocrinology and Metabolism*, *14*(7), 303–309.

Chen, S., Oikonomou, G., Chiu, C. N., Niles, B. J., Liu, J., Lee, D. A., Antoshechnik, I., Prober, D. A. (2013). A large-scale in vivo analysis reveals that TALENs are significantly more mutagenic than ZFNs generated using context-dependent assembly. *Nucleic Acids Research*, *41*(4), 2769–2778.

Chiba, S. (2006). Notch signaling in stem cell systems. *Stem cells*, *24*(11), 2437–2447.

Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., Hummel, A., Bogdanove, A. J., Voytas, D. F. (2010). Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics*, *186*(2), 756–761.

Ireland, H., Kemp, R., Houghton, C., Howard, L., Clarke, A. R., Sansom, O. J., Winton, D. J. (2004). Inducible Cre-Mediated Control of Gene Expression in the Murine Gastrointestinal Tract: Effect of Loss of ??-Catenin. *Gastroenterology*, *126*(5), 1236–1246.

Jaks, V., Barker, N., Kasper, M., van Es, J. H., Snippert, H. J., Clevers, H., Toftgård, R. (2008). Lgr5 marks cycling, yet long-lived, hair follicle stem cells. *Nature genetics*, *40*(11), 1291–1299.

Jiang, W., Zhou, H., Bi, H., Fromm, M., Yang, B., Weeks, D. P. (2013). Demonstration of CRISPR/Cas9/sgRNA-mediated targeted gene modification in Arabidopsis, tobacco, sorghum and rice. *Nucleic Acids Research*, *41*(20), 1–12.

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., Charpentier, E. (2012). A Programmable Dual-RNA – Guided, *337*(August), 816–822.

K??hl, M., Sheldahl, L. C., Park, M., Miller, J. R., Moon, R. T. (2000). The Wnt/Ca2+ pathway A new vertebrate Wnt signaling pathway takes shape. *Trends in Genetics*, *16*(7), 279–283.

Kato, S., Matsubara, M., Matsuo, T., Mohri, Y., Kazama, I., Hatano, R., Umezawa, A., Nishimori, K. (2006). Leucine-rich repeat-containing G protein-coupled receptor-4 (LGR4, Gpr48) is essential for renal development in mice. *Nephron - Experimental Nephrology*, *104*(2), 63–76.

Kazanskaya, O., Glinka, A., del Barco Barrantes, I., Stannek, P., Niehrs, C., Wu, W. (2004). R-Spondin2 is a secreted activator of Wnt/??-catenin signaling and is required for Xenopus myogenesis. *Developmental Cell*, *7*(4), 525–534.

Kengaku, M., Capdevila, J., Rodriguez-Esteban, C., De La Peña, J., Johnson, R. L., Izpisúa Belmonte, J. C., Tabin, C. J. (1998). Distinct WNT pathways regulating AER formation and dorsoventral polarity in the chick limb bud. *Science (New York, N.Y.)*, *280*(5367), 1274–1277.

Kim, K.-A. (2005). Mitogenic Influence of Human R-Spondin1 on the Intestinal Epithelium. *Science*, *309*(5738), 1256–1259.

Klaus, A., & Birchmeier, W. (2008). Wnt signalling and its impact on development and cancer. *Nat Rev Cancer*, *8*(5), 387–398.

Kohn, A. D., & Moon, R. T. (2005). Wnt and calcium signaling: ??-Catenin-independent pathways. *Cell Calcium*, *38*(3–4 SPEC. ISS.), 439–446.

Kolterud, Å., Grosse, A. S., Zacharias, W. J., Walton, K. D., Katherine, E., Madison, B., Waghray, M., Ferris, J. E., Hu, Ch., Merchant, J. L., *et al.* (2009). for Hedgehog in gastrointestinal patterning. *Gastroenterology*, *137*(2), 618–628.

Kolterud, Å., & Toftgård, R. (2007). Strategies for Hedgehog inhibition and its potential role in cancer treatment. *Drug Discovery Today: Therapeutic Strategies*, *4*(4), 229–235.

Komiya, Y., & Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis*, *4*(2), 68–75.

Koo, B. K., Spit, M., Jordens, I., Low, T. Y., Stange, D. E., van de Wetering, M., van Es, J. H., Mohammed, S., Heck, A. J. R., Maurice, M. M., Clevers, H. (2012). Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. *Nature*, *488*(7413), 665–669.

Kopan, R., & Ilagan, M. X. G. (2009). The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. *Cell*, *137*(2), 216–233.

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

Kosinski, C., Li, V. S. W., Chan, A. S. Y., Zhang, J., Ho, C., Tsui, W. Y., Chan, T., L., Mifflin, R. C., Powell, D. W., Yuen, S. T., *et al.* (2007). Gene expression patterns of human colon tops and basal crypts and BMP antagonists as intestinal stem cell niche factors. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(39), 15418–23.

Krausova, M., & Korinek, V. (2012). Signal transduction pathways participating in homeostasis and malignant transformation of the intestinal tissue. *Neoplasma*.

Krausova, M., & Korinek, V. (2014). Wnt signaling in adult intestinal stem cells and cancer. *Cellular Signalling*, *26*(3), 570–579.

Lander, A. D., Kimble, J., Clevers, H., Fuchs, E., Montarras, D., Buckingham, M., Calof, A. L., Trumpp, A., Oskarsson, T. (2012). What does the concept of the stem cell niche really mean today? *BMC biology*, *10*(1), 19.

Lehoczky, J. a., & Tabin, C. J. (2015). Lgr6 marks nail stem cells and is required for digit tip regeneration. *Proceedings of the National Academy of Sciences*, *2015*(43), 201518874.

Li, D., Qiu, Z., Shao, Y., Chen, Y., Guan, Y., Liu, M., Li, Y., Gao, N., Wang, L., Lu, X., *et al.* (2013). Heritable gene targeting in the mouse and rat using a CRISPR-Cas system. *Nature Biotechnology*, *31*(8), 681–683.

Li, X., Madison, B. B., Zacharias, W., Kolterud, A., States, D., Gumucio, D. L. (2007). Deconvoluting the intestine: molecular evidence for a major role of the mesenchyme in the modulation of signaling cross talk. *Physiological genomics*, *29*(3), 290–301.

Lin, F.-L., Sperle, K., Sternberg, A. N. (1985). Recombination in mouse L cells between DNA introduced into cells and homologous chromosomal sequences (homologous recombination/thymidine kinase gene/gene transfer/DNA rearrangement). *Biochemistry*, *82*(March), 1391–1395.

Liu, J., Li, C., Yu, Z., Huang, P., Wu, H., Wei, C., Zhu, N., Shen, Y., Chen, Y., Zhang, B., *et al.* (2012). Efficient and Specific Modifications of the Drosophila Genome by Means of an Easy TALEN Strategy. *Journal of Genetics and Genomics*, *39*(5), 209–215.

Liu, S., Qian, Y., Li, L., Wei, G., Guan, Y., Pan, H., Guan, X., Zhang, L., Lu, X., Zhao, Y., *et al.* (2013). Lgr4 gene deficiency increases susceptibility and severity of dextran sodium sulfate-induced inflammatory bowel disease in mice. *Journal of Biological Chemistry*, *288*(13), 8794–8803.

Logan, C. Y., & Nusse, R. (2004). the Wnt Signaling Pathway in Development and Disease. *Annual Review of Cell and Developmental Biology*, *20*(1), 781–810.

Luo, C. W., & Hsueh, A. J. (2006). Genomic analyses of the evolution of LGR genes. *Chang Gung Med J*, *29*(1), 2–8.

Luo, J., Zhou, W., Zhou, X., Li, D., Weng, J., Yi, Z., Cho, S. G., Li, Ch., Yi, T., *et al.* (2009). Regulation of bone formation and remodeling by G-protein-coupled receptor 48. *Development (Cambridge, England)*, *136*(16), 2747–56.

Luo, W., Rodriguez, M., Valdez, J. M., Zhu, X., Tan, K., Li, D., Siwko, S., Xin, L., Liu, M. (2013). Lgr4 is a key regulator of prostate development and prostate stem cell differentiation. *Stem Cells*, *31*(11), 2492–2505.

Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P. M., Birchmeier, W., Behrens, J. (2002). Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors. *Molecular and cellular biology*, *22*(4), 1184–93.

Madison, B. B., Braunstein, K., Kuizon, E., Portman, K., Qiao, X. T., Gumucio, D. L. (2005). Epithelial hedgehog signals pattern the intestinal crypt-villus axis. *Development (Cambridge, England)*, *132*(2), 279–289.

Mak, A. N.-S., Bradley, P., Cernadas, R. A., Bogdanove, A. J., Stoddard, B. L. (2012). The crystal structure of TAL effector PthXo1 bound to its DNA target. *Science*, *335*(6069), 716–9.

Makarova, K. S., Grishin, N. V, Shabalina, S. A., Wolf, Y. I., Koonin, E. V. (2006). A putative RNA-interference-based immune system in prokaryotes: computational analysis of the predicted enzymatic machinery, functional analogies with eukaryotic RNAi, and hypothetical mechanisms of action. *Biology direct*, *1*(1), 7.

Mansour, S. L., Thomas, K. R., Capecchi, M. R. (1988). Disruption of the proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. *Nature*, *336*(6197), 348–52.

Mao, J., Kim, B.-M., Rajurkar, M., Shivdasani, R. A., McMahon, A. P. (2010). Hedgehog signaling controls mesenchymal growth in the developing mammalian digestive tract. *Development (Cambridge, England)*, *137*(10), 1721–9.

Matsuyama, M., Aizawa, S., Shimono, A. (2009). Sfrp controls apicobasal polarity and oriented cell division in developing gut epithelium. *PLoS Genetics*, *5*(3).

Mazerbourg, S., Bouley, D. M., Sudo, S., Klein, C. A., Zhang, J. V, Kawamura, K., Goodrich, L. V., Rayburn, H., Tessier-Lavigne, M., Hsueh, A. J. (2004). Leucine-rich repeat-containing, G protein-coupled receptor 4 null mice exhibit intrauterine growth retardation associated with embryonic and perinatal lethality. *Mol Endocrinol*, *18*(9), 2241–2254.

Mendive, F., Laurent, P., Van Schoore, G., Skarnes, W., Pochet, R., Vassart, G. (2006). Defective postnatal development of the male reproductive tract in LGR4 knockout mice. *Developmental Biology*, *290*(2), 421–434.

Merlos-Suárez, A., Barriga, F. M., Jung, P., Iglesias, M., Céspedes, M. V., Rossell, D., Sevillano, M., Hernado-Momblona, X., da Silva-Diz, V. Munoz, P., *et al.* (2011). The intestinal stem cell signature identifies colorectal cancer stem cells and predicts disease relapse. *Cell Stem Cell*, *8*(5), 511–524.

Mikels, A. J., & Nusse, R. (2006). Purified Wnt5a protein activates or inhibits beta-catenin-TCF signaling depending on receptor context. *PLoS Biology*, *4*(4), 570–582.

Miller, J. C., Tan, S., Qiao, G., Barlow, K. A., Wang, J., Xia, D. F., Meng, X., Paschon, D. E., Leung, E.,Hinkley, S. J., *et al.* (2011). A TALE nuclease architecture for efficient genome editing. *Nature Biotechnology*, *29*(2), 143–148.

Miller, J., McLachan, a D., Klug, A. (1985). Repetitive zinc-binding doains in the protein transcription factor IIIA from Xenopus oocytes. *EMBO Journal*, *4*(6), 1609–1614.

Miller, J. R. (2001). The Wnts. *Genome biology*, *3*(1), REVIEWS3001.

Mishina, Y. (2002). Function of bone morphogenetic protein signaling during mouse development. *Front Biosci*, 855–869.

Mishina, Y. (2003). Function of bone morphogenetic protein signaling during mouse development. *Frontiers in bioscience : a journal and virtual library*, *8*, d855-69.

Miyoshi, H. (2017). Wnt-expressing cells in the intestines: guides for tissue remodeling. *Journal of Biochemistry*, *161*(1), 19–25.

Morita, H., Mazerbourg, S., Bouley, D. M., Luo, C.-W., Kawamura, K., Kuwabara, Y., Baribault, H., Tian, H., Hsueh, A. J. W. (2004). Neonatal lethality of LGR5 null mice is associated with ankyloglossia and gastrointestinal distension. *Molecular and cellular biology*, *24*(22), 9736–43.

Moscou, M. J., & Bogdanove, A. J. (2009). A simple cipher governs DNA recognition by TAL effectors. *Science*, *326*(5959), 1501.

Mumm, J. S., Kopan, R. (2000). Notch signaling: from the outside in. *Dev Biol*, *228*(2), 151–165.

Mustata, R. C., Van Loy, T., Lefort, A., Libert, F., Strollo, S., Vassart, G., Garcia, M.-I. (2011). Lgr4 is required for Paneth cell differentiation and maintenance of intestinal stem cells ex vivo. *EMBO reports*, *12*(6), 558–64.

Najdi, R., Proffitt, K., Sprowl, S., Kaur, S., Yu, J., Covey, T. M., Virshup, M., Waterman, M. L. (2012). A uniform human Wnt expression library reveals a shared secretory pathway and unique signaling activities. *Differentiation*, *84*(2), 203–213.

Nakano, Y., Guerrero, I., Hidalgo, a, Taylor, a, Whittle, J. R., Ingham, P. W. (1989). A protein with several possible membrane-spanning domains encoded by the Drosophila segment polarity gene patched. *Nature*, *341*(6242), 508–13.

Ng, A., Tan, S., Singh, G., Rizk, P., Swathi, Y., Tan, T. Z., Huang, R. Y.-J., Leushacke, M., Barker, N. (2014). Lgr5 marks stem/progenitor cells in ovary and tubal epithelia. *Nature cell biology*, *16*(8), 745–57.

Nishita, M., Enomoto, M., Yamagata, K., Minami, Y. (2010). Cell/tissue-tropic functions of Wnt5a signaling in normal and cancer cells. *Trends in Cell Biology*, *20*(6), 346–354.

Oeztuerk-Winder, F., Guinot, A., Ochalek, A., Ventura, J.-J. (2012). Regulation of human lung alveolar multipotent cells by a novel p38α MAPK/miR-17-92 axis. *The EMBO Journal*, *31*(16), 3506–3506.

Palmiter, R. D., Brinster, R. L., Hammer, R. E., Trumbauer, M. E., Rosenfeld, M. G., Birnberg, N. C., Evans, R. M. (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature*, *300*(5893), 611–5.

Papkoff, J., Brown, A. M., Varmus, H. E. (1987). The int-1 proto-oncogene products are glycoproteins that appear to enter the secretory pathway. *Molecular and cellular biology*, *7*(11), 3978–84.

Pattanayak, V., Lin, S., Guilinger, J. P., Ma, E., Doudna, J. a, Liu, D. R. (2013). High-throughput profiling of off-target DNA cleavage reveals RNA-programmed Cas9 nuclease specificity. *Nature biotechnology*, *31*(9), 839–43.

Plaks, V., Brenot, A., Lawson, D. A., Linnemann, J. R., Van Kappel, E. C., Wong, K. C.,de Sauvage, F., Klein, O. D., Werb, Z. (2013). Lgr5-Expressing Cells Are Sufficient And Necessary for Postnatal Mammary Gland Organogenesis. *Cell Reports*, *3*(1), 70–78.

Potten, C. S., Booth, C., Pritchard, D. M. (1997). The intestinal epithelial stem cell: the mucosal governor. *International journal of experimental pathology*, *78*(4), 219–43.

Potten, C. S., Gandara, R., Mahida, Y. R., Loeffler, M., Wright, N. A. (2009). The stem cells of small intestinal crypts: Where are they? *Cell Proliferation*, *42*(6), 731–750.

Ramalho-Santos, M., Melton, D. a, McMahon, a P. (2000). Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development (Cambridge, England)*, *127*(12), 2763–2772.

Ren, W., Lewandowski, B. C., Watson, J., Aihara, E., Iwatsuki, K., Bachmanov, A. A., Margolskee, R. F., Jiang, P. (2014). Single Lgr5- or Lgr6-expressing taste stem/progenitor cells generate taste bud cells ex vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(46), 16401–6.

Rocha-martins, M., & Cavalheiro, G. R. (2015). From Gene Targeting to Genome Editing : Transgenic animals applications and beyond, *87*, 1323–1348.

Romay, G., & Bragard, C. (2017). Antiviral Defenses in Plants through Genome Editing. *Frontiers in Microbiology*, *8*(January), 1–11.

Roose, J., & Clevers, H. (1999). TCF transcription factors: Molecular switches in carcinogenesis. *Biochimica et Biophysica Acta - Reviews on Cancer*, *1424*(2–3).

Ruffner, H., Sprunger, J., Charlat, O., Leighton-Davies, J., Grosshans, B., Salathe, A., Zietzling, S., Beck, V., Therier, M., Isken, A., *et al.* (2012). R-spondin potentiates Wnt/beta-Catenin signaling through orphan receptors LGR4 and LGR5. *PLoS ONE*, *7*(7).

Sander, J. D., Cade, L., Khayter, C., Reyon, D., Peterson, R. T., Joung, J. K., Yeh, J.-R. J. (2011). Targeted gene disruption in somatic zebrafish cells using engineered TALENs. *Nature biotechnology*, *29*(8), 697–698.

Sangiorgi, E., & Capecchi, M. R. (2008). Bmi1 is expressed in vivo in intestinal stem cells. *Nature genetics*, *40*(7), 915–20.

Sato, T., van Es, J. H., Snippert, H. J., Stange, D. E., Vries, R. G., van den Born, M., Barker, N., Shroyer, N. F., van de Wetering, M., Clevers, H. (2011). Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature*, *469*(7330), 415–418.

Sheldahl, L. C., Park, M., Malbon, C. C., Moon, R. T. (1999). Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. *Current Biology*, *9*(13), 695–698.

Shen, B., Zhang, J., Wu, H., Wang, J., Ma, K., Li, Z., Zhang, X., Zhang, P., Huang, X. (2013). Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell research*, *23*(5), 720–3.

Shimizu, H., Julius, M. a, Giarré, M., Zheng, Z., Brown, a M., Kitajewski, J. (1997). Transformation by Wnt family proteins correlates with regulation of β-catenin. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research*, *8*(12), 1349–1358.

Schambony, A., & Wedlich, D. (2007). Wnt-5A/Ror2 Regulate Expression of XPAPC through an Alternative Noncanonical Signaling Pathway. *Developmental Cell*, *12*(5), 779–792.

Schepers, A., & Clevers, H. (2012). Wnt signaling, stem cells, and cancer of the gastrointestinal tract. *Cold Spring Harbor perspectives in biology*, *4*(4), 1–14.

Schulte, G. (2010). International Union of Basic and Clinical. *Pharmacological Reviews*, *62*(4), 632–667.

Slusarski, D. C., Yang-Snyder, J., Busa, W. B., Moon, R. T. (1997). Modulation of embryonic intracellular Ca2+ signaling by Wnt-5A. *Developmental biology*, *182*(1), 114–20.

Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A., Kucherlapati, R. S. (1985). Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature*, *317*(6034), 230–4.

Snippert, H. J., Haegebarth, A., Kasper, M., Jaks, V., van Es, J. H., Barker, N.,van de Wetering, M., van den Born, M., Begthel, H., Vries, R. G. *et al.* (2010). Lgr6 Marks Stem Cells in the Hair Follicle That Generate All Cell Lineages of the Skin. *Science*, *327*(5971), 1385–1389.

Sone, M., Oyama, K., Mohri, Y., Hayashi, R., Clevers, H., Nishimori, K. (2013). LGR4 expressed in uterine epithelium is necessary for uterine gland development and contributes to decidualization in mice. *FASEB Journal*, *27*(12), 4917–4928.

Song, H., Luo, J., Luo, W., Weng, J., Wang, Z., Li, B., Li, B., Li. D., Liu, M. (2008). Inactivation of G-protein-coupled receptor 48 (Gpr48/Lgr4) impairs definitive erythropoiesis at midgestation through down-regulation of the ATF4 signaling pathway. *Journal of Biological Chemistry*, *283*(52), 36687–36697.

Sugi, T. (2016). Genome editing in c. Elegans and other nematode species. *International Journal of Molecular Sciences*, *17*(3).

Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T., Takada, S. (2006). Monounsaturated Fatty Acid Modification of Wnt Protein: Its Role in Wnt Secretion. *Developmental Cell*, *11*(6), 791–801.

Takahashi, H., Ishii, H., Nishida, N., Takemasa, I., Mizushima, T., Ikeda, M., Yokobori, T., Mimori, K., Yamamoto, H., Sekimoto, M., *et al.* (2011). Significance of Lgr5(+ve) cancer stem cells in the colon and rectum. *Annals of surgical oncology*, *18*, 1166–1174.

Tanese, K., Fukuma, M., Yamada, T., Mori, T., Yoshikawa, T., Watanabe, W., Ishiko, A., Amagai, M., Nishikawa, T., Sakamoto, M. (2008). G-protein-coupled receptor GPR49 is up-regulated in basal cell carcinoma and promotes cell proliferation and tumor formation. *The American journal of pathology*, *173*(3), 835–843.

Tanneberger, K., Pfister, A. S., Brauburger, K., Schneikert, J., Hadjihannas, M. V, Kriz, V., Schulte, G., Bryja, V., Behrens, J. (2011). Amer1/WTX couples Wnt-induced formation of PtdIns(4,5)P2 to LRP6 phosphorylation. *The EMBO journal*, *30*(8), 1433–43.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, Ch. C., Lin, X., Heasman, J. (2005). Maternal Wnt11 activates the canonical Wnt signaling pathway required for axis formation in Xenopus embryos. *Cell*, *120*(6), 857–871.

Terns, M. P., & Terns, R. M. (2011). CRISPR-Based Adaptive Immune Systems. *Current Opinions in Microbiology*, *14*(3), 321–327.

Tesson, L., Usal, C., Ménoret, S., Leung, E., Niles, B. J., Remy, S., Santiago, Y., Vincent, A. I., Meng, X., Zhang, L., *et al.* (2011). Knockout rats generated by embryo microinjection of TALENs. *Nature biotechnology*, *29*(8), 695–696.

Tetsu, O., & McCormick, F. (1999). Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature*, *398*(6726), 422–426.

Uchida, H., Yamazaki, K., Fukuma, M., Yamada, T., Hayashida, T., Hasegawa, H., Kitajima, M., Kitagawa, Y., Sakamoto, M. (2010). Overexpression of leucine-rich repeat-containing G protein-coupled receptor 5 in colorectal cancer. *Cancer Science*, *101*(7), 1731–1737.

Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S., Gregory, P. D. (2010). (13) Genome editing with engineered zinc finger nucleases. *Nature reviews. Genetics*, *11*(9), 636–46.

Valenta, T., Gay, M., Steiner, S., Draganova, K., Zemke, M., Hoffmans, R., Cinelli, P., Aguet, M., Sommer, L., Basler, K. (2011). Probing transcription-specific outputs of beta-catenin in vivo. *Genes and Development*, *25*(24), 2631–2643.

van Amerongen, R., & Nusse, R. (2009). Towards an integrated view of Wnt signaling in development. *Development (Cambridge, England)*, *136*(19), 3205–14.

Van de Wetering, M., Sancho, E., Verweij, C., De Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batle, E., Coudreuse, D., Haramis, A.-P., *et al.* (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell*, *111*(2), 241–250.

van den Bosch, M., Lohman, P. H., Pastink, a. (2002). DNA double-strand break repair by homologous recombination. *Biol Chem*, *383*(6), 873–892.

van den Brink, G. R. (2007). Hedgehog Signaling in Development and Homeostasis of the Gastrointestinal Tract. *Physiol Rev*, *87*, 1343–1375.

van den Brink, G. R., Bleuming, S. A., Hardwick, J. C. H., Schepman, B. L., Offerhaus, G. J., Keller, J. J., Nielsen, C., Gaffield, W., van Deventer, S. J. H., Roberts, D. J., Peppelenbosch, M. P. (2004). Indian Hedgehog is an antagonist of Wnt signaling in colonic epithelial cell differentiation. *Nature genetics*, *36*(3), 277–282.

van der Flier, L. G., & Clevers, H. (2009). Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol*, *71*, 241–260.

van der Flier, L. G., Haegebarth, A., Stange, D. E., van de Wetering, M., Clevers, H. (2009). OLFM4 Is a Robust Marker for Stem Cells in Human Intestine and Marks a Subset of Colorectal Cancer Cells. *Gastroenterology*, *137*(1), 15–17.

Van Dop, W. A., Heijmans, J., Büller, N. V. J. A., Snoek, S. A., Rosekrans, S. L., Wassenberg, E. A., van den Bergh Weerman, M. A., Lanske, B., Clarke, A. R., Winton, D. J., *et al.* (2010). Loss of Indian hedgehog activates multiple aspects of a wound healing response in the mouse intestine. *Gastroenterology*, *139*(5), 1665–1676.e10.

Van Schoore, G., Mendive, F., Pochet, R., Vassart, G. (2005). Expression pattern of the orphan receptor LGR4/GPR48 gene in the mouse. *Histochemistry and Cell Biology*, *124*(1), 35–50.

VanDussen, K. L., & Samuelson, L. C. (2010). Mouse atonal homolog 1 directs intestinal progenitors to secretory cell rather than absorptive cell fate. *Developmental Biology*, *346*(2), 215–223.

Vanuytsel, T., Senger, S., Fasano, A., & Shea-Donohue, T. (2013). Major signaling pathways in intestinal stem cells. *Biochimica et biophysica acta*, *1830*(2), 2410–26.

Vogel, G. (2007). A Knockout Award in Medicine. *Science*, *318*(5848), 178–179.

Wah, D. a, Bitinaite, J., Schildkraut, I., Aggarwal, a K. (1998). Structure of FokI has implications for DNA cleavage. *Proc Natl Acad Sci U S A*, *95*(18), 10564–10569.

Waite, K. a, & Eng, C. (2003). From developmental disorder to heritable cancer: it's all in the BMP/TGF-beta family. *Nature reviews. Genetics*, *4*(10), 763–73.

Wang, H., Yang, H., Shivalila, C. S., Dawlaty, M. M., Cheng, A. W., Zhang, F., Jaenisch, R. (2013). One-step generation of mice carrying mutations in multiple genes by CRISPR/cas-mediated genome engineering. *Cell*, *153*(4), 910–918.

Wang, Y., & Nathans, J. (2007). Tissue/planar cell polarity in vertebrates: new insights and new questions. *Development (Cambridge, England)*, *134*(4), 647–58.

Wefers, B., Ortiz, O., Wurst, W., Kühn, R. (2014). Generation of targeted mouse mutants by embryo microinjection of TALENs. *Methods (San Diego, Calif.)*, *69*(1), 94–101.

Weiss, M. J., & Mullighan, C. G. (2016). Welcoming a new age for gene therapy in hematology. *Blood*, *127*(21), 2523–2524.

Wiedenheft, B., Sternberg, S. H., Doudna, J. a. (2012). RNA-guided genetic silencing systems in bacteria and archaea. *Nature*, *482*(7385), 331–338.

Wijshake, T., Baker, D. J., van de Sluis, B. (2014). Endonucleases: New tools to edit the mouse genome. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1842*(10), 1942–1950.

Willert, K., Brown, J. D., Danenberg, E., Duncan, A. W., Weissman, I. L., Reya, T., Yates III, J. R., Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature*, *423*(6938), 448–452.

Wodarz, A., & Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu.Rev.Cell Dev.Biol.*, *14*, 59–88.

Wolfe, S. A., Nekludova, L., Pabo, C. O. (2000). DNA recognition by Cys2His2 zinc finger proteins. *Annual review of biophysics and biomolecular structure*, *29*, 183–212.

Woodford-Richens, K., Williamson, J., Bevan, S., Young, J., Leggett, B., Frayling, I., Thway, Y., Hodgson, S., Kim, J. Ch., Iwama, T., *et al.* (2000). Allelic loss at SMAD4 in polyps from juvenile polyposis patients and use of fluorescence in situ hybridization to demonstrate clonal origin of the epithelium. *Cancer Research*, *60*(9), 2477–2482.

Xiao, A., Wang, Z., Hu, Y., Wu, Y., Luo, Z., Yang, Z., Li, W., Huang, P., Tong, X., Zhu, Z., *et al.* (2013). Chromosomal deletions and inversions mediated by TALENs and CRISPR/Cas in zebrafish. *Nucleic Acids Research*, *41*(14), 1–11.

Yamashita, R., Takegawa, Y., Sakumoto, M., Nakahara, M., Kawazu, H., Hoshii, T., Araki, K., Yokouchi, Y., Yamamura, K. I. (2009). Defective development of the gall bladder and cystic duct in Lgr4- hypomorphic mice. *Developmental Dynamics*, *238*(4), 993–1000.

Yee, K. K., Li, Y., Redding, K. M., Iwatsuki, K., Margolskee, R. F., Jiang, P. (2013). Lgr5-EGFP marks taste bud stem/progenitor cells in posterior tongue. *Stem Cells*, *31*(5), 992–1000.

Yi, J., Xiong, W., Gong, X., Bellister, S., Ellis, L. M., Liu, Q. (2013). Analysis of LGR4 Receptor Distribution in Human and Mouse Tissues. *PLoS ONE*, *8*(10), 1–11.

Ying, J., Li, H., Yu, J., Ka, M. N., Fan, F. P., Wong, S. C. C., Chan, A. T. C., Sung, J. J. Y., Tao, Q. (2008). WNT5A exhibits tumor-suppressive activity through antagonizing the Wnt/beta-catenin signaling, and is frequently methylated in colorectal cancer. *Clinical Cancer Research*, *14*(1), 55–61.

Zacharias, W. J., Li, X., Madison, B. B., Kretovich, K., Kao, J. Y., Merchant, J. L., Gumucio, D. L. (2010). Hedgehog Is an Anti-Inflammatory Epithelial Signal for the Intestinal Lamina Propria. *Gastroenterology*, *138*(7).

Zhang, S., Li, L., Kendrick, S. L., Gerard, R. D., Zhu, H. (2014). TALEN-mediated somatic mutagenesis in murine models of cancer. *Cancer Research*, *74*(18), 5311–5321.

Zhang, Y., Chen, Y., Ni, W., Guo, L., Lu, X., Liu, L., Li, W., Sun, S., Wang, L., Li, H. (2015). Dynamic expression of Lgr6 in the developing and mature mouse cochlea. *Frontiers in cellular neuroscience*, *9*(May), 165.

Zucman-Rossi, J., Benhamouche, S., Godard, C., Boyault, S., Grimber, G., Balabaud, C., Cunha, A. S., Bioulac-Sage, P., Perret, C. (2007). Differential effects of inactivated Axin1 and activated b-catenin mutations in human hepatocellular carcinomas. *Oncogene*, *26*, 774–780.