Tampere University

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ACADEMIC DISSERTATION
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The Man in the Arena

It is not the critic who counts; nor the one who points out how the strong person stumbled, or where the doer of a deed could have done better.

The credit belongs to the person who is actually in the arena; whose face is marred by dust and sweat and blood who strives valiantly; who errs and comes short again and again, because there is no effort without error and shortcoming; who does actually strive to do deeds; who knows the great enthusiasms, the great devotion, spends oneself in a worthy cause; who at the best knows in the end the triumph of high achievement; and who at worst, if he fails, at least fails while daring greatly.

Far better it is to dare mighty things, to win glorious triumphs even though checkered by failure, than to rank with those timid spirits who neither enjoy nor suffer much because they live in the gray twilight that knows neither victory nor defeat.

Theodore Roosevelt, "Citizenship in a Republic" 1910

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Tampere, December 2021

Joel Johnson

ABSTRACT

The intestinal tract is subject to multiple antigens that are consumed with food, detrimental foreign pathogens, and antigens from symbiotic bacteria. The mucosal lining of the intestinal tract is a well-equipped combatant against these invasions since it employs multiple layers of defense. Physical barriers are set up using tight junction barriers to physically hinder the invasion of pathogenic molecules. Microvilli generate an electrostatic zeta charge to impair pathogen binding and goblet cells in the intestinal tract generate a mucous layer that physicochemically inhibits the attachment of harmful antigens. Inductive immune sites in the gut called Peyer's patch are found in the Gut-associated lymphoid tissue, these sites directly sample mucosal antigens via the use of specialized epithelial cells in the follicular associated epithelium known as Microfold cells or M cells. M cells form a part of the adaptive immunity barrier as they house B cells, T cells, and other antigen-presenting cells such as dendritic cells. This immune ecosystem is required for producing secretory immunoglobulin A (SIgA). SIgA production is dependent on the uptake of commensal particles and antigens and subsequent activation of B cells, T cells, and dendritic cells. M cells serve as a portal for the entry of foreign antigens to induce an antigen-specific immune response.

The population of M cells in the gut is low in number, they only consist of 8% of epithelial cells in the follicle-associated epithelium and they reside in 6-7 Peyer's patch in the entire intestinal tract in mouse. Due to the low population, factors regulating differentiation and development of M cell and its function remains yet to be fully elucidated. Polycomb group (PcG) proteins are critical for embryonic stem cell self-renewal and pluripotency. They have also been found to be responsible for intestinal cell differentiation, development, and functionality. Polycomb repressive complex 2, a subunit of PcG, is a critical factor in maintaining intestinal homeostasis and also contributes to conditions instigating stemness and differentiation. Previous work has indicated PRC2's indispensable role in regulating stemness and differentiation in the intestinal epithelium and since PRC2's role in M cell differentiation remained to be elucidated, we set out to study how PRC2 regulates genome-wide regulation in M cell differentiation and development in mouse (Mus

musculus). Our Chip-seq and Gro- seq revealed 12 novel transcription factors that were regulated by the PRC2 that could be responsible for M cell development. We further characterized three transcription factors from our analysis Estrogen related receptor gamma (Esrrg), Atonal BHLH Transcription factor 8 (Atoh8) and Musculoaponeurotic fibrosarcoma (Maf) to understand how they regulate M cell development.

In our work characterizing Esrrg, we observed it to be upregulated in M cell differentiation. Our analysis found that it was PRC2 regulated and explored further its effect on M cell development. We noticed a significant decrease in functionality and development of M cells without Esrrg activation. Atoh8 was another transcription factor revealed to be PRC2 regulated. Atoh8 was observed to be necessary for regulating the population of M cells. In contrast to Esrrg, Loss of Atoh8 led to an increase in M cell population, and increased transcytosis. Maf a PRC2 regulated gene during M cell differentiation demonstrated its role to be critical for the development of M cells in the follicle-associated epithelium.

This thesis identifies the previously unknown PRC2 regulated transcription factors essential for the differentiation and development and functionality of M cells. We further characterize the roles of Esrrg, Atoh8, and Maf in M cell differentiation and elucidate their signaling pathway network with previously identified regulators of M cell differentiation.

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ABBREVIATIONS

4OHT	4-Hydroxytamoxifen
APC	Antigen-presenting cell
BALT	Bronchus-associated lymphoid tissue
CALT	Conjunctiva-associated lymphoid tissue
CBC	Crypt base columnar cells
ChIP-seq	Chromatin immunoprecipitation sequencing
CMIS	Connected mucosal immune system
DC	Dendritic cells
DSS	Dextran sodium sulfate
EGF	Epidermal Growth Factor
EHS	Engelbreth-Holm-Swarm
ENRI	Epidermal growth factor – Noggin – R-spondin – IWP2 Wnt Inhibitor
Esrrg	Estrogen Receptor Related Gamma
FAE	Follicle associated epithelium
GALT	Gut-associated lymphoid tissue
GRO-seq	Global Run-On sequencing
H3K27me3	Histone trimethylation of K27
Gro-seq	Global Run-on nuclear sequencing
Hsp60	Heat shock protein 60
iBALT	Induced bronchus-associated lymphoid tissue
IκB kinase-β	Inhibitor of nuclear factor kappa B
IBS	Irritable bowel syndrome
ISC	Intestinal stem cells
KRAB	Krüppel-associated box
LPS	Lipopolysaccharide
LT	Lymphotoxin
LT-βR	Lymphotoxin-β receptor
LT-α	Lymphotoxin alpha
LT-β	Lymphotoxin beta
LRCs	Label retaining cells
MALT	Mucosa Associated Lymphoid Tissue
MCi	M cell inducer
MHC	Major histocompatibility complexes
NALT	Nasopharynx-associated lymphoid tissue

NF-ϰB	Nuclear factor kappa B
OPG	Osteoprotegerin
PP	Peyer's patch
PRR	Pattern recognition receptors
RANK	Receptor activator NF – Kappa
RANKL	Receptor activator NF – Kappa B Ligand
SED	Sub-epithelial dome
Th1	T- helper 1 cells
Th2	T- helper 2 cells
Th17	T- helper 1 cells
TNF	Tumor necrosis factor
TNFα	TNF alpha
TNFR	TNF receptor
TNFR1	TNF receptor 1
TNFR2	TNF receptor 2
WENRC	Wnt – Epidermal growth factor – Noggin – R-spondin – Chir99021

LIST OF ORIGINAL COMMUNICATIONS

This thesis is based on the following original communications, referred to in the text by Roman numerals (I-III).

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AUTHOR'S CONTRIBUTIONS

The author of this dissertation contributed to all three original publications as the main author. None of the original publications have been a part of another academic dissertation. The original study protocol of this long-term follow-up was designed by Docent Keijo Viiri. The coauthors collaborated in the planning and gave their expertise for proceeding with the studies

The additional contributions of the authors in the original publications were as follows:

I The author of this dissertation contributed to planning the study, designing the methodology, classifying the previously collected data, performing the statistical analyses, writing the manuscript and being responsible for the publication process.

II The author of this dissertation contributed to planning the study, designing the methodology, classifying the previously collected data, performing the statistical analyses, writing the manuscript and being responsible for the publication process.

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1 INTRODUCTION

Peyer's patches are inductive sites that initiate mucosal immune responses in the intestine and since they lack afferent lymphatics, PP's directly sample luminal antigens through the epithelial barrier via Microfold cells (M cells). M cells are primarily located among follicle-associated epithelium (FAE) on Peyer's patches that are located in the distal portion of the small intestine that comprises the jejunum and ileum. M cells allow for the transport of microbes, antigens, and foreign pathogens across the epithelial cell layer from the gut lumen to the lamina propria where they interact with lymphoid cells such as B cells, T cells, and other antigen-presenting cells. Microfold cells differ in morphology from neighboring epithelial cells by their irregular and shorter microvilli on the apical surface. They have an inverted pocket-shaped invagination on the basolateral surface. Mature M cells sample antigens through their receptor Glycoprotein 2 (Gp2), this receptor contributes to uptake of Salmonella typhimurium by recognizing the bacteria flagella protein FimH. Lack of Gp2 receptor impairs transcytosis capacity and antigen-specific T cell responses in the PP's (Dillon and Lo 2019).

M cells, while they are responsible for initiating mucosal immune responses, some pathogens like orally acquired prions have been able to exploit the transcytosis capacity of M cells to infect the host. Accumulation of prions in the FAE enables its spread through the nervous system and M cells act as gatekeepers against oral prion infection whose density and the rate of differentiation directly ameliorates or mitigates disease susceptibility; indicating that M cell differentiation and its population is tightly regulated. M cells differentiate from cycling Lgr5+ intestinal stem cells present in intestinal crypts. Lgr5+ progenitor cells differentiate into M cells after being stimulated by the cytokine nuclear receptor activator NF κB ligand (RANKL)(W. de Lau et al. 2012). The identity of stromal cells that produce RANKL secretion has been under debate for some time, but recent studies have shown M cell inducer cells (MCi) under the sub-epithelial dome (SED) are responsible for the secretion of the cytokine. RankL binds to Rank receptors on progenitor cells and enabling differentiation into M cells.

Once RANKL binds to Rank receptors, a cascade of signaling events involving NF-ϰB signaling and activation of various transcription factors take place. Traf6 is activated upon RANKL stimulation which in turn activates the classical NF-ϰB-RelA/p50 pathway. The classical pathway is essential for the expression of early M cell markers and activation of the non-canonical pathway RelB/p50. Non-canonical RelB/p50 has been demonstrated to be essential for activation of critical transcription factors like Spi-B and Sox8 which are critical for differentiation of a mature M cell with Gp2 receptor (Takashi Kanaya et al. 2018; Shunsuke Kimura, Kobayashi, et al. 2019). However, studies have shown that M cell differentiation might not have a single master regulator but probably requires the activation of multiple genes for differentiation function and development.

Polycomb repressive complexes (PRCs) play a major role in regulating gene expression during development and differentiation. PRCs are a group of protein complexes that covalently modify histone tails to modulate transcriptional silencing and chromatin compaction. PRCs are broadly divided into subclasses PRC1 and PRC2, each of these complexes reassemble chromatin by explicitly defined mechanisms that involve variable configurations of core and accessory subunits (Schuettengruber and Cavalli 2009). This configuration is demonstrated by the way PRC2 catalyzes trimethylation of histone H3 lysine 27 (H3K27me3) and presents a binding site for PRC1 in embryonic stem cells. Previous research demonstrated that PRC2 played a repressive role in the expression of developmental regulators necessary for cell differentiation. Interestingly, genes critical for cell identity lose their methylation on H3 lysine K27 whereas genes that regulate alternate cell types keep their methylation and remain repressed. To gain insight into the structure, function, and conservation of chromatin, we used ChIP-Seq to acquire genome-wide maps of H3K27me3 histone modifications in their role of enabling differentiation. Our Chip-seq and Gro-seq revealed twelve previously unknown novel PRC2 regulated transcription factors activated for M cell differentiation. Among the twelve, 3 identified transcription factors- Estrogen-related receptor gamma (Esrrg), Atonal BHLH transcription factor 8 (Atoh8), and musculoaponeurotic fibrosarcoma (Maf) were characterized further to understand their role in differentiation, development, and function of M cells.

Esrrg also known as NR3B3 is a nuclear receptor that is encoded by Esrrg gene in humans (Eudy et al. 1998). Loss of Esrrg in intestinal organoids resulted in an immature phenotype of M cells lacking Gp2 receptors. Esrrg deficit also led to a loss

of Sox8 activation, another critical transcription factor for M cell differentiation. Atoh8, a BHLH transcription factor was previously identified to play a regulatory role in myogenic and osteoblastic differentiation (Yahiro et al. 2020). Mice lacking intestinal expression of Atoh8 exhibited a higher density of mature M cell population. They also demonstrated a higher transcytosis capacity compared to their wildtype counterpart. This could mean that Atoh8 plays a pivotal role in controlling the population of M cells which is critical as increased M cell numbers in a system is a feature that orally acquired prions could target to initiate an infection. Maf, another transcription factor identified in the analysis also known as proto-oncogene c-Maf or V-maf musculoaponeurotic fibrosarcoma oncogene homolog was previously identified to be an oncogene that regulated various cellular differentiation and development processes particularly lens development, renal development, and γδ T cell (Xie et al. 2016). We observed that complete knockout of Maf in mice was lethal and the pups did not survive for more than 4 hours. however, we were able to observe that invitro intestinal organoids from Maf KO mice grown in RANKL lacked mature M cells with Gp2 receptors. This indicates that Maf is critical for the development of mature M cells with transcytosis capacity.

This thesis aims to understand how PRC2 regulates M cell differentiation, development, and function. We also aim at understanding how identified PRC2 regulated genes, Esrrg, Atoh8, and Maf fit in the signaling pathway leading to differentiation and how it interplays with previously identified players in the M cell differentiation network.

2 REVIEW OF THE LITERATURE

2.1 Gastrointestinal Structure and Function

The gastrointestinal tract is a group of tissues that play a critical role in countering pathogens, aiding in digestion, absorbing nutrients from food and coordinating the delicate balance between microbes in the gut lumen and the immune system. The intestinal tract broadly comprises of two segments, the small intestine and the colon; each of these segments consist of 4 layers of tissue, known as tunics with their own distinct function- the mucosa, submucosa, muscular layer and the serosa (Delaunoit et al. 2005). The small intestine is divided into 3 regions based on their anatomy and physiology: duodenum, jejunum and ileum. Nutrient digestion and absorption are localized in the duodenum and jejunum while Vitamin B12 and bile salt reabsorption is processed in the ileum. The colon is located in the distal part of the gastrointestinal tract and exhibits a flat, non-villous surface due to functions characteristic of this section (Mowat and Agace 2014). The colon is responsible for water resorption, maintaining and housing the commensal bacteria population, immune response against pathogens and waste elimination. Epithelial cells, size of the organ and anatomical structure vary depending on the region-specific physiology and function of the small intestine and colon. Due to the absorptive nature of this region, the primary tissue in this region is organized in to brush like projections known as villi. At regular interspaced regions of the villi, the epithelium forms invaginations to form crypts of Lieberkühn (Delaunoit et al. 2005).

The crypts of Lieberkühn house three different epithelial cells- Lgr5+ intestinal stem cells, progenitor transit amplifying (TA) cells and Paneth cells. The crypts of Lieberkühn are found in both the small intestine and the colon and are situated over an underlying connective tissue known as lamina propria. The crypts of Lieberkühn lead to tiny finger like projections called intestinal villi. Intestinal villi line the entire length of the small intestine and are comprised of enterocytes, Goblet cells enteroendocrine cells and chemosensory cells. Enterocytes arise from transit amplifying progenitor cells and comprises of several major lineages that aid in

absorptive and secretory function. Goblet cells, enteroendocrine cells and Paneth cells are some of the secretory cells (Cheng and Leblond 1974). Other specialized cells in the gastrointestinal tract with specialized function are chemosensory tuft cells and microfold cells (M cells) in the Peyer's patches (Clevers 2013).

The intestinal epithelium comprises of one of the most rapidly regenerating tissues with a high turnover rate in the body. To maintain homeostasis in the gut- specialized epithelial cells with short lifespans are regularly turned over and replaced through differentiation of Lgr5+ stem cells that reside in the invaginating crypts (Cheng and Leblond 1974; Clevers 2013; Sato. T et al. 2009). Lgr5+ stem cells give rise to transit-amplifying progenitor cells that differentiate to goblet cells, Paneth cells, enteroendocrine cells, tuft cells, enterocytes, and M cells. Several differentiated lineages move up towards the luminal plane through their maturation and eventually undergo apoptosis, shedding into the lumen within 4 days. Paneth cells are the exception as they migrate down to the crypt position turning over every 6-8 weeks (Barker.N et al. 2012)

Depending on the identity of the epithelial cell, the regulatory mechanisms that contribute to maintaining the multi-layer regulatory control in intestinal epithelial cells vary.

2.1.1 Cell cycling mechanisms in the intestinal epithelium

Historically 2 parallel theories have competed to describe intestinal stem cell identity. Leblond, Chend and Bjerkens put forth the 'stem cell zone' model which suggests that the columnar cells that reside at the crypt base are the resident stem cells that are responsible for maintaining the intestinal epithelial ecosystem (Cheng and Leblond 1974). Potten et al proposed the '+4 model', which proposed that stem resided within a ring of 16 cells that were localized above the Paneth cells (Potten. 1977). Recent research has confirmed the presence of both these groups of stem cells and assigned different conditions for when these two groups are active. For regular maintenance of the epithelial cell ecosystem, the stem cell zone propagates and differentiates whereas in stress conditions like injury or radiation the +4 model takes over (HUA et al. 2012).

The stem cell model identified mitotically active undifferentiated crypt base columnar cells (CBC) cells intercalated among Paneth cells. Critical experiments

involving lineage tracing studies demonstrated actively cycling CBC's in in the small intestine and the colon and are specifically marked by Wnt target gene Lgr5. CBC cells are adult stem cells that require a specialized stem cell niche at the crypt base also named as stem cell permissive zone. Daughter cells that originate exit the niche to commit to differentiation at the +5 position the 'common origin of differentiation'. These Lgr5+ stem cells demonstrate all the classic characteristics of stem cells functions such as self-renewal and multi-lineage differentiation. These cells in the stem cell model are known as intestinal stem cells (ISCs). In contrast, Paneth cell progenitors migrate into a downward position to mature into a functional lysozyme-secreting cells (Barker et al. 2007; T Sato et al. 2009).

Cell tracking experiments demonstrated the existence of intestinal stem cells at the +4 position (4th position from the crypt) immediately above the Paneth cell compartment. These cells shared similar attributes as that of CBC cells; they are capable of dividing and could retain labels in their DNA when new ones originated. Though these label retaining cell (LRCs) features are characteristic of quiescent cells, they also identified secretory precursor cells dominant in the +3 position. The LRCs are able to differentiate into Lgr5+ cells, demonstrate multi lineage stem cell potential and provide the required impetus to differentiate especially after injury (AS et al. 2011). The +4 cells act like a reserve population of stem cells or quiescent group of cells to restore the intestinal stem cell zone after injury. To preserve intestinal integrity after injury it is possible that the proposed +4 cells are radiation resistant populations and proliferate right after injury. More evidence supporting the +4 model came from lineage tracing experiments that utilized a newly generated Bmi-Cre-ER knock-in allele. After induction for 24 hours, the cells that expressed Cre-reporter were shown to be located at the +4 position, directly above the Paneth cells. Through lineage tracing several expression markers of +4 population such as Hopx, mTert, Bmi1 and Lrig have been identified (Ö. H. Yilmaz et al. 2012; Cheng and Leblond 1974; Gerbe, Legraverend, and Jay 2012; Watson and Hughes 2012). However, these markers have also been identified in Lgr5+ intestinal stem cells and transit amplifying progenitors giving rise to many unanswered questions about the +4 population (Barker 2013).

2.1.2 Niche signaling that regulates intestinal homeostasis

Several niche regulatory signaling mechanism maintain the balance between stem cell propagation, self-renewal and differentiation. In addition to maintaining intestinal

stem cell identity and the identity of epithelial cells, juxtacrine niche factors also adapt their signaling to injury and stress condition to interpret stimuli from pathogenic luminal antigens and translate them into regeneration of the epithelium (Beumer and Clevers 2016). Existence of stem cell niche microenvironment is paramount to maintaining stem cell self-renewal and proliferation, the intestinal stem cell niche provides a range of signals necessary for the nourishment of stem cells that support tissue homeostasis which enables a sufficient epithelial cell turnover to form an effective tight barrier versus detrimental neoplastic overgrowth. The diverse paracrine signaling factors that regulate intestinal stem cell niche are Wnt, R-spondin, Notch, BMP and Hedgehog (Sailaja, He, and Li 2016) (See Figure. 1).

Wnt signaling - One of the major drivers of intestinal stem cell proliferation is the Wnt/β-catenin signaling. Wnt ligands are encoded by 19 related genes that are obligately palmitoylated by the enzyme Porcupine (Porcn) from the endoplasmic reticulum; this aids in secretion and binding of WNT to Frizzled receptors. Various knockout and overexpression and pharmacological experiments point to a canonical Wnt signaling's critical role as knockout of *Tcf4* an important gene critical for Wnt signaling and proliferation exhibited crypt/villus/Lgr5+ ISC loss. Further experiments involving deletions of mediators of Wnt biosynthesis and secretions like Wntless (WIs) or Porcn and overexpression of Wnt inhibitor Dickkopf-1 (DKK1) (Kabiri et al. 2014; San Roman et al. 2014; K. S. Yan et al. 2012; Pinto et al. 2003) or small molecule PORCN inhibitors depleted Lgr5+ ISC via their premature lineage commitment.

The source of Wnt ligands have been identified to numerous stromal cells as well as epithelial cells. Paneth cells are the main source of Wnt3a and demonstrate its activity within a short range in the intestinal crypt in vivo (Farin et al. 2016). Deletion of Paneth cells was observed to not alter maintenance, proliferation or intestinal homeostasis of intestinal stem cells this pointing to the critical role of intestinal stromal cells (Durand et al. 2012; T. H. Kim, Escudero, and Shivdasani 2012). Wnt2b, Wnt4 and Wnt5a are localized and secreted by intestinal stroma such as Foxl1+ mesenchymal stromal cells, this subpopulation is also known as telocytes (Shoshkes-Carmel et al. 2018). Ablation of Foxl1+ cells demonstrated a lack in expression of Wnt2b, Wnt4 and Wnt5a in the crypt/villus axis and a severe impaired development of the intestinal epithelium (Aoki et al. 2016). However, the loss of crypts and short villi did not affect Paneth cell in mice without Foxl1-expressing cells. Wnt2B expression is also sourced from 2 different mesenchymal stromal cells,

αSMA+ and Gli1+ stromal cells. Injection of αSMA+ and Gli1+ stromal cells in mice lacking global Wnt secretion restored the intestinal epithelial homeostasis (Valenta et al. 2016).

R-spondins - In addition to Wnt ligands, R-spondins are another critical family that regulates intestinal stem cell homeostasis. R-spondins do not possess intrinsic Wnt signaling activity but accentuate the downstream activity of Wnt ligand-receptor binding to activate β-catenin-dependent transcription and canonical Wnt signaling (W. B. M. de Lau et al. 2012). R-spondins comprise of R-spondin1 (RSPO1), R-spondin2 (RSPO2), R-spondin3 (RSPO3) and R-spondin4 (RSPO4); theses ligands are secreted glycoproteins with Furin domains and can be broadly classified into 2 receptor classes - the leucine-rich repeat seven-pass transmembrane proteins that comprise of Lgr4/5/6, and the transmembrane E3 ligases that comprise of RNF43 and ZNRF3 (Carmon et al. 2011; Glinka et al. 2011; Schuijers et al. 2015). Genetic ablation of Rnf43 and Znrf3 led to crypt hyper-proliferation and intestinal overgrowth and overexpression of in vivo R-spondin signaling led to Lgr5+ expressing cells (Koo et al. 2012; K. S. Yan et al. 2017; 2012; Ootani et al. 2009; K. A. Kim et al. 2005). Deletion of Lgr4 and Lgr5 in mouse led to the complete loss in crypt-villus integrity (Wim De Lau et al. 2011). Blockage of Rspo2 and Rspo3 with anti-Rspo2 and anti-Rspo3 neutralizing monoclonal antibodies led to a loss of Lgr5+ expressing stem cells and poor recovery post radiation (Storm et al. 2016).

The sources of R-spondins within the niche remain to be elucidated, however Foxl1+ and other mesenchymal cells do express R-spondins along with Wnts (Stzepourginski et al. 2017; E. Kang et al. 2016). This evidence was further corroborated by research which showed that Pdgfrα+ myofibroblasts were sufficient to support the growth of enteroids without exogenous R-spondin in the medium (Greicius et al. 2018).

Notch- Notch signaling uses a unique lateral inhibition feature to maintain the undifferentiated status of intestinal stem cells. Notch ligands (Jag1-2, Dll1-4) bind to Notch receptors (Notch1-4) to activate Notch signaling and downstream transcriptional activity through cell-cell contact (Kopan and Ilagan 2009). Notch receptor upon binding to Notch ligands undergo conformational changes that lead to a series of proteolytic cleavages to give rise to Notch intracellular domain (ICD)s which then translocates to the nucleus and forms a complex with RBP-Jκ (CSL in humans) to activate transcription of target genes (Kovall et al. 2017). In contrast to

Wnt signaling which originates from mesenchymal/stromal cells, Notch signaling requires cell-contact; Lgr5+ adjacent epithelial cells and stromal cells in contact with the intestinal stem cells plays a critical role in Notch signaling (Carulli et al. 2015). These stromal cells and Lgr5+ adjacent epithelial cells express Notch receptors and ligands, Notch1 and Notch2 receptors are localized in the intestinal crypts including Lgr5+ cells and Notch ligands Dll1 and Dll4 are observed to be expressed in intestinal secretory lineages expressing Atoh1+, such as Paneth cells or c-Kit+/ Reg4+ expressing goblet cells (Rothenberg 2012; Sasaki et al. 2016).

Disruption of Notch signaling leads to Lgr5+ intestinal stem cell loss and lack of transit amplifying cells as they convert to secretory cells. Deletion of Notch1 or Notch2 did not lead to any significant changes in the stem cell niche however, combined deletions of Notch1 and Notch2 led to loss of Lgr5+ and secretory hyperplasia (Riccio et al. 2008; Wu et al. 2010). Transcriptional profiling of Atoh1 expressing cells revealed Notch ligands Dll1 and Dll4 as direct Atoh1 targets indicating a positive feedback signaling in populations within the niche to bolster Notch-mediated lateral inhibition (Y. H. Lo et al. 2017). To corroborate this, research involving deletion of *Dll1* and *Dll4* in intestinal epithelium initiated intestinal stem cell differentiation to secretory lineages suggesting that they act as primary Notch ligands (Pellegrinet et al. 2011).

Hedgehog (Hh) - Hedgehog signaling comprise of 2 ligands; Sonic Hedgehog (Shh) expressed in crypts and Indian Hedgehog (Ihh) expressed in villi. Hedgehog ligands maintain intestinal stem cell niche by binding with Patched (Ptc1) receptor which in turn leads to the de-repression of Smoothened and its nuclear translocation to bind to Gli transcription factors (Mao et al. 2010; Kolterud et al. 2009; Huang et al. 2013). Deletion of Sonic hedgehog signaling leads to obstruction of the duodenal tract and impaired intestinal innervation while deletion of Indian Hedgehog leads to reduction in crypt proliferation and differentiation (Ramalho-Santos et al. 2000). Autocrine Hedgehog signaling is found to be expressed in Paneth cells and Intestinal stem cells (Varnat et al. 2009; 2006; Regan et al. 2017)

Bone Morphogenetic Protein (BMP) – BMP signaling are opposing signaling pathways to Wnt/β-catenin signaling. They play an important role in regulating the Wnt/β-catenin signaling in the crypt-villi axis with increase in BMP signaling in higher gradient towards the villi. Smad expression in Lgr5+ cells mediate the repression of genes involved in BMP signaling thus inhibiting differentiation in the

crypt stem cell zone (Haramis et al. 2004; Qi et al. 2017; He et al. 2004). To counter the inhibitory role of BMP signaling various BMP antagonists like Noggin, Gremlin-1 and Gremlin-2 are enriched in the stem cell niche. Facilitation of BMP agonists is carried out by sub-epithelial myofibroblasts and smooth muscle cells situated adjacent to crypt cells

Hippo – Hippo signaling is a conserved mechanism that was first discovered in Drosophila. Mechanosensory stress activates the core Hippo pathways that activate a cascade which leads to the phosphorylation of transcriptional co-activators YAP and TAZ. This cascade leads to the phosphorylated complex leaving the nucleus which inhibits transcriptional activity of intestinal stem cell marker Olfm4 expression (Mo, Park, and Guan 2014). Overexpression of the active form of YAP1 led to the inhibition of intestinal proliferation and deletion of YAP1 did not lead to changes in homeostasis but recovery after radiation lead to massive intestinal overgrowth and Lgr5+ ISC expansion (Barry et al. 2013). Early intestinal regeneration is thought to be dependent on YAP1 signaling while late hyperproliferation is independent of YAP and TAZ (Gregorieff et al. 2015).

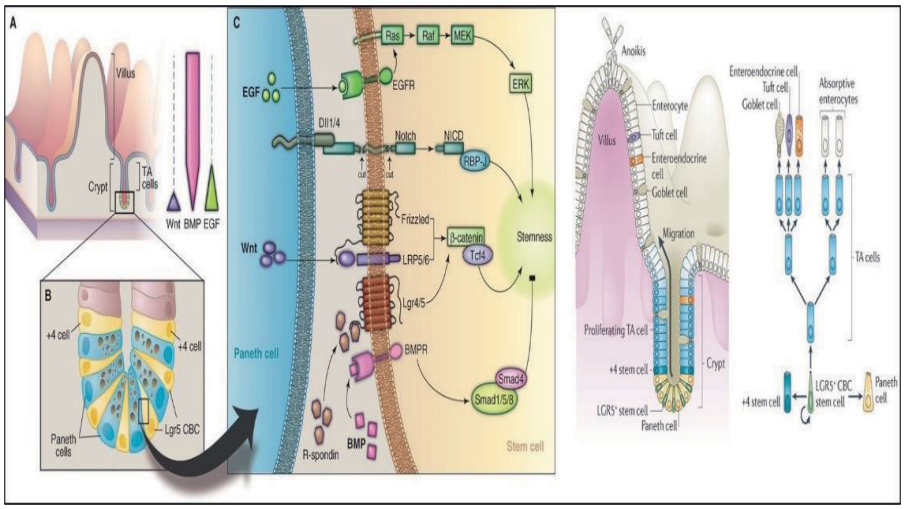

Figure 1. Niche factors that regulate homeostasis in the intestinal epithelium. Opposing gradients of stem cell promoting stemness and differentiation inhibiting signals maintain homeostasis in the intestinal epithelium A) Overlay scheme the intestinal crypts and villous epithelium with spatial gradients of Wnt, BMP, and EGF. B) Scheme depicting the stem cell niche. Paneth cells adhere and support intestinal CBC cells to maintain the stem cell niche. Radioresistant cells +4 cells serve as stem cells reservoir cells as they turn into cycling, Lgr5+ stem cell CBC cells upon tissue damage C) EGF, Notch and Wnt are critical for to regulate epithelial stemness whereas BMP serves to negatively regulate stemness in order to promote

differentiation. D) Schematic depiction of intestinal differentiation from the crypt. Stem cells continuously generate transit amplifying TA cells that differentiate into various functional cells are they translocate up the villi. (Modified from Sato and Clevers, Science, 2013 and Nick Barker.2013 Nat Rev Mol Cell Biol.)

2.1.3 Intestinal organoid culture

Having elucidated various signaling mechanisms that regulate the intestinal stem cells niche, the possibility of isolating tissues and growing cells in a reliable and accurate ex vivo model has raised interest among the cell development community. An organoid is defined as a miniature organ that can be grown in vitro. They are usually generated from induced pluripotent cells that are cultured in a scaffolding material that mimics the stroma. In the context of intestinal organoids, the source of the harvested tissue plays a role in defining the structure and characteristic of the resulting organoids; for instance, intestinal biopsies grown in vitro will recapitulate itself into a small intestinal structure resembling its function *in vivo* and is termed as an enteroid (Stelzner et al. 2012). The structure assumes a 3-dimensional model of epithelial cells that mimic the structural organization presented in in vivo when supplied with appropriate exogenous growth factors and basement membrane scaffolding.

Intestinal crypts isolated from mice are able to recapitulate into enteroids that contained *lgr5*+ ISCs (Sato. T et al. 2009). Harvested crypts exhibit self-renewal and differentiation properties induced by the addition of exogenous Epidermal Growth Factor (EGF), R-spondin and Noggin to standard growth media. The enteroids assume a 3-dimensional assemblage that resembles the macroscopic structure of the intestine with polarized epithelial cells organizing itself to distinct crypt and villous domains. The growth media optimal for the culture of intestinal organoids have been well studied and characterized. One of the first extracellular matrix used to grow enteroids was produced by Engelbreth-Holm-Swarm (EHS) tumor cell line. This matrix mimicked the native stoma found In vivo; Present day matrixes are combinations of EHS produced basement membrane-like matrix with laminin proteins, collagen IV, heparin sulfate proteoglycans and a number of growth factors. The basement membrane-like matrix provides support for the ISC's to attach to and provides cues for epithelial cell survival via integrin signaling which suppresses anoikis. Exogenous R-spondin is added as it mimics signaling from subepithelial fibroblasts. R-spondin binds to lgr5 receptor inhibiting the degradation of Wnt

receptors and accentuating Wnt activation (Hao 2012; Koo et al. 2012). *In vivo* studies in mouse have demonstrated Noggin to be a secreted glycoprotein and BMP antagonist, Noggin is added exogenously to aid in maintenance of intestinal enteroid; crypt stem cells lose *lgr5* expression and cease proliferation after 2 weeks without Noggin. Along with R-spondin and noggin, Epidermal Growth factor is also added to the cocktail as it is a strong mitogen that promotes the proliferation of Lgr5+ stem cell and aids in long term culture and epithelial cell survival. This intestinal organoid model is composed of all major intestinal epithelial cells including *lgr5*+ crypt-based columnar stem cells, +4 quiescent stem cells, transit-amplifying cells (TA), absorptive enterocytes, goblet cells, Paneth cells and enteroendocrine cells (Wallach and Bayrer 2017). Rare cells like M cells can also be grown in organoid cultures; intestinal crypts isolated from the duodenum are cultured in vitro for a week till they develop into intestinal organoids. After which they are grown in 100 μg of RANKL for 4 days in which Lgr5+ cells in the organoids fully differentiate to M cells (W de Lau 2012)

Human intestinal and colon organoids growth conditions are a bit more complex that intestinal organoids from mouse as they require a more multi targeted set of growth factors to support propagation and self-renewal. In addition to EGF, Noggin and R-spondin, human cultures are supplemented with nicotinamide, Gastrin, a p38 inhibitor, a TGF-βinhibitor, and Wnt3 (Toshiro Sato et al. 2011). These organoids help in modeling various gastrointestinal diseases and also aid in characterizing signaling networks required for each of the 6 differentiated cell type in the gut (See Figure. 2.).

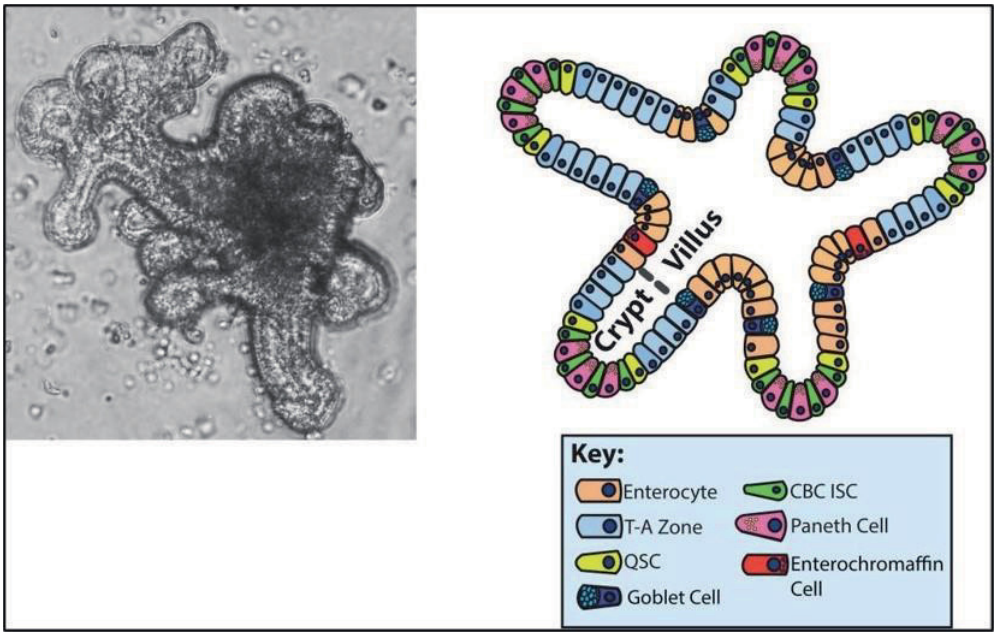

Figure 2. Small intestinal organoid culture- Biopsies or crypt isolated from the gastrointestinal tract can take on a 3-dimensional structure when grown in a growth factor rich basement membrane matrix with the exogenous addition of Wnt, R-spondin, EGF and Noggin. (Adapted from Wallach. T et al 2017 J Pediatr Gastroenterol Nutr. 2017 Feb; 64(2): 180–185).

2.1.4 Polycomb group complex's epigenetic regulation of crypt stem cells and villus differentiated cells

Regulation of intestinal homeostasis, differentiation, development and functionality are overseen by many factors as mentioned above but recent research has strongly indicated at the major role of Polycomb group proteins (PcG). They have been observed to be critical for embryonic stem cell renewal and pluripotency; several cell fates and identities require the overview of Polycomb group proteins throughout life (Schuettengruber and Cavalli 2009). The Polycomb group protein comprises of 3 groups of Polycomb-repressive complexes (PRCs)- Polycomb repressive complex 1 (PRC1), Polycomb repressive complex 2 (PRC2) and polycomb repressive DeUBiquitinase; each of these complexes reorganize chromatin by their own characteristically defined mechanisms with specific configurations of core and accessory subunits (Cao et al. 2002). For instance, PRC2 catalyzes the trimethylation of H3 lysine 27 (H3K27me3) to form a binding site for PRC1. In embryonic cells

the PRC2 represses the expression of developmental transcription factors required for cell differentiation and in differentiated cells, regulators required for the given cell identity lose H3K27me3 while genes that are required for alternate cell types remain repressed and methylated (T. I. Lee et al. 2006; Bernstein et al. 2006). Transcription repression is a crucial mechanism for establishing cell identity and maintaining it for homeostasis (Chiacchiera F et al. 2013; Chen. T and Dent SY. 2014).

PRC2 dependent methyl transferase activity is overseen by one of the 2 paralogs EZH2 and EZH1, both these catalytic subunits require 2 other structural subunits, EED and SUZ12 to be functionally active. EZH2 and EZH1 are critical for intrinsic enzymatic activity and functional loss of these subunits exhibited the complete loss of H3K27me3 expression and disruption of the PRC2 complex. While the loss of EZH2 function only produced a mild phenotype in some cells, the loss of Eed or inactivation of EZH1 and EZH2 simultaneously affected the integrity of homeostasis. (IH et al. 2003; Chen et al. 2009; Ezhkova. E et al. 2011; Juan et al. 2011; Xie et al. 2014).

In the intestine, global PRC1 activity was observed to preserve intestinal stem cell identity by specific repression of transcriptional factors of non-lineage-specific-some of which were found to interfere with Wnt signaling which thereby lead to the loss of Lgr5+ intestinal stem cells (Chiacchiera et al. 2016).

PRC2 activity was observed to be dispensable for preserving the stemness in the intestinal stem cell zone in regular physiological conditions. However, PRC2 plays a dual role in restricting differentiation of the secretory lineage and maintaining proliferation in the transit-amplifying progenitor cells. Passini et demonstrated that while the loss of PRC2 was dispensable for homeostatic intestinal regeneration, the lack of PRC2 activity led to a loss in epithelial regeneration in a radiation induced condition. This indicates that PRC2 plays a critical role in maintaining precursor cell plasticity in the crypt base. The conditional deletion of catalytic subunit Eed showed that PRC2 activity is required to preserve the proliferation of the transit amplifying zone while also controlling the fine balance between absorptive and secretory lineage differentiation (Chiacchiera et al. 2016). Further evidence of Chip-seq with intestinal organoids grown in stem cell conditions and enterocyte differentiated condition demonstrated that the function of PRC2 at transit-amplifying zone at the crypt-villus axis is to selectively put an epigenomic identity by labelling genes with repressive

H3K27me3 mark and, thereby enforce and maintain the dichotomy for crypt and villus identities governed by Wnt-signaling. 90 genes were identified to be regulated by the PRC2 along the crypt-villus axis and many of them have defined role in intestinal homeostasis and maintenance of the cells in the crypt base (Oittinen et al. 2017).

2.2 Mucosal Immunity

The immune system can be viewed as an organ, not limited by location as it is distributed throughout the body. The immune system finds itself localized throughout the body defending against foreign antigens and pathogens wherever they may be. Several anatomically unique compartments can be distinguished within the immune system, these compartments play a distinct role to mount a defensive response to pathogens present in a particular set of body tissues. For instance, the compartment made of peripheral lymph nodes and spleen constitutes the adaptive immune response to antigens that have entered tissues and the bloodstream. The mucosal immune system commonly described as MALT (Mucosal-associated lymphoid tissue) is commonly located at surfaces where most pathogens invade. These mucosal surfaces of the body are particularly sensitive to infection. These thin and permeable barriers to the interior of the body serve a purpose to physiological activities such as food absorption (the gut), gas exchange (the lungs), sensory activities (eyes, mouth, throat, and nose), and reproduction (uterus and vagina)(Bienenstock and McDermott 2005). This necessity for such important physiological functions creates an obvious potential portal for a diverse range of pathogens to invade the human body.

The different compartments of mucosal immunity can be defined by their location and structure. MALT provides a unique defensive mechanism as its defense response is based on its location and the site of infection. In the gut, the MALT is made of structures such as Peyer's patches (PPs) in the gut-associated lymphoid tissue (GALT) The function of these particular lymphoid tissues is the quick uptake and initiation of a suitable immune response against antigens (Bienenstock and McDermott 2005), with the help of macrophages, effector lymphocytes, and dendritic cells (DC's) localized underneath the epithelial surfaces.

The different mucosal compartments of the body are well connected through a complex network of effector cells that communicate mucosal responses. Following the circulation, activated lymphocytes are conditioned to return to their compartments and function on the mucosal surfaces. For example, a lymphocyte primed and activated in the PP is capable of binding to the MAdCAM-1 (a mucosal homing molecule) present in the airway vasculature and entering the mucosal tissue of the lungs. This binding results in directing lymphocyte traffic into PP's and the intestinal lamina propria (Berlin et al. 1993; Mora et al. 2003). The mucosal immune system at different anatomical locations works as a single unit so that it can provide protective immunity at multiple sites when required (Holmgren and Czerkinsky 2005).

The human gut is home to a community of diverse microbiota comprising approximately 40 trillion microorganisms whose roles are to prevent colonization by pathogens, metabolism of non-digestible nutrients, detoxification of bile acids, and the generation and breakdown of key metabolites critical for human health (Sender, Fuchs, and Milo 2016; Pickard et al. 2017; Staley et al. 2017; Gill et al. 2006; Bäckhed et al. 2005). This presents a unique challenge to mucosal surfaces in the human gut (GALT) as they try to differentiate between commensal bacteria and harmful pathogenic antigens. Pathogens contain numerous virulent elements in their physiology that can alert and activate a mucosal immune response, whereas a soluble non-replicating antigen would not induce the same strong immune response but instead activate a state of antigen-specific hypo-responsiveness(Akbari, DeKruyff, and Umetsu 2001). The induction of tolerance to non-pathogenic antigens on the mucosal surface involves distinct mechanisms such as anergy, suppression by T regulatory cells (Treg), and clonal deletion. Treg cells function by secreting anti-inflammatory cytokines such as TGF- β, and IL-10 (Hawrylowicz and O'Garra 2005). An immunoregulatory subtype B10 cells have been found to dull inflammation through the production of IL-10 and play a big part in mucosal response (Dilillo, Matsushita, and Tedder 2010).

In conclusion, the defense mechanisms of the body have evolved to protect the body from pathogens and can generate a diverse variety of cells and molecules to specifically recognize and terminate a vast and varied range of foreign invaders. The mucosal immune system has 2 parts; an innate system that comprises of varying recognition molecules and natural killer cells and an adaptive system that comprises

of various antigen-presenting cells and the T and B lymphocytes (Singh and Lillard 2008).

When the immune system requires immediate removal of an infectious pathogen, the innate immune response takes over and its specific responses lead to the shaping of the adaptive immune response. The mucosal innate immune system is responsible to maintain a balance between defending mucosa from infection and deterring an inflammatory response which can harm the structural integrity of the mucosal surfaces. Pattern recognition receptors (PRRs), antigen-presenting cells (APCs), and epithelial cells are some of the components that form the essential mucosal innate responses (Martin and Frevert 2005).

When the innate immune system is incapable of handling an invading pathogen by itself, it is critical for a more potent mechanism such as adaptive immunity to step in, attack, and defend against invading pathogens. Adaptive immunity, on the other hand, is more antigen-specific and distinctly tailored to target the attacking microbe. The main salient feature of adaptive immunity is the production of high titer volume of antigen-specific IgA antibodies and the targeted localization of effector cells, these cells are often found in high populations in mucosal lymphoid tissues (MacPherson et al. 2008; Cerutti 2008).

Isolated lymphoid follicles are organized lymphoid structures found in the small intestine; they are diffused through the gut tube. In the gut, the inductive tissues, gut-associated lymphoid tissues (GALT), include the Peyer's patches, cecal patches, colonic patches, isolated lymphoid follicles (ILFs), and cryptopatches, which, along with the gut-draining mesenteric lymph nodes (MLNs), organize themselves as inductive sites for adaptive immune responses to commensal bacteria and other antigens in the gut. Apart from inductive sites, effector sites play an import role in maintaining immunity in the gut. The cellular basis of immune response in the gastrointestinal tract is formed by the migration of immune cells from the mucosal inductive sites to effector tissues via the lymphatic system. Mucosal effector sites which include the lamina propria regions of the gastrointestinal tract, upper respiratory tract, female reproductive tracts and secretory glandular tissues containAg-specific mucosal effector cells such as IgA-producing plasma cells, and memory B and T cells

In the gastrointestinal tract adaptive immunity is localized in lymphoid tissue known as Peyer's patches. Peyer's patches are dome-shaped localized areas in the intestine

that house lymphoid cells. They originate on the 15th day of prenatal development in murine embryos (Satoko Adachi et al. 1997; Hashi et al. 2001). Signaling in the PP are under the overview of TNFα, lymphotoxin (LT), and their cognate receptors (LT-βR and TNFRp55/TNFRp75) to combinedly generate a "controlled inflammatory program" that drives the differentiation of PP as well as the secondary lymphoid organs associated with it (Fütterer et al. 1998; De Togni et al. 2014). Various chemokines such as CCL19, CCL21, and CXCL13 which are induced by NF-ϰB, facilitate the function of the lymphoid follicle (Honda et al. 2001; Cyster 1999). The epithelial cells covering the Peyer's patches are known as follicle-associated epithelium. The follicle associated epithelium comprises of a specialized epithelial cell line known as Microfold cell or M cell. Due to M cell's unique function of immune surveillance and antigen transcytosis, they make an excellent site for initiation of mucosal immunity

2.3 M cells and their specialized role in the intestine

As the immune initiation site, the GALT's responsibility is to sample luminal antigens to commence an immune response against them (MacPherson et al. 2008). The Peyer's patch has a similar structure and function when compared to lymph nodes of the system immune system, however, Peyer's patches lack an afferent lymphatic system through which antigens and lymphoid cells are processed and transported. For instance, dendritic cells captured antigens at various sites of infection are transported into the lymph nodes for further processing whereas, in the GALT antigens are transcytosed directly from the intestinal epithelium through the follicle-associated epithelium overlaying the GALT (Rios et al. 2016; Owen 1999; Kraehenbuhl and Neutra 2000; Brandtzaeg et al. 2008). Immunity in the villous epithelium is carried out by specific cells like absorptive enterocytes; 20% of cells in the colon are comprised goblet cells that produce mucus which impair attachment of antigens on the colon tract (Shunsuke Kimura, Kobayashi, et al. 2019; Kato and Owen 2005; Vijay-Kumar and Gewirtz 2005). Another significant immune cell type in the gut residing at the base of the crypt next to Lgr5+ cells is the Paneth cell. Paneth cells secrete anti-microbial peptides to stave off bacterial contamination. Furthermore, IgA secreted from mucosa to intestinal lumen plays a important role in protecting the gut ecosystem. For transport, IgA binds to polymeric

immunoglobulin receptors expressed on basolateral surfaces of enterocytes followed by transcytosis to lumen (Kraehenbuhl and Neutra 2000; Kato and Owen 2005; Vijay-Kumar and Gewirtz 2005; Brandtzaeg et al. 2008). The FAE is vastly different from the villous epithelium due to the lack of Paneth cells, lower number of enterocytes, and significantly lower expression of IgA-transporting polymeric immunoglobulin receptors. Due to the lack of specialized immune cells in the FAE, evolutionary mechanisms have enabled close proximity of luminal microorganisms with FAE and thereby increase the rate of transcytosis of antigens. This characteristic trait is made possible by the unique feature that FAE possesses which is the presence of M cells (See Figure. 3.).

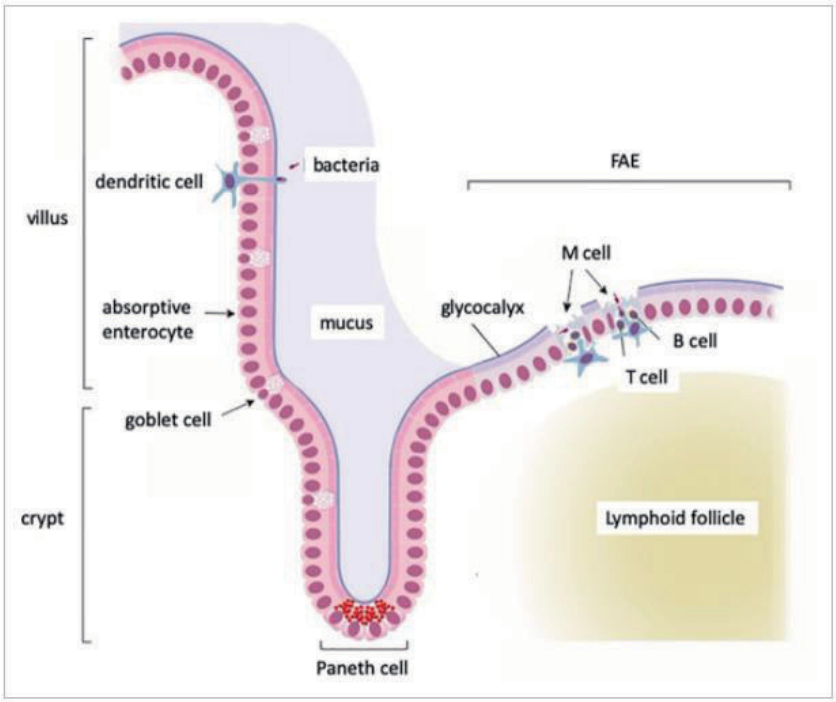

Figure 3. Graphical abstract of the small intestinal epithelium with villous epithelium on the left and follicle associated epithelium on the right with the intestinal crypt between them. Absorptive cells are found on the villous epithelium. The villous epithelium overlays the lamina propria which comprises of lymphocytes and, in murine intestine, specifically dendritic cells sampling antigens from the lumen. The crypt epithelium mainly consists of Paneth cells and Lgr5+ stem cells and they travel upward to the villous epithelium and the follicle-associated epithelium as they differentiate. M cells are localized on the follicle-associated epithelium which overlay the sub epithelial dome (SED). The sub-epithelial dome houses dendritic

cells, B cells and T cells. Distinct feature between the follicle associated epithelium and the rest of the intestinal epithelium is the presence of a thin glycocalyx layer that lines over it allowing for antigens to attach whereas the rest of the epithelium exhibits a mucous layer secreted by goblet cells. (Adapted from Takashi Kanaya, Williams, and Ohno 2019)

M cells (Microfold or membranous cells) are specialized intestinal epithelial cells subtype that is responsible for the immune-surveillance of luminal bacterial and viral particles (Kato and Owen 2005; Vijay-Kumar and Gewirtz 2005). 99 years ago, in 1922, Kenzaburo Kumagi, a Japanese microbiologist found out that the mycobacterium in the gut could only be taken up by the FAE. He observed that local epithelial cells mediated the antigen transport of carmine pigments and lyophilized red blood cells through them. However, due to limitations in technology back then, Kenzaburo thought all the cells in the FAE took up antigens. Robert Owen and D.E Bockman independently discovered and characterized M cells based on their morphology and antigen uptake. They noticed that M cells lack microvilli on their luminal side, exhibited invaginated fold-like structures or micro folds that developed into a large sac-like structure. They found that M cells with basolateral pocket, formed by the invagination of surfaces housed B cells, dendritic cells, and sometimes T cells inside accommodated the pocket (Bockman and Cooper 1973; Owen and Jones 1974). They also observed that M cells were significantly rare in number and only accounted for 5-10% FAE cells in humans and mice. Soon M cells were discovered in many other species including mice, rats, rabbits, guinea pigs, bovines, and chickens, although humans demonstrated a more pronounced invagination in M cell morphology (RL and DK 1983; AJ and DF 1984; Gebert, Rothkötter, and Pabst 1994; M. E et al. 1988; Befus et al. 1980).

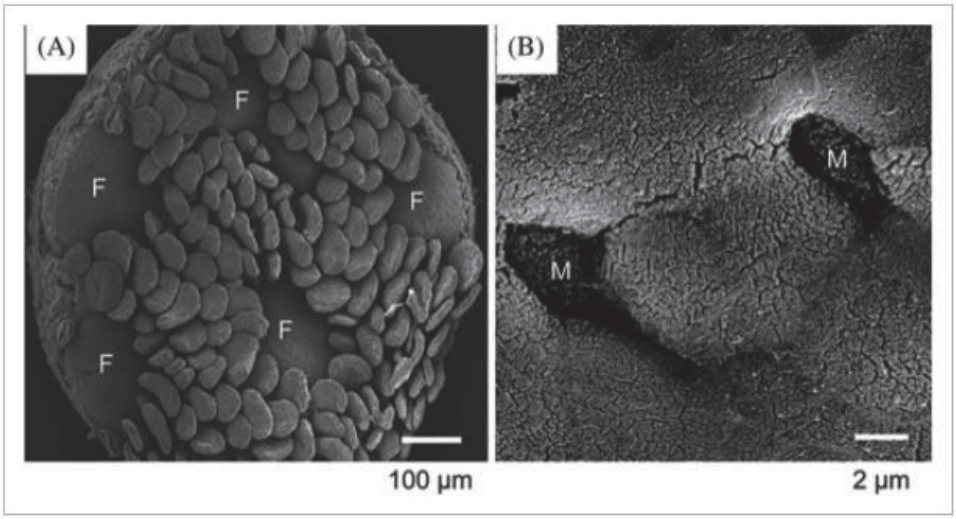

Figure 4. Scanning electron microscopy pictures of small intestinal mucosa showing lymphoid follicles, follicle-associated epithelium and M cells. A) 5 lymphoid follicles (F) can be observed among villi. B) M cell surface is observed to be invaginated with a concave like surface and it is surrounded by absorptive enterocytes. M cell's distinct morphology of irregular brush border with low number of microvilli can be observed. (adapted from Hiroshi Ohno et al)

The composition of FAE is related to its sampling of luminal antigens and induction of antigen-specific immune response function in the gastrointestinal tract. M cells allow for sampling of antigens by taking advantage of several of its morphological features. M cells have the short irregular microvilli that make the apical surface down and hollow which allows antigens to transcytose (SC, CC, and C 2008) To abet this feature M cells also possess a thin glycocalyx layer that promotes luminal antigen influx and for bacteria is easy to attach to. (Neutra, Frey, and Kraehenbuhl 1996)

2.3.1 M cells in other locations

M cells were also observed in FAE in various other MALT locations. after first being observed in Peyer's patch in humans, they were also discovered in isolated lymphoid follicles, lymphoid tissues in the caecum, colon, and rectum(M. Ann Clark, Hirst, and Jepson 1998; Owen, Piazza, and Ermak 1991; Jacob, Baker, and Swaminathan 1987). Surprisingly, they were also discovered in low incidence in the villous

epithelium as well as in the nasal-associated lymphoid tissue (NALT), Bronchus-associated lymphoid tissue (BALT), and Conjunctiva-associated lymphoid tissue (CALT) (Guliano E, Cecil P, and Thomas E 2002; Gebert. A and Hach G 1992). . The population density of M cells has been found to be dependent on the species and locations of MALT. For instance, in mice M cells only comprise 8% of Peyer's patch epithelial cells and 1% of intestinal epithelial cells whereas in rabbits M cell numbers are higher, approximately 50% in Peyer's patches (M A Clark et al. 2017).

M cells are predominantly found in the FAE at various mucosal sites. However, recent research has discovered M cell presence in murine nasal passages, the new subset of M cell's role is to primarily take up respiratory antigens and its presence was detected in the single-layer epithelium that covers regions of the nasal cavity. Regardless of location, their characteristic morphology of microfold invagination on its basolateral sides and its function of antigen uptake remains the same (Owen and Jones 1974; Kiyono and Fukuyama 2004). However, there are some subtle differences the immune mechanisms differ depending on the location of M cells. Peyer's patch targeted immunization enables an intestine-specific immune response (Yuki and Kiyono 2003; Nochi et al. 2007a) whereas oral immunizations which target the NALT site lead to antigen-specific immune responses in the respiratory (Kurono et al. 1999; Velge-Roussel et al. 2000) and reproductive tracts (Balmelli et al. 1998; Imaoka et al. 1998). This difference in antigen-specific response could be due to the activation of B cells that have different homing receptors. Different chemokine receptors and alpha integrins are expressed depending on the M cell site of antigen uptake. When the uptake of antigens or the site of immunization is the Peyer's patch CCR9, CCR10, as well as α4β7 and α4β1-integrins IgA-committed B cells, are activated whereas in the nasal M cell site of antigen uptake CCR10 and α4β1-integrin IgA-committed B cells are activated (Kunkel et al. 2003; Kunkel and Butcher 2003)

Besides the difference in the mode of immunity, there are clear differences in organogenesis and cytokines that contribute to M cell development in the Peyer's patches and M cells in the NALT. Along with the intestinal crypt, M cells in the Peyer's patches develop during the gestational period (Hashi et al. 2001) and organogenesis of M cells in the NALT begins after postnatally. M cell differentiation in the NALT is independent of the lymphotoxin βreceptor (LTβR) and interleukin-7 receptor (IL-7R)-mediated tissue-genesis program which is specific to the M cell differentiation mechanism found in Peyer's patch (S Adachi et al. 1998; Gebert, Rothkötter, and Pabst 1994).

Other external factors such as the NALT ecosystem and the Peyer's patch ecosystem play a part in the different functions between the 2 M cell types. Peyer's patch M cells exhibit a rapid turnover, and they originate from the Lgr5+ stem cells in the crypt while M cells in the NALT originate from a diverse basal cell population and have relatively longer longevity up to weeks or months (Rawlins and Hogan 2006).

Other than the Peyer's patch in the intestine, M cells are also present in the upper regions of the intestinal villi in mice. Though they are few in terms of population (approximately 1 in 50 villi) they are reactive to stimuli such as cholera toxin or pathogens and can induce Ag-specific IgG response (Jang et al. 2004). Intestinal villous M cells develop differently from their Peyer's patch counterparts as exhibited by the presence of villi M cells and the absence of Peyer's patch M cells in LT-α knockout mice (Mach et al. 2005). Transcriptomic profiling of villous M cells and Peyer's patch M cells showed a distinct difference in gene expressions for the development of both M cell subtypes (Terahara et al. 2008a) even though functionally they retain some of the same characteristics. This could be due to villous M cells only responded to antigenic stimuli from pathogenic but not from commensal bacteria.

Respiratory M cells were recently explored to understand their origin, development, and function. Respiratory M cells express the typical early and late markers of M cell differentiation and showed a high capacity for the uptake of antigens. The airway M cells were located along with the pathological induced bronchus-associated lymphoid tissue (iBALT) in mouse models. Lymphoid tissue of the iBALT B-cells and the T-cell zone are organized as a conventional secondary lymphoid organ. However, they do not exhibit the typical FAE organization with underlying dendritic cells that process trancytosed antigens (S.Kimura et al. 2019).

2.3.2 Role of M cells

M cells have a high capacity for transcytosis and phagocytosis and as a results aid in the uptake of commensal bacteria and other luminal antigens to dendritic cells in the underlying M cell pocket to initiate an immune response (Rios et al. 2016; Brandtzaeg et al. 2008; Owen 1999; Kraehenbuhl and Neutra 2000; Kato and Owen 2005). Since M cells have low lysosomal enzyme activity, they also exhibit fewer lysosomes, as a

result, the antigens that are taken up by M cells are transferred intact to antigen-presenting cells such as dendritic cells. This ability to take up intact and inert antigens is also exhibited in its capacity to take up inert particles such as latex beads. However, their ability to phagocytose can also be extended to live cells. M cell-mediated specific recognition only uptakes live Vibrio cholerae and not killed Vibrio cholerae (Owen et al. 1986).

2.3.2.1 M cell function of transcytosis and endocytosis in steady-state

In an environment that is constantly frequented by nutritional antigens, pathogenic and commensal bacteria, the primary role of M cells is to transcytosis particles from the gastrointestinal tract. M cells envelop bacteria (Owen et al. 1986; M. Ann Clark, Hirst, and Jepson 1998; M Ann Clark et al. 1996) viruses (Wolf et al. 1983), microparticles (Smith et al. 1995) as well as inert and non-infectious particles such as nanobeads (J Pappo and Ermak 1989). These transcytosed particles are presented to the dendritic cells and B cells inside the follicle. For transcytosis to commence, the first step is lumen particles binding to an M cell receptor; various membrane-bound macromolecule experiments have shown that tracer proteins on antigen surfaces adhere to the apical membrane of M cells and are taken up more efficiently than tracers that do not adhere (Neutra et al. 1987). Size also seems to be a factor by which M cells choose to uptake particles, experiments in rabbits demonstrated that 30nm particles were able to bind to M cells exclusively and were not able to bind to other neighboring enterocytes (Andreas Frey et al. 1996.). Neighboring enterocytes are not able to bind and uptake antigens probably due to the presence of thick glycocalyx layer or presence of mucus that covers the apical surface. The wavy motion and the morphology of villous epithelium also make it inaccessible for antigens to bind to enterocytes (Andreas Frey et al. 1996).

Some antigens prefer to only bind to M cells; antigens such as Salmonella enterica (Jones, Ghori, and Falkow 1994) , Escherichia coli (Inman and Cantey. 1983) , and Shigella sonnei (Sansonetti et al. 1996) have shown an exclusive binding affinity to M cells. This affinity has likewise been reported with certain viruses such as human pathogens poliovirus (Sicip et al. 1990), HIV-1 (Fotopoulos et al. 2002). These findings suggest that that the morphology of M cells along with the specific binding receptors of M cells play a huge role in the transcytosis of antigens. M cells have multiple antigen-binding receptors on their apical surfaces that enable them to sample a diverse range of lumen particles.

A receptor that is highly expressed on the surface of M cells is cellular prion protein (PrPC) and it has been implicated in the uptake of microorganisms from the gastrointestinal tract. The cellular prion protein is attached to the membrane by a glycosyphosphatidylinositol (GPI-) anchor and is crucial for the transfer of prion viral particles that leads to neurodegenerative and fatal prion diseases. Brucella abortus a gram-negative bacterium is a pathogen that expresses heat shock protein 60 (Hsp60) on its apical surface. Analysis has shown that PrPC binds with Hsp 60 and aid in the binding of, and internalization of B. abortus into M cells (Nakato et al. 2012; 2009; Kaufmann 1990)

ANXA5 has been demonstrated to be expressed by both immature and mature M cells and they frequently bind to gram-negative bacteria with high affinity via lipid A domain of lipopolysaccharide (LPS) (Rand 2012). ANXA5 performs an important anti-inflammatory role by blocking the activity of LPS to protect the host against LPS-mediated endotoxemia. Invitro study of M cells exhibited ANXA5 binding to LPS act as an uptake receptor for gram-negative bacteria possibly mediated by the lipid A domain of LPS accessing the M cell receptors (Verbrugghe et al. 2006).

Peptidoglycan recognition protein (PGLRP)-1 is a receptor that is encoded by Pglyrp1 and also known as PGRP-S; it is an innate pattern recognition protein actively expressed in M cells to bind to bacterial peptidoglycan. In vitro analysis exhibited PGLRP's binding affinity to trap gram-positive bacteria signifying a protective M cell mechanism. However, it is yet unknown if in vivo M cells can demonstrate the same anti-pathogenic activity (J Wang et al. 2011; Osanai et al. 2011).

β-1 integrin has been recently shown to be integral in the uptake of Yersinia enterocolitica. Aif1 gene function was found to be critical for this uptake process; lack of Aif1 disrupted the β-1 integrin complex and disabled its capacity to uptake Yersinia enterocolitica. β-1 integrin is primarily expressed at the basolateral surface in enterocytes however, in M cells due to the anatomy and uptake function they are found at the apical and lateral surface. β-1 integrin significance was also observed in mice as experiments with an antagonist that blocked the β-1 integrin complex showed a loss of transcytosis capacity (M. Ann Clark, Hirst, and Jepson 1998; Kishikawa et al. 2017)

Toll-like receptors (TLRs) are also widely expressed on the apical surface of M cells and are responsible for identifying antigens from the luminal surface. They are

members of the pattern-recognition receptors family and comprise of at least 13 members in the TLR family. Each member of the TLR family is responsible for binding to different ligands. For instance, bacteria secreted lipoproteins, peptidoglycan, and lipoteichoic acid are recognized exclusively by TLR2 (Yoshioka et al. 2016; Chang et al. 2004; Nishimura and Naito 2005). Double-stranded RNA from viruses are recognized and bind to TLR3 (Alexopoulou et al. 2001). Studies have shown that M cells express all the TLR's except for TLR3 and TLR6; while specific roles of all the TLR's on M cells have yet to be studied, TLR2 and TLR4 have been examined closely. Dose-dependent transcytosis efficiency has shown to be dependent on TLR2 activation even though TLR2 is expressed in both FAE and villous epithelium. TLR2 receptor redistribution has been found exclusive to FAE. Nontypeable Haemophilus influenzae uptake through the FAE has been shown to be TLR4 dependent. In-vitro M cell models have shown that the specific blocking of TLR4 receptors leads to a significant loss of the uptake of Nontypeable Haemophilus influenzae and the uptake of the virus was 3 fold higher compared to the controlled group with TLR4 silenced (Chabot et al. 2006).

M cells must differentiate between the different kinds of lumen antigen it has to uptake. It is detrimental to the whole microbial ecosystem if M cells were to uptake critical commensal bacteria. Regulation of commensal microflora is necessary to maintain a healthy and functional microbial ecosystem in the gut. This regulation is carried out by secretory IgA (NJ Mantis et al. 2011). The role of secretory IgA is to block pathogenic particles binding to the apical surface of villous epithelial cells and the FAE so as to provide protection against intestinal infection (KJ Silvey et al. 2001). Another role of secretory IgA is to regulate the trapping of pathogenic bacteria within the mucus layer and holding them for further breakdown (Boullier et al. 2009). Mice lacking secretory IgA have shown higher rates of infection with Salmonella typhimurium (P Michetti et al. 1992). Furthermore, targeted binding of immune complexes formed of secretory IgA and microbes to M cells has shown that they are subsequently transcytosed through to mononuclear phagocyte (MNP) population and follicle cells in the sub-epithelial dome; this process is aimed towards antigen sampling and modulation of the mucosal immune response (J Rey et al. 2004; KA Kadaoui et al. 2007). It remains to be seen if this process is antigen specific as M cells have not shown any evidence of the presence of putative IgA receptor and additionally it can also be inferred that the transcytosis function of M cells helps to internalize secretory IgA and IgA containing immune complexes. Further analysis of these complexes demonstrates that they are not disseminated beyond mesenteric

lymph nodes once uptake occurs (KA Kadaoui et al. 2007). Therefore, secretory IgA plays an important function to recognize antigens under neutralizing and non-inflammatory conditions without enabling an inflammatory response that requires tissue dissemination.

Glycoprotein 2 (GP2) has been identified as an M cell-specific marker; M cells are the only cells that express Gp2 receptors when compared to other cell types in the gut epithelium. An immature M cell does not express Gp2 thereby indicating a mature M cell with Gp2 has a fully functional transcytosis capacity (Hase et al. 2005; Terahara et al. 2008). Gp2 is a glycosylphosphatidylinositol-anchored protein and it binds specifically to the FimH unit of the type I pili on the outer membrane of certain commensal antigens and pathogenic bacteria such as S. typhimurium and Escherichia coli. Mice lacking Gp2 have shown impaired transcytosis and an increased rate of infection with S. typhimurium (Koji Hase et al. 2009) . Similar results were observed with FimH- strain; additionally, Gp2-dependent uptake of type 1-pilli pathogens was important for immunosurveillance in the gastrointestinal tract (Koji Hase et al. 2009).

Transcriptomic analysis of cells in the FAE shows that the homolog of Gp2, Uromodulin (UMOD, Tamm–Horsfall protein) is expressed specifically on the apical surface of M cells (S. Sato et al. 2013; W. de Lau et al. 2012). Similar to Gp2, it binds to type 1 pili part of pathogenic bacteria such as E.coli and since UMOD is a secretory protein receptor, its function is to prevent the binding of bacteria to uroepithelial cells (J Pak et al. 2001). Even though the expression of Gp2 is more prevalent than that of UMOD, their similar features indicate that each protein aids in the sampling of FimH+ gram-negative bacteria.

Apart from conventional receptors, other factors play a major role in the functioning of M cells apart from transcytosis. Chemokines and chemokine receptors are critical for attracting leukocytes and lymphocytes to the lymphoid tissue. CCL20 is expressed by other cells in the FAE including M cells; CCL9 and C-X-C motif chemokine ligand 16 (CXCL16) are expressed specifically on M cells (Tahoun et al. 2012; Terahara et al. 2008; Nakato et al. 2009; A Iwasaki et al. 2000; K Hase et al. 2006). CXCL11 has been demonstrated to be expressed by M cells, the FAE, and the RANKL-stimulated villous epithelia (Terahara et al. 2008b; Nakato et al. 2009) . This induction by RankL stimulation shows that a specific set of chemokines are regulated by an active transcription network, this was further confirmed by

experiments with RankL null mice showing a significant decrease in expression of CXCL16 and CCL9. Previous research demonstrated a significant amount of redundancy with chemokine and chemokine receptor functions. However, certain receptors like CCL20 (CCR6−/− mice) have been found critical for the development and maturation of M cells in the FAE as mice lacking CCL20 showed a significant decrease of M cells, this suggests that M cell and FAE expressed chemokines play a role in attracting specific lymphoid cell population to the basolateral section to sample antigens and initiate a mucosal response (Westphal et al. 2008; Ebisawa et al. 2011). CCL9 functions as a recruiting agent for CD11b+ MNP to the subepithelial dome and CCL16 promotes interactions between T cells that express CXCR6 and the cells in the FAE, this aids in the secretion of an antimicrobial peptide in the gut lumen (K Hase et al. 2006; Kotarsky et al. 2010) . Other chemokine receptors such as CXCL11 and CCL25 help in recruiting CXCR3-expressing Th1 cells and the homing of IgA antibody-secreting cells (Hieshima et al. 2004; Lee et al. 2008). Previous research has shown that HIV can be transported by M cells through lactosyl cerebroside and CXCR4 receptors in a 2-dimensional M cell in-vitro model (Fotopoulos et al. 2002)

Tight junction proteins are present in all enterocytes and secretory cells in the gut epithelium including villous epithelium and FAE cells. However, the distribution of tight junction proteins in M cell are quite distinct. Recent research using in situ hybridization and antibody staining has shown that claudin 4, a tight junction protein, is not restricted to tight junctions but also observed in the cytoplasm. Claudin 4 being a bacterial enterotoxin receptor may play a part in M cell-mediated endocytosis Clostridium perfringens binds specifically to the external domain of claudin 4 (Rajapaksa et al. 2010; D. D. Lo, Ling, and Holly Eckelhoefer 2012). Adhesion molecule-A is a tight junction protein that has been known to uptake reovirus. This capacity has been observed with M cells (Barton et al. 2001)

2.3.2.2 Antigen targeting to M cells to aid in the development of Oral Vaccines.

Vaccines are means by which a biological preparation can provide acquired immunity to a particular infectious disease. The biological preparation comprises of an agent (mRNA, live attenuated virus, DNA, protein, etc.) that resembles or codes for a protein of the infecting microorganism or resembles the microorganism itself to stimulate the body's immune system to identify the threat and initiate an immune

response against it. Vaccination has been dependent on parenteral administration and is hampered by logistical issues, handling, storage temperature, and instability. However, recent studies have shown administration of vaccines via oral means is efficient in inducing an immune response not only systemically but also in the mucosal compartment where many pathogenic infections are initiated. Mucosal vaccinations require the need for an adjuvant or a binding agent that can bind to receptors for the efficient delivery of vaccine into lymphoid tissues that are populated by dendritic cells, T cells, and B cells. These cells in turn initiate a mucosal immune response. Since M cells have the unique functionality of being a portal for uptake of luminal antigens and their ability to initiate an antigen-specific immune response there is an interest in research into the development and function of M cells. However, the low frequency of M cells and the lack of characterization of M cell-specific receptor makes it difficult to isolate M cells for targeted delivery of vaccines (Azizi et al. 2010).

The immune response from mucosal vaccination is initiated by presenting exogenous antigens in mucosa-associated lymphoid tissue aggregates that can absorb or transcytose these elements (Brandtzaeg et al. 2008). Once antigens are transcytosed through to the sub-epithelial dome, they bind to dendritic cells. These loaded dendritic cells with the introduced antigen migrate to intrafollicular T-cell areas which lead to the specific activation of T cells which, in turn, facilitate the somatic hypermutation in B cells through the CD40-CD40 ligand signaling pathway. They also facilitate the antigen-specific dimeric IgA class switch recombination (Bemark, Boysen, and Lycke 2012) . Once the recombination is complete, antigen-specific dimeric IgA produced from IgA-expressing plasma cells is transported through the lamina propria into the lumen via polymeric Ig receptors and becomes SIgA (Pabst 2012). The routes of mucosal vaccination are an important factor to consider when vaccinating orally since different routes induce different immune responses. Intranasal immunization of the upper respiratory, genital and gastric tracts leads to the secretion of secretory IgA (SIgA) by initiating an immune response in the nasopharynx-associated lymphoid tissue. Oral immunization is carried out by introducing an antigen into the Peyer's patches of the gut-associated lymphoid tissue which leads to the induction of IgA response in the salivary glands, mammary glands, and gastrointestinal tract (Holmgren and Czerkinsky 2005) For instance, when cholera toxin subunit B (CTB) is delivered nasally into humans, CTB-specific IgA is especially induced in the nasal cavity, large intestine, and genital tracts but no IgA activity is observed in the small intestine and salivary glands (Kozlowski et al. 1997;

2002). Even though oral vaccination is an efficient mode of vaccination, its effectiveness is hampered by several limitations; the lack of an effective antigen delivery system that can bind specifically to cells on the follicle-associated epithelium is one of the reasons for the limited availability of oral vaccines. Due to the intrinsic nature of the lack of a lymphatic system in the mucosal immune system, the immune responses have lower efficiency in antigen delivery into the inductive site and a lower tendency to induce oral tolerance (Azizi et al. 2010b). However, research in M cells as a target for oral vaccination has increased lately particularly due to their high capacity to transcytose antigens including vaccines targeted to recently characterized M cell receptors

In the past, developing vaccines that target M cells have been difficult due to limitations in understanding the mechanistic working of surface molecules on the apical area of M cells. However, recent research has been vital to understanding specific markers of M cells for example the establishment of in vitro human M-like cell culture model led to the generation of M cell-specific antibody NKM 16-2-4 by the immunization of UEA-1+ WGA− cells (Nochi et al. 2007). In addition to understanding different receptors on M cells, recent research has also focused on different pathways luminal antigen sampling. The 3 pathways identified are 1) nonspecific endocytosis 2) specific receptor-mediated endocytosis and 3) extension of transcellular dendritic processes by lysozyme-expressing dendritic cells that exhibit a sturdy antigen sampling and phagocytic activity (Belkaid, Bouladoux, and Hand 2013). Given that specific M cell receptors are targeted by specific antigens to induce pathogenic infection, it is possible to design vaccines that can be targeted towards specific M cell receptors to provide specific mucosal immune responses.

2.4 M cell Development and Differentiation

The origin and lineage of M cell development used to be a controversial subject. For some time, scientists postulated that M cells were converted from fully differentiated enterocytes. They believed that lymphocytes from underlying follicles provide the niche and initiate the lineage switching from enterocytes to an M cell phenotype. An attempt to confirm this speculation was carried out by Kerneis et al, B cell lymphocytes from Peyer's patches were injected into the duodenal mucosa of recipient mice; 9-days post-injection, a Peyer's patch-like structure with M cells and other enterocytes were found at the site of the injection (Kernéis et al. 1997).

However, considering that the turnover rate of intestinal epithelial cells is 4-6 day, it is not sufficient to prove that the new M cells at the injection site were converted from enterocytes (Potten 1998). It is possible that the newly differentiated M cells may have arisen from an Lgr5+ crypt cell that differentiated under the influence of the injected Peyer's patch B cells. Coculturing of Caco- 2 cells and Raji B cells were one of the earlier in-vitro experiments used to study M cell differentiation (Hilgers, Conradi, and Burton 1990) . After 14 days of co-culturing, Caco-2 cells were able to differentiate into M cell phenotype. However, Caco-2 cells are an immortal cell line that originates from cancer cells and is poorly differentiated crypt-like cells. Caco-2 cells are known to differentiate into different epithelial cell types and express many crypt cell markers, therefore even though they attain some characteristics of an M cell phenotype when co-cultured with Raji B cells, it does not necessarily indicate that B lymphocytes directly induces trans-differentiation of fully matured enterocytes into M cells.

Over the years, it has been observed in mice and humans that M cells have increased in population in an inflammation/infectious state over a steady physiological state. Streptococcus, a non-intestinal bacterium has been recorded to increase M cell population (C Borghesi et al. 1999; Meynell 1999) , a similar increase in M cell population was also observed with exposure to Salmonella (Savidge et al. 1991) . It could be hypothesized that inflammation and immunological pathways lead to the differentiation and development of M cells, however, it was observed that an increase in the number of M cells was correlated and localized to the surface area and upregulation of microsphere uptake (Meynell 1999) . The study also showed that M cell numbers increase 12 hours after exposure and last 5 days which aligned with the doubling rate of Lgr5+ crypt stem cell differentiation and its migration towards the apex of follicle-associated epithelium. This suggests that the newly differentiated M cells might possibly arise from stem cells. The newly differentiated M cells from infection only shared one characteristic with regular M cells that existed previously in the system- the lack of microvilli which is not sufficient to prove that these were functional M cells. The mice used in this experiment were also germ-free mice which are recorded to have a reduced number of Peyer's patches, the increase in the number of M cells could be the effect of lymphoid follicles organizing to combat the increasing number of microorganisms. All of these pieces of evidence point to the fact that stem cells localized in the intestinal play a major role in the differentiation of M cells.

2.4.1 Differentiation of M cells from Lgr5+ stem cells in the crypt

In 1984 Bye et al observed immature M cells at the base of the follicle-associated epithelium close to the intestinal crypts based on their characteristic morphology (Bye et al. 1984). Various research groups confirmed that these cells were M cells by observing expressions of different markers of M cell receptors and morphological similarities (A Gebert, G Hach, and H Bartels 1992; Siciński et al. 1986; Jacques Pappo 1989) . Confirmation of M cells deriving from Lgr5+ stem cells from the crypt was confirmed in 2012 by de Lau et al (W. de Lau et al. 2012). They generated mice carrying an Lgr5 knock-in allele expressing an enhanced green fluorescent protein (EGFP)-ires-creERT2 cassette. In these transgenic mice, the EGFP and cre ERT2 are specifically expressed in Lgr5 stem cells. These mice were crossed with Rosa26-LacZ reporter mice to closely observe the progression of Lgr5+ stem cell to M cell as it travels up the apex of follicle-associated epithelium. Excision of the Rosa26-LacZ site in Lgr5+ cells was induced by tamoxifen injection. Three days after injecting the mice with tamoxifen, mice were sacrificed, and Peyer's patch samples showed ribbons of LacZ+ cells in the follicle-associated epithelium emanating from crypts adjacent to the Peyer's patches. This experiment was repeated with a 6-month gap from the time of Tamoxifen injection to the preparation of the sample, the follicle-associated epithelium cells were still able to retain LacZ+ cells. This experiment proved without a doubt that M cells arise from Lgr5+ stem cells that arise from intestinal crypts. All the 6 cell subtypes of the gastrointestinal tract arise from Lgr5+ stem cells however, M cells are very few in number and not observed in other locations like the zone above the transit-amplifying zone or en masse in the intestinal villous epithelium when compared to the follicle associated epithelium. This goes on to show that more factors are required for M cells to specifically differentiate from Lgr5+ stem cells. Various researchers have proposed that specific factors/ligands released in the subepithelial dome immediately below the follicle associated epithelium can initiate M cell differentiation (Owen and Jones 1974; Kernéis et al. 1997)

2.4.2 Ligands required for M cell differentiation.

Given that the M cell population is specific to the follicle-associated epithelium in the Peyer's patches, it is possible that the lymphoid environment in the sub-epithelial dome could play a part in M cell differentiation and function. Various experiments that involved the editing of genes that are responsible for the integrity of Peyer's

patches revealed reduced M cell numbers. B cell knockout mice exhibit amongst various other immune deficiencies, a severe lack of M cells in their Peyer's patches (Golovkina et al. 1999). In-vitro studies of M cell differentiation with characteristic transcytosis capacity involved co-culturing of Caco-2 cells with Raji B lymphocytes provided further evidence of specific signaling mediators from lymphocytes underneath the follicle region (des Rieux et al. 2007; Kernéis et al. 1997). However, experiments with in-vitro analysis of M cell differentiation failed to reveal a specific mechanism so did experiments involving analysis of in vivo experiments with Peyer's patches from B cell-deficient mice.

An important cytokine that is selectively expressed by stromal cells present in the sub-epithelial dome in the Peyer's patch is the receptor activator of NF-κB ligand (RANKL). RANKL expression by stromal cells beneath the follicle-associated epithelium indicates a possible function for RANKL for M cell differentiation. RANKL is a member of the tumor necrosis family (TNF) specifically tumor necrosis factor superfamily 11 (TNFSF11) (Bachmann et al. 1999); another name for RANKL is TNF-related activation-induced cytokine. RANKL is freely secreted under Peyer's patches but initially, it is produced as a transmembrane protein that is attached to a stromal cell surface which gets cleaved off by several metalloproteases (Bachmann et al. 1999; Lum et al. 1999). RANKL specifically binds to 2 receptors; the specific receptor RANK (receptor activator NF-κB) and to its soluble decoy receptor Osteoprotegerin (OPG), these 2 receptors allow for a tight regulation for RANKL-RANK signaling (Wong et al. 1997; Galibert et al. 1998; Simonet et al. 1997) . RANKL-RANK signaling was first discovered when studying normal osteoclast function, RANKL null mice and RANK null mice were both found to have osteopetrosis and other skeletal abnormalities (Kong et al. 1999; N. Kim et al. 2000) . Other critical physiological developments and functions that require RANK-RANKL signaling are medullary thymic epithelial cells (mTEC) development, dendritic cell signaling, mammary gland lactation, and lymph node development (Kong et al. 1999; Kim et al. 2000; Akiyama et al. 2008; Hikosaka et al. 2008; Wong et al. 1997; Fata et al. 2000) . RANKL null mice demonstrated the absence of lymph nodes which indicated that it was critical for lymphoid organogenesis; though RANKL deficient mice still showed the presence of Peyer's patches, they were comparably smaller in size compared to their control counterparts. Thus, it might be possible that RANKL plays a significant role in normal Peyer's patch development (Kong et al. 1999; N. Kim et al. 2000).

Utilizing transgenic RANKL null mice Knoop et al showed the significance of RANK signaling in Peyer's patches; the absence of RANKL was associated with a decrease in the expression of UEA-I + M cells. Peyer's patch of RANKL KO mice stained with UEA-I lectin antibody demonstrated a significant reduction in UEA-I + expressing M cell population in the gut almost less than 2% of the population found in wildtype. RANKL null mice also exhibited defects in transcytosis of antigen uptake of Salmonella enterica serovar Typhimurium, Yersinia enterocolitica, and latex nanobeads. However, systemic administration of RANKL orally restored the M cell population in RANKL null mice; these restored M cells also retained their transcytosis capacity as well towards luminal antigens. This observation was not restricted to the follicle-associated epithelium but the intestinal epithelium as well. This provides further insights into the mechanistic action of RANKL as RANK expressing progenitor cells are located in crypts next to the Peyer's patches as well as in crypts that grow vertically into villous from the transit-amplifying zones. If RANK receptors exposed to extraneous RANKL ligand can initiate differentiation, this might be sufficient stimulation for Lgr5+ crypt cells to differentiate into M cells. These observations point to RANKL RANK signaling's pivotal role in establishing M cell-mediated antigen processing (Knoop et al. 2009). A significant milestone in M cell research was achieved when de Lau et al showed crypt organoids isolated from mice duodenum and cultured in vitro were able to differentiate into functional Gp2+ M cells upon treatment with recombinant RANKL for 4 days (W. de Lau et al. 2012).

A key speculative point in RANKL signaling has been the kind of stromal cells in the Peyer's patches responsible for secreting RANKL. For several years RANKL secreting cells were referred to as stromal cells. Researchers found three major sources of RANKL expressed by a number of cell types: A) group 3 innate lymphoid cell (ILC3s); B) T cells; C) mesenchymal cells (Cella, Miller, and Song 2014; Cella, Otero, and Colonna 2010; Totsuka et al. 2009; Taylor 2007; Katakai et al. 2008; Katakai 2012). Cells close to the dome of the follicle-associated epithelium were identified as the cells responsible for secreting RANKL specifically for the development of M cells and Peyer's patch. These mesenchymal cells were referred to as M cell inducer cells or MCi, additionally, these cells also played a role in regulating bacteria-specific IgA production (Nagashima, Sawa, Nitta, Tsutsumi, et al. 2017). Additional experiments involving the targeted deletion of RANKL in M cell inducer cells leading to a decreased population of M cells provided further insight

into the role MCi plays in the development of Peyer's patches and M cells (Nagashima, Sawa, Nitta, Prados, et al. 2017).

Interestingly, RANKL null mice still exhibited a small number of residual UEA-I+ M cells in some of the Peyer's patches especially in increasing numbers in the distal locations (still comparably lesser than B cell-deficient mice), this could mean that other signaling mechanisms exist in parallel with RANKL-RANK signaling. Metastasis-promoting protein S100A4 was found to be essential for the maturation of M cells. S100A4 was secreted by a heterogeneous population that includes group3 innate lymphoid cells and lysozyme expressing dendritic cells; this heterogeneous population was regulated by a Cdc42 activator cell called Dock8. DOCK8 is a conserved guanine nucleotide exchange factor (GEF) for Cdc42 (Harada et al., 2012). Dock8 impairment in humans is documented to be related to immunodeficiency that lead to recurring viral infections, atopic dermatitis and early-onset malignancy (Engelhardt et al., 2015, Sanal et al., 2012, Zhang et al., 2009, Zhang et al., 2010). Dock8 knockout mice exhibited a reduced expression of S100A4 expressing cells and impaired development of mature M cells. S100A4 is a member of the S100family proteins their characteristic feature are the 2 Ca2+-binding sites that includes a canonical EF-hand motif (Schneider, Hansen, and Sheikh 2008). Previous research has demonstrated S100A4's presence in leukocytes such as macrophages and DC and metastatic tumor lines (Boye and Mælandsmo 2010; Österreicher et al. 2011; Zhang et al. 2018) . Targeted deletion of Dock8 in the intestine using *VilCre* mice still retained Gp2+ M cells due to active RANKL expression in the Peyer's patches, however, targeted deletion of DOCK8 expression in S100A4-producing cells exhibited a lower population of mature M cells expressing Gp2 receptors. Functional assays of these mice also exhibited a severe reduction in the transcytosis capacity and uptake of latex nanobeads. S100A4 was able to promote a higher rate of differentiation of crypt organoids into mature M cells in-vitro in combination with RANKL than RANKL alone. S100A4 deficient mice demonstrated impaired development of mature M cells indicating its crucial function in differentiation. S100A4 knockout mice showed poor uptake of orally administered latex nanobeads and S. choleraesuis; secretory IgA levels were severely diminished too indicating a lack of mucosal immune response. Quantitative analysis of M cell numbers showed a 48% reduction in S100A4 null mice when compared to its wildtype counterparts (Kunimura et al. 2019) .

Apart from these 2 main factors RANKL and S100A4, various other factors play a role in M cell differentiation such as lymphotoxin (LT) and tumor necrosis factor α (TNFα). LT and TNF belong to the family of tumor necrosis factor (TNF) superfamily of cytokines; they are responsible for the organization and development of lymphoid cells and lymphoid follicles (Nedwin et al. 1985). Lymphotoxin is found in 2 forms Lymphotoxin alpha (LT-α) and Lymphotoxin beta (LT-β), both of them have distinctive functions and specific structural characteristics (Weinstein and Storkus 2015; Ruddle 2014). LT-α and TNFα bind together to form a homotrimer that binds to TNFR1 and TNFR2 while the heterotrimer LTα1β2 or LTα2β1 binds to LTβR. This leads to TNF receptors binding to TNFR associated factors such as TRAF2 and TRAF5 which in turn initiates the nuclear factor kappa B (NFκB) signaling pathway. TRAF2, TRAF3, and TRAF6 activate P100/RelB which is responsible for activating a slew of transcriptional factors that are responsible for Peyer's patch development. Consequently, LTα1β2 treatment of organoids in vitro leads to the activation of several genes essential for M cell (Takashi Kanaya et al. 2018). The significance of LTβR was observed when mice injected with LTβR-Ig during the mice's gestational period showed a lack of lymphoid follicles in the intestinal tract (Rennert et al. 1996; Rennert, Browning, and Hochman 1997). Blocking TNFR with TNFR-Ig did not impair Peyer's patch development which points to the LTαβ heterotrimer or LTβR's important role in Peyer patch and M cell development.

TNFα was shown to augment M cell differentiation in enteroid cultures in vitro. Enteroids grown in RANKL alone showed significantly less expression of M cell-associated genes by 3 to 6-fold when compared to TNF-α + RANKL treatment (Wood, Rios, and Williams 2016). It is known that TNF-α activates the canonical and not the non-canonical NF-κB pathway (Oeckinghaus. A, Mathew S, and Sankar G 2011). This is indicative of the importance of activation of the canonical pathway (RelA/P50's) role in supporting RANKL mediated M cell differentiation. An increase in infection and inflammation increases the number of Peyer's patches and M cells to counter the invading elements. This increase is usually found in the distal regions of the gastrointestinal tract especially in the colonic region, these M cells are called inducible colonic M cells. Expression of TNFα is significantly higher in the colon; recent research demonstrated that inducible colonic M cells are dependent on TNFα. Experiments were carried out with TNF receptor 1 (TNFR1) knockout mice and TNF receptor 2 (TNFR2) knockout mice and for the induction of colitis, mice were treated with dextran sodium sulfate (DSS) which led to an increase in M cell

numbers. Though DSS was able to induce colitis in both TNFR1 KO and TNFR2 KO, TNFR1 KO mice showed a significant increase in overall M cell numbers in the colon and TNFR2 KO showed no significant increase in M cell population in the colon. Interestingly RANKL induction and its function in the Peyer's patch was completely independent of both TNFR1 and TNFR2 signaling. Thus, it can be concluded that inducible M cells in the colon are strictly dependent on the TNF-α signaling and specifically dependent on TNFR2 signaling and not TNFR1. Furthermore, it goes on to show that signaling mechanisms in inflammation-inducible M cells are different from constitutively differentiating M cells as inducible M cell functions to influence specific immune responses related to infection and inflammation at hand (Parnell, Walch, and Lo 2017) (See Figure 5).

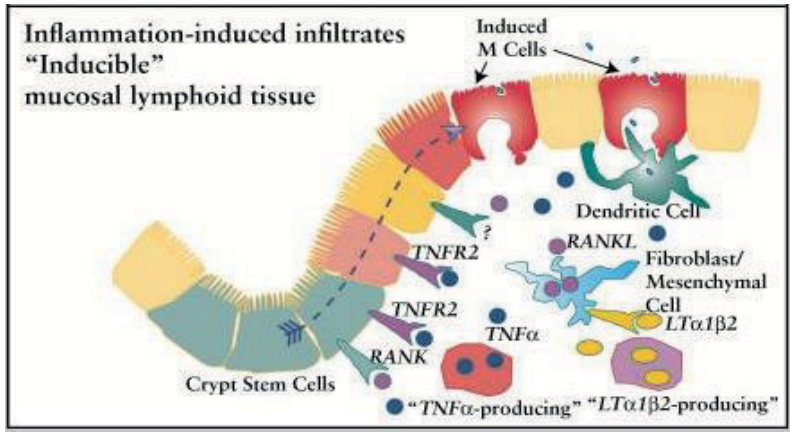

Figure 5. Graphical model for inducible M cell subsets. Inducible M cells in addition to RANKL require the binding of TNFα to TNFR2 receptor to aid in the differentiation of M cells in the cecal patches. (Adapted from Erinn A.P et al)

2.4.3 Factors necessary for M cell differentiation

RANKL-RANK signaling is the first step in initiating M cell differentiation in the follicle-associated epithelium of the Peyer's patches. Once RANKL bind to RANK receptors, a cascade of regulatory gene network is activated which aids the stem cell to its new fate; distinct transcription factors control the expression of set genes that determine the differentiation of Lgr5+ crypt stem cell-derived progenitor cells into terminally differentiated intestinal M cells. Recent research and advances in transcriptomic profiling techniques have revealed the identity of several of these

genes. These genes have been characterized in length and many of them play a significant role in the maintenance of M cell's immune function of antigen uptake and secretion of secretory IgA.

2.4.3.1 TRAF6 activation is required for NF-κB signaling

Canonical and non-canonical NF-ϰB signaling pathways can be activated by the ligation of the RANK receptor (Akiyama et al. 2008). TRAFs are regulatory molecules of the RANK-mediated classical/canonical NF-ϰB signaling pathway. Once RANKL binds to the RANK receptor, TRAFs are recruited to the cytoplasmic domain of TNFRs. An important member of the family TRAF6 is critical for the activation of RANK mediated canonical NF-ϰB signaling (Walsh et al. 2014). Experiments conducted with TRAF6 KO localized in the intestine using VilCre mice showed a severe decrease of Gp2+ M cells in the FAE. These mice also demonstrated reduced antigen uptake capacity and reduced expression of M cell-associated transcription factors and other follicle-associated molecules such as Ccl20 and Cxcl16. A previous study revealed that TRAF6 was needed for RelB expression in Medullary thymic epithelial cells (mTECS) (Akiyama et al. 2005). RelB is documented to be activated under non-canonical NF-ϰB signaling; previous research involving inhibition of NF-ϰB by the deletion of ReLB and RelA impaired the development of lymphoid tissues including Peyer's patches, thereby resulting in a lack of follicle associated epithelium or M cells (Weih and Caamaño 2003).

In vitro experiments involving organoids were able to decipher the specific role TRAF6 played in NF-ϰB signaling mediated M cell differentiation. TRAF6 KO organoids treated with RANKL revealed that classical P50/RELA signaling pathway that activates inflammatory genes such as Cxcl10, Nfbia and Ccl20 was severely affected. In vitro experiments using a pharmacological inhibitor of canonical NF-ϰB pathway – SC514 demonstrated that expression of RelB decreased significantly in organoids. Thus, proving that canonical NF-ϰB (RelA/P50) is essential to evoke a non-canonical NF-ϰB signaling pathway(Takashi Kanaya et al. 2018).

Non-canonical (P52/RelB) plays an important role in M cell differentiation as well. RelB KO intestinal organoids treated with RANKL failed to differentiate to Gp2+ M cells and demonstrated a significant decrease in expression of other M cell-associated transcription factors. RelB deficient mice also completely lacked PP's and therefore follicle-associated epithelium (Miyawaki et al. 1994; Z. B. Yilmaz et al.

2003). Interestingly, organoids transduced with RelB/P52 with a lentiviral vector showed significant expression of key transcription factor Spi-B and other early markers of M cell differentiation without RANKL but not Gp2 which is a marker for mature M cells. This shows that epithelium intrinsic non-canonical NF $\varkappa$B itself is not sufficient for differentiation of M cells but essential for the initiation of differentiation(Takashi Kanaya et al. 2018).

In conclusion, canonical NF-$\varkappa$B signaling is involved in inflammatory responses and is fast and transient whereas non-canonical NF- $\varkappa$B signaling is slow and continuous and is critical to cellular differentiation. Since TRAF6 is required to activate the canonical NF-$\varkappa$B pathway which in turn activates the non-canonical pathway, it can be inferred that TRAF6 plays a significant role upstream of NF-$\varkappa$B signaling

2.4.3.2 Spi-B is a master regulator of M cell differentiation

Transcriptional profiling at various times by different researchers for RANKL stimulated intestinal organoids using microarrays revealed Spi-B to be highly expressed. Spi-B belongs to the ETS family of transcription factors (Ray D et al. 1992). Spi-B plays a significant role in many physiological functions such as human plasmacytoid dendritic cells, B cell antigen receptor signaling, germinal center formation, antigen-antibody responses, and a critical role in B cell development (HSu et al. 1997; DeKoter et al. 2010; Garrett-Sinha et al. 1999; Schotte et al. 2004). Identification of Spi-B in the RANKL induced epithelium was in sharp contrast to previously held thought that Spi-B was specific for lymphoid and myeloid cell types; Spi-B was not known to be expressed outside the immune system (Dahl et al. 2002). For characterizing Spi-B's critical role, SpiB KO mice were analyzed and even though constitutive knockouts of Spi-B caused minor defects in B cell receptor signaling, no major abnormalities in development were observed (HSu et al. 1997). Earlier experiments studying NFKB signaling role in M cell development revealed that non-canonical RelB/p52 was sufficient for Spi-B expression (Takashi Kanaya et al. 2018).

Mice that underwent systemic ingestion of RANKL orally exhibited ectopic M-cell differentiation in their villous epithelium. Whole-genome expression profiling of the villous epithelium showed a significant linear increase in expression of Spi-B shortly after RANKL treatment. Spi-B expression peaked at its highest one hour after RANKL treatment. Spi-B expression was also confirmed by Lau et al, 2012,

intestinal crypt organoids cultured in vitro were treated with RANKL, Spi-B expression was distinctly up 6 hours after treatment. From whole-mount staining and immunohistochemistry of Peyer's patches from Spi-B null mice, a lack of GP2+ M cells and CCL9+ M cells was observed. However, the expression of MarcksL1 which is an early marker significant of immature M cells did not seem to be affected by the lack of Spi-B. Organoids from SpiB null mice were not able to differentiate into Gp2+ mature M cells either upon treatment with RANKL. Electron microscope imaging of the Peyer's patch from SpiB null mice showed a complete absence of M cells in the follicle-associated epithelium. Spi-B null mice were given an oral administration of green, fluorescent latex nanobeads (200 nm in diameter) which they failed to transcytose into the subepithelial dome of the Peyer's patches indicating a failure in M cell functionality. SpiB- null mice further exhibited impaired mucosal response as well upon oral treatment with S. typhimurium; defects were observed in S. typhimurium-specific activation of T cells in Spi-B knockout mice. Spi-B alone wasn't enough to induce M cell differentiation indicating Spi-B expression wasn't sufficient for complete maturation of M cells (W. de Lau et al. 2012; Takashi Kanaya et al. 2012).

These studies point to the fact that Spi-B is a critical transcription factor required for mature, functional M cell differentiation and development. Furthermore, a tight linkage was established between the need for the expression of Spi-B to activate other M cell-associated genes.

2.4.3.3 Sox8 is needed to accelerate IgA response and M cell maturation

Since Spi-B was identified to not be sufficient for the maturation of M cells. Researchers looked for other regulatory transcription factors that were activated alongside Spi-B or required the activation of Spi-B first. Using 3 different transcriptome analyses that profiled M cells, Sox8 was identified as a transcription factor highly expressed in the datasets (K Hase et al 2005; T Kanaya et al 2012; Lau et al 2012). Molecular experiments such as GST-RANKL oral administration confirmed the RANKL-mediated induction of Sox8. qPCR analysis of follicle-associated epithelium of Peyer's patch showed that Sox8 expression was exclusive to the follicle-associated epithelium along with GP2 expression when compared to the villous epithelium. Sox8, Sox9, and Sox10 encode a member of the SRY-related HMG box family of transcription factors, in particular the SOX-E subgroup. The members of this subgroup are master regulators of various physiological

developments like sex determination, neural crest development, and gliogenesis (Weider and Wegner 2017; Jiang et al. 2012). For instance, Sox9 was demonstrated to be pivotal in the differentiation of Paneth cells which provide the niche for Lgr5+ stem cells to proliferate (Mori–Akiyama et al. 2007).

Sox8 was found to be constitutively expressed during the development of M cells. Experiments involving whole-mount immunofluorescence staining along with quantitative image cytometry revealed that Sox8 to be expressed in both mature M cells as well as immature M cells. The phenotype from the loss of Sox8 was similar to the phenotype of Spi-B null mice. Sox8 null mice exhibited a profound lack of GP2+ M cells in the follicle-associated epithelium when compared to its wildtype counterparts. Other M cell-associated factors such as CCL9, Ccl20 showed reduced expression in the Sox8 KO follicle-associated epithelium, except for MarcksL1 which remained comparable to its wildtype counterpart, suggesting that immature M cells did not need Sox8 expression but was required for maturation. Spi-B expression was slightly impaired in the Sox8 KO mice and Sox8 expression was still present in the Spi-B KO mice indicating that Spi-B was not needed for Sox8 expression. The concentration of fecal S-IgA was significantly diminished in 4-week-old Sox8 null mice but reached the same concentration as its wildtype counterpart by week 6 indicating that IgA response was delayed and dependent on Sox8 expression in young mice. Functionals assays to study the antigen uptake capacity of Sox8 null mice revealed that the loss of Sox8 impaired the mice's transcytosis capacity to uptake latex nanobeads and S.Typhimurium. Interestingly, similar to Spi-B, Sox8 was activated upon RelB/P52 activation; ChIP-seq analysis provided additional evidence of RelB/P52 directly interacting with Sox8 promoter and Sox8 directly binding to Gp2 promoter. Sox8 is a critical transcription factor for M cell development however, the presence of Sox8 in Spi-B knockout mice and lack of mature M cells proved that Sox8 alone isn't sufficient for M cell maturation.

2.4.3.4 OPG required for M cell self-regulation to balance infection and immunity

The functional role of M cells is to conduct surveillance and uptake luminal antigens in the gastrointestinal tract. However, M cells can also function as vulnerable gatekeepers that allow several pathogenic bacteria, toxins, and virus-like oral prions to exploit them as entry portals to bypass tight junctions and epithelial barriers to induce systemic infection. This indicates that the M cell population in the gut is

tightly regulated to balance immunity and infection (Mabbott et al. 2013). Stem/progenitor cells from the crypt express RANK receptors and follicle-associated cells are continuously subjected to RANKL stimulation from M cell inducer cells (Nagashima, Sawa, Nitta, Tsutsumi, et al. 2017) however, only a small percentage of those cells differentiate to M cells (~10–20%) and this number decreases much further in the distal locations like the colon even though RANKL secretion is still active (S Kimura et al. 2014). These observations indicate the existence of a regulatory mechanism to ensure controlled M cell differentiation. Previous research has demonstrated the hindrance of RANKL signaling by the binding of soluble decoy receptor osteoprotegerin (OPG) in the bone. OPG exhibits the capacity to bind to RANK receptors in place of RANKL and negatively regulate osteoclast differentiation affecting the RANKL-OPG balance resulting in osteoporosis, periodontal disease, and rheumatoid arthritis (Walsh et al. 2014; Simonet W.S et al. 1997; Yasuda et al. 1998). OPG is also found in higher concentrations in distal Peyer's patches and is a biomarker for inflammatory bowel disease, Crohn's disease, and ulcerative colitis (Moschen et al. 2005; Franchimont et al. 2004).

Advances in transcriptomic profiling revealed the expression of OPG directly under the follicle associate epithelium in the subepithelial dome. qPCR analysis confirmed the upregulated OPG expression in the follicle-associated epithelium when compared to the villous epithelium. Immunostaining and transmission electron microscopy confirmed that OPG-expressing cells exhibited physical characteristics of an M cell with the irregular microvilli and brush border. Mice administered with GST-RANKL demonstrated that OPG expression transpired concomitantly with that of Spi-B indicating that OPG expression occurs at an early stage of the M cell differentiation process. Since RANKL secreted by MCi cells could bind to OPG and impede the RANKL-mediated NF-$\varkappa$B pathway for M cell differentiation, this would mean that OPG tightly regulates the number of M cells. This hypothesis was confirmed when OPG null mice exhibited an increased M cell population in the Peyer's patch. Further evidence of transmission electron microscope and whole-mount staining confirmed the prominent increase in M cell numbers. OPG deficient mice also demonstrated an increase in expression of M cell-associated genes such as Spi-B, Sox8, CCL9, and GP2. Cecal patches in the colon exhibited a higher number of Gp2+ M cells which was in stark contrast to wildtypes with fewer Gp2 cells in cecal patches. These Gp2+ M cells in the colon Peyer's patches were functional and were able to transcytose nanoparticles 13-fold higher than their wildtype

counterparts. The higher number of Gp2+ M cells in the proximal Peyer's patch did not exhibit increased antigen uptake indicating that OPG played a suppressive role in M cell maturation in cecal patches. In addition, to an increase in RANKL signaling in the epithelia, an increase in mucosal immune response was observed as well. Under inflammatory conditions, the production of specific high-affinity SIgA was significantly increased. Interestingly, an increase in M cell numbers led to increased susceptibility to M cell targeting pathogenic elements such as S.typhimurium in OPG null mice (Kimura et al. 2020).

In the bone, RANKL/OPG balance is critical for the maintenance and regulation of osteoclast differentiation; OPG plays a similar role in M cell self-regulation of their differentiation. A controlled M cell population is critical for the neutralization of invading mucosal antigens and for the maintenance of homeostasis of the mucosal immune systems. Evidence proving OPG's role in cecal patches and that OPG is a benchmark marker for Crohn's disease and ulcerative colitis, it is reasonable to conclude that RANKL/OPG imbalance could play a pivotal role in driving these diseases.

2.4.3.5 Other factors involved in differentiation and function of M cells.

Several transcriptional profiling datasets at various stages of M cell differentiation identified various genes that serve as specific markers at different stages of M cell maturation. An important factor that indicates an early marker of an immature M cell is Marcksl1, expression of Marcksl1 reaches a peak around day 1 along with Spi-B upon RANKL treatment. However, Marcksl1 expression is independent of Spi-B expression as Spi-B knockout mice still exhibited intact MarcksL1 expression (Takashi Kanaya et al. 2012). Anxa5 is another marker indicating early-stage M cells, they are independent of Spi-B expression and are expressed in immature M cells leaving the intestinal crypt for the follicle-associated epithelium (Verbrugghe et al. 2006). Since Marcksl1 and Anxa5 are intact in the absence of Spi-B, this means that there is an earlier commitment towards M-cell lineage in the RANKL-RANK pathway before the activation of Spi-B (Kanaya et al. 2012).

Allograft inflammatory factor 1 (Aif1) is another factor identified by RNA-seq analysis. Aif1 was specifically expressed by M cells and further evidence from whole-mount staining and qPCR analysis corroborated the RNA-seq findings. Previous research identified Aif1 also known as calcium-binding adapter molecule 1 (Iba1), as

a cytoplasmic protein with calcium-binding domains in the macrophages of rats with heart allografts undergoing chronic rejection (Utans et al. 1995). Aif1 is prominently expressed in monocytic lineages which include microglia and macrophages and is critical for their phagocytic function. To further characterize Aif1's role in M cells, Aif1 deficient mice were used; Aif1 deficient mice showed no changes in M cell morphology. Scanning electron microscope showed M cells in the follicle-associated epithelium with the appropriate invagination and an intact brush-border with a thin glycocalyx layer on top; Aif1 deficiency did not affect the lymphocyte population in the subepithelial dome either indicating that Aif1 did not play a role in the development and differentiation of M cells. However, Aif1 deficiency affected the functional transcytosis capacity of M cells, Aif1 deficient mice exhibited a significant drop in uptake of latex nanobeads and Lactobacillus *reuteri* when compared to the wildtype. Interestingly, reduction in transcytosis capacity was only restricted to a few antigens, Aif1 null mice were able to transcytose S.*typhimurium* in comparable levels to the wildtype indicating that Aif1 deficiency played a role in the specific receptor development on M cells. Yersinia *eneterocolitica* is a microorganism that is found in the gastrointestinal tract, they bind to the receptor β1 integrin on the apical surfaces of M cells with Invasin which is one of the outer proteins of the Y.*enterocolitica* (Isberg and Leong 1990; Grutzkau et al. 1990; M. Ann Clark et al. 1998). Aif1 deficient mice demonstrated impaired uptake of Y.*enterocolitica* indicating that Aif1 played a role in the development of active β1 integrin receptors on M cells. Whole-mount staining confirmed the hypothesis as Aif1 deficient mice exhibited significantly less active β1 integrin receptors than Aif1 wildtype controls. In conclusion, Aif1 is not required for the differentiation and maturation of Gp2+ M cells however, they play a significant role in the M cell functionally as they are required for proper development of β1 integrin receptor(Kishikawa et al. 2017).

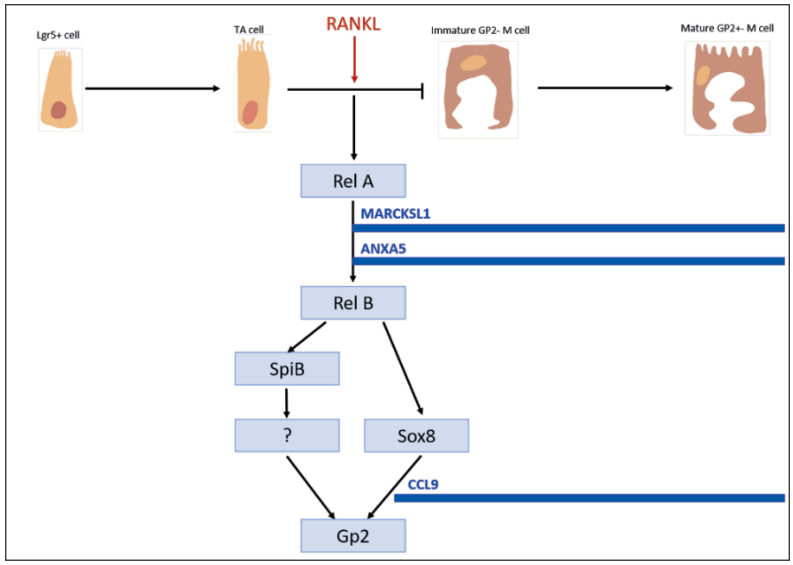

Figure 6. Major transcription factors involved in M cell differentiation; TRAF6 activates canonical (P50/RelA) and non-canonical NF-kB (P52/RelB) signaling. Non-canonical NF-kB (P52/RelB)Bl is sufficient for activation of SpiB and Sox8. Blue lines indicate first incidence of protein expression; activation of canonical (P50/RelA) is sufficient for MARCKSL1 and ANXA5 and activation of sox8 is sufficient for CCL9 expression. Gp2- M cells are immature M cells incapable of transcytosis of FIMH+ expressing bacteria whereas Gp2+ M cells are fully functional M cells

3 AIMS OF THE STUDY

M cell plays an important role in immune surveillance and transcytosis of antigens and pathogens in the gastrointestinal tract. Impaired maturation of M cells has led to poor mucosal responses and increased susceptibility to infections by Salmonella *Typhimurium* and other pathogenic bacteria. Previous research identified unique genes critical for M cell differentiation and development however, these were not sufficient for induction of M cell differentiation and the complete differentiation pathway remains to be elucidated. Polycomb repressive complex 2 (PRC2) has been previously identified to be critical for regulating proliferation and differentiation in the intestinal epithelium. Considering how important PRC2's role is we set out to elucidate its role in M cell differentiation

The specific aims of this thesis were:

1) Identify if Polycomb repressive complex 2 activity played a role in M cell differentiation and to identify PRC2 regulated genes in M cell differentiation.

2) Investigate if the identified PRC2-regulated Esrrg played a role in M cell differentiation and development

3) Explore if the identified PRC2-regulated Atoh8 regulated M cell population in Peyer's patches

4) Explore if PRC2-regulated Maf played role in M cell differentiation.

4 MATERIALS AND METHODS

4.1 Animals (I-III)

4.1.1 Sox8 mice (I)

Wildtype C57BL/6JRj mice were procured from Janvier Labs (Le Genest-Saint-Isle, France) and were maintained with constant breeding in a germ-free facility. The Bac-Cre-ERT2;Sox9f/f;Sox8-/-;Rosa26Eyfp mice were a gift from Raphael Jimenez (University of Granada, Granada, Spain). To isolate the Sox8-/- allele Bac-Cre-ERT2;Sox9f/f;Sox8-/-;Rosa26Eyfp mice were backcrossed with C57BL/6JRj. Sox8 heterozygous were bred to obtain Sox8-/- and littermate controls: Sox8+/- and Sox8+/+. Sox8 heterozygous were bred to obtain Sox8 knockout and littermate controls: Sox8+/- and Sox8+/+. F1 to F4 progeny mice were used for validating our computational analysis with gene or protein expressions. Wildtype mice, heterozygous, and deleted alleles were genotyped by PCR with the following primers: F1, 5'-GTCCTGCGTGGCAACCTTGG-3'; R1, 5'-GCCCACACCATGAAGGCATTC-3'; and F3, 5'-TAAAAATGCGCTCAGGTCAA-3'. Mice were caged and bred by following conventional protocols for the maintenance of these mice at the pathogen-free animal facility of the faculty of Medicine and Health Technology. All animal experiments (I-III) and permits were approved by the Finnish National Animal Experiment Board (permit ESAVI/5824/2018).

4.1.2 Atoh8 mice (II)

B6.Cg-Tg(Vil1-cre)1000 Gum/J mice (Cat No: 021504) were procured from Jackson Laboratories. Atoh8 *lox/lox* mice, in which exon 1 is flanked by two loxP sites, and was generously gifted by Rosa Gasa (Rawnsley et al. 2013). To breed intestinal-specific deletion of Atoh8, Atoh8 *lox/lox* were bred with Vil1-cre mice. The F1

generation was backcrossed with Atoh8 *lox/lox* to breed mice homozygous for floxed Atoh8 allele carrying the Vil1-cre transgene. Littermates with Vil1-cre allele were used as control. Atoh *lox/lox* and Atoh8 *lox/VilCre* were confirmed by PCR with genotyping primers 5' ATTGGAGGAAGGCTCGGTGAA 3' and 5' TTGGCATTCGTCGTGCTGTC 3'. Mice were maintained on standard light-dark conditions, with food and water ad libitum at the pathogen-free animal facility of the Faculty of Medicine and Health Technology.

4.1.3 Maf mice (III)

All animal experiments were approved by the Finnish National Animal Experiment Board (Permit: ESAVI/5824/2018). B6.129-Maftm1Gsb/J heterozygous mice were procured from Jackson laboratories (Cat number: 004158 | MaflacZ). B6.129-Maftm1Gsb/J heterozygous mice can generate Maf LacZ mice. Maf lacZ mice were bred with conventional protocols in standard light-dark conditions at the pathogen-free animal facility. A regular schedule of food and water ad libitum was followed for the mice. To generate Maf wildtype line, heterozygous line, and homozygous line, B6.129-Maftm1Gsb/J heterozygous were mated with B6.129-Maftm1Gsb/J heterozygous, F1 progeny was backcrossed with heterozygous mice. Littermates with Maf wildtype were utilized as control. Wildtype mice, heterozygous, and deleted alleles were genotyped by PCR. Maf deficient mice with C57BL/6 background are lethal embryonically or perinatally Maf KO pups are often born stillborn or only survive up to 4 hours after birth (Kawauchi et al. 1999, J. I. Kim et al. 1999). For our experiments to characterize Maf in M cell differentiation, we isolated littermates towards the end of the mother's pregnancy to isolate crypt organoids from the intestines.

4.2 In vitro Studies (I-III)

4.2.1 Intestinal organoid culture (I-III)

Intestinal crypt isolation and in vitro culture techniques were followed as previously established by the protocols of Sato and Clevers and de Lau et al (Toshiro Sato and Clevers 2013; W. de Lau et al. 2012) . Duodenal samples were isolated from the mice; the piece of the duodenum was cut longitudinally, and the villous epithelium was

scraped off. The cleaned tissue was washed with phosphate-buffered saline (PBS) buffer and then cut into 2-mm pieces and using mechanical forces of a pipette, they were washed in an up and down manner 5 times in 15 mL PBS with a 10-mL pipette, this step was repeated 3 times with fresh PBS. This step helps to remove the more villous epithelium. The intestinal pieces were left rocking in 10 mmol/L EDTA in PBS for 20 mins at room temperature. The pieces were vigorously shaken in cold PBS after and strained through a 70-μm cell strainer (cat no: 22363548; Fisher Scientific, Waltham, MA). This tissue atop the strainer was enriched to crypt fraction through centrifugation at 150 × g for 5 minutes. Matrigel was used to embed the crypt fraction (cat no: 356255, lot 9119006; Corning, Corning, NY), and 30 uL of the crypt-matrigel mix was plated on a 24-well plate. The organoids were cultured in an optimal medium comprising of advanced Dulbecco's modified Eagle medium/F12 (cat no: 12634010; Thermo Fisher Scientific, Waltham, MA) that contained HEPES (10 mmol/L, cat no: 15630-080; Sigma-Aldrich, St. Louis, MO), Glutamax (2 mmol/L, cat no: 35050-038; Thermo Fisher Scientific), penicillin-streptomycin (100 U/mL, cat no: 11659990; Sigma-Aldrich), B-27 supplement minus vitamin A (cat no: 17504-044; Thermo Fisher Scientific), N-2 supplement (cat no: 17502-001; Thermo Fisher Scientific), N-acetylcysteine (1 mmol/L, cat no: A9165, lot SLCB3719; Sigma-Aldrich), recombinant murine epidermal growth factor (50 ng/mL, cat no: PMG8043, lot 2135273; Gibco, Waltham, MA), recombinant murine Noggin (100 ng/mL, cat no: 250-38; PeproTech), and recombinant mouse R-spondin 1 (1 μg/mL, cat no: 120-38; R&D Systems, Minneapolis, MN). Media were changed every 2 days. For M-cell differentiation, recombinant mouse RankL (100 ng/mL, cat no: 315-11; PeproTech, Cranbury, NJ) was added to the media and grown for 4 days.

4.2.2 HEK cells (I-III)

To generate viral particles for the knockdown of genes, we used HEK cells to aid in the propagation of lentiviral particles. Human embryonic kidney 293 cells are often referred to as HEK293 cells. HEK293 cells were received as a gift from Pekka Katajisto's lab, HEK293 were grown in Opti-MEM media and passaged every time they reached 80% confluency with regular media change. For viral generation, HEK cells were plated at 70% confluency and transduced with the suitable vector and lipofectamine 2000 after 24 hours. The media was collected 48 hours later and 2 ml of Lenti-X concentrator (cat number: 631231; Clontech, Mountain View, CA). This

mixture was kept overnight in the fridge and spun at 700g for 1 hour. The viral pellet was collected and mixed with EGF, Noggin, Chir, Y-27632; epidermal growth factor, Noggin, Chir-99021 (cat no: S1263; Selleckchem) and Y-27632 (cat no: 72304; Selleckchem) and stored in -80 freezer.

4.3 Drug treatments and inhibitions (I-II)

The Polycomb repressive complex 2 was inhibited in organoids by the treatment of 5 μm of Ezh2 inhibitor (cat no: CAS 1418308-27-6 C; Calbiochem Chemicals, San Diego, CA). NF-κB was activated with human LTα1β2 (1 μg/mL, cat no: 8884-LY; R&D Systems) that was added to organoid cultures. Restriction of IκB kinase-β (RelA/P50) activity was achieved by adding SC-514 (125 μmol/L, cat no: 354812-17-2; Selleckchem, Houston, TX) to organoids for 3 days (Akiyama et al. 2008). Esrrg receptors were blocked with a non-specific antagonist (Z)-4-Hydroxytamoxifen (1 μm, cat no: 68392-35-8; Sigma-Aldrich) by adding it to organoids. Esrrg receptors were activated in organoids with a non-specific ligand- GSK 4716 (10 μm, cat no: 101574-65-6; Sigma-Aldrich). To investigate Atoh8 expression via BMP2/BMP6, organoids were grown in EGF, R-spondin, and BMP2 (100 ng/mL, R&D Biosystems) and EGF, R-spondin, and BMP6 (100 ng/mL, R&D Biosystems) according to the protocol of Calpe et al (Calpe et al. 2015). Noggin was not part of the culturing media of the BMP2/BMP6 experiments as Noggin is a known antagonist against BMP signaling.

4.4 Modification of gene expression

4.4.1 CRISPR-Cas9 gene editing of intestinal organoids (I-III)

Rank, Spi-B, and Esrrg knockouts were carried out by guide RNAs that were designed with the CRISPR design tool (http://crispr.mit.edu) (Shalem et al. 2014). The Rank, Spi-B, and Esrrg guides were cloned into lentiCRISPR v2 vector (52961; Addgene, Watertown, MA). Multiple guides were designed to account for off-target activities. The cloned vector was transduced into HEK293FT cells (cat no: R7007; ThermoFisher) and the supernatant was collected after 48 hours and concentrated with Lenti-X concentrator (cat number: 631231; Clontech, Mountain

View, CA). Intestinal organoids were cultured in Epidermal Growth Factor, Noggin, Chir, Y-27632; cat no: S1263; Selleckchem) and Rock inhibitor Y-27632 (cat no: 72304; Selleckchem) 2 days before transduction. Organoids were dissociated into single cells mechanically along with TrypLE Express (Thermo Fisher Scientific) supplemented with 1000 U/mL DNase I for 5 minutes at 32°C. The single-cell suspension was washed with Advanced Dulbecco's modified Eagle medium(ADMEM) and resuspended in transduction medium (Egf, Noggin, R-spondin media supplemented with 1 mmol/L nicotinamide, Y-27632, Chir99021, 8 μg/mL polybrene, cat no: 28728-55-4; Sigma-Aldrich) and mixed with the concentrated virus. The cell–virus mixture was centrifuged for 60 minutes at 600 × g at 32°C followed by a 2- to 4-hour incubation at 37°C, after which they were collected and plated on 60% Matrigel overlaid with transduction medium without polybrene. Transduced organoids were selected after 2 rounds of 2 μg/mL of puromycin selection (cat no: P8833, 10 mg; Sigma-Aldrich) on day 2 and day 4, after which surviving clones were expanded in maintenance ENR medium. Knockout of the genes was validated by Western blot to check for the expression of deleted gene.

Table 1. Sequence of Guide RNAs used in studies I, II and III.

Target gene	Primers 5' -> 3'/ guide RNA seq	Origin/Source	Guide Efficiency	Study
Esrrg 1 fwd	CACCGTCTGTCAAGACGGACCCCTG	Own/Sigma	90%	I
Esrrg 1 rev	AACCAGGGGTCCGTCTTGACAGAC	Own/Sigma	90%	I
Esrrg 2 fwd	CACCGTGGCGTCGGAAGACCCACCA	Own/Sigma	80%	I
Esrrg 2 rev	AAACCAGGGGTCCGTCTTGACAGAC	Own/Sigma	80%	I
Spi-B fwd	CACCGAGACTCCTTCTGGGTACTGG	Own/Sigma	86%	I
Spi-B rev	AAACCCAGTACCCAGAAGGAGTCTC	Own/Sigma	86%	I
Rank fwd	CACCGAAAGCTAGAAGCACACCAG	Own/Sigma	90%	I,II,III
Rank rev	AAACCTGGTGTGCTTCTAGCTTTC	Own/Sigma	90%	I,II,III

4.4.2 Lentivirus infection for overexpression (I)

RelA, p50, RelB, and p52 plasmids were a generous gift from Hiroshi Ohno's laboratory (RCIMS, Kangawa, Japan), and Esrrg, Spi-B, and Sox8 complementary DNA were designed and cloned by Twist Bioscience (San Francisco, CA). These cDNAs were cloned into CSII-CMV-MCS-IRES2-Bsd overexpression vectors,

which were procured from the RIKEN Bioresource Center (Ibaraki, Japan) and Hiroyuki Miyoshi. The protocol for Crispr-Cas9 lentiviral generation and transduction into HEK293FT were observed and overexpressed cells were embedded with Matrigel and incubated for 2–3 days in Epidermal Growth Factor, Noggin, Y-27632, R-spondin, Chir-99021.

4.5 Gene expression analysis

4.5.1 RNA extraction (I-III)

Total RNA extractions from intestinal organoids, follicle associated epithelium, and villous epithelium were carried out by using either TRIZOL LS RNA extraction protocol or GeneJET RNA Purification Kit (Thermo Fisher Scientific), or PureLink™ RNA Mini Kit with On-Column PureLink® DNase Treatment Protocol (Ambion® by Life Technologies and Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The concentration and quality of total RNA extractions were analyzed with a nanodrop spectrophotometer (Thermo Fischer Scientific).

4.5.2 Quantitative reverse-transcription PCR (I-III)

Around 0.25-1 µg of total RNA extracted was utilized for reverse transcription synthesis of complementary DNA (cDNA) with iScpript (Bio-Rad). RT-qPCR was performed by using synthesized cDNA which was mixed with SsoFast EvaGreen® Supermix (Bio-Rad) according to the manufacturer`s instructions. BioRad CFX96TM Real-Time System (Bio-Rad) was run with the following program; initial denaturation step for 30 seconds at 96°C, 39 cycles of denaturation at 96°C for 2 seconds, annealing and extension at 60°C for 5 seconds, and the final plate read (melt curve 60°C → 95°C). RT-qPCR measurement was carried out in triplicate, and the relative 2-ΔΔCT method was used for quantification (Livak et al., 2001). RT-qPCR primers and their sequences are listed in Table 2.

Table 2. RT-qPCR primer sequences.

Target	Primers 5' -> 3'	Study
Gapdh_fwd	TGTGTCCGTCGTGGATCTG	I, II, III
Gapdh_rev	CCTGCTTCACCACCTTCTTGA	I, II, III
Suz 12_fwd	GATGAGAAAGATCCAGAATGGC	I
Suz12_rev	ATAATTTTCTACAAACAGCATACAGGC	I
Ezh2_fwd	GTCTGATGTGGCAGGCTGG	I
Ezh2_rev	GCCCTTTCGGGTTGCATC	I
Spi-B_fwd	GGAGTCTTCTACGACCTGGACAG	I, II, III
Spi-B_rev	GCAGGATCGAAGGCTTCATAGG	I, II, III
Sox8_fwd	GGACCAGTACCCGCATCTCC	I, II, III
Sox8_rev	TTCTTGTGCTGCACACGGAGC	I, II, III
GP2_fwd	GTGTACAAGTTACAGGGTACCCC	I, II, III
GP2_rev	GACAAGTAATCTCACAATTCTTGG	I, II, III
CCL9_fwd	GCCCAGATCACACATGCAAC	I, II
CCL9_rev	AGGACAGGCAGCAATCTGA	I, II
MarcksL1_fwd	CCCGTGAACGGAACAGATGA	I, II, III
MarcksL1_rev	CCCACCCTCCTTCCGATTTC	I, II, III
Esrrg_fwd	GTGTCTCAAAGTGGGCATGC	I
Esrrg_rev	GCTGTTCTCAGCATCTATTCTGC	I
Aif1_fwd	GGATTTGCAGGGAGGAAAA	I, II
Aif1_rev	TGGGATCATCGAGGAATTG	I, II
CCL20_fwd	TGTACGAGAGGCAACAGTCG	I, II
CCL20_rev	TCTGCTCTTCCTTGCTTTGG	I, II
TNFAIP2_fwd	GTGCAGAACCTCTACCCCAATG	I, II, III
TNFAIP2_rev	TGGAGAATGTCGATGGCCA	I, II, III
18s rRNA_fwd	GTAACCCGTTGAACCCCATT	I, II
18s rRNA_rev	CCATCCAATCGGTAGTAGCG	I, II
Atoh8_fwd	CGGGGGAAAGTTCCTACTCGTC	II
Atoh8_rev	CGGAAGAATCCGGGTGGTTATT	II
OPG_fwd	ACCCAGAAACTGGTCATCAGC	II
OPG_rev	CTGCAATACACACACTCATCACT	II
c-Maf_fwd	AGGAGGTGATCCGACTGAAGCA	III
c-Maf_rev	TCTCCTGCTTGAGGTGGTCTAC	III

4.5.3 Global Nuclear Run-On sequencing (I)

Intestinal organoids grown in maintenance condition Egf, Noggin and R-spondin and intestinal organoids grown in RANKL conditions to differentiate to M cell were used for global run-on sequencing (GRO-seq). GRO-seq measures all active transcriptional activity while RNA-seq measures steady-state bulk transcriptional activity. Nuclei extractions and GRO-seq reactions were carried out as previously described in materials and methods in the paper I and Kaikkonen et al (Kaikkonen et al. 2014; George et al. 2021).

4.5.4 Chromatin immunoprecipitation (I)

DNA-protein interactions were analyzed by ChIP assay where DNA fragments were immunoprecipitated from crosslinked cells by using antibodies against H3K27me3 (cat no: ab6002; Abcam, Cambridge, UK) or H3 antibody (cat no: ab1791; Abcam), as described in T I Lee et al (T. I. Lee et al. 2006). 2 biological replicates were collected for Maintenance conditions (Efg, noggin, R-spondin) and M cell differentiated (Egf, noggin, R-spondin, and RANKL). Collected ChIP samples were sent to EMBL Germany for sequencing.

4.6 Protein expression analysis

4.6.1 Protein extraction and western blotting (I-III)

Cultured organoids were collected and lysed and proteins were extracted from the cell lines with M-PER reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The total protein concentrations were calculated using DC Protein Assay (Bio-rad). 15-20 μg of was measured from the total protein was put in the pre-casted 10% Mini-PROTEAN® TGX Stain-FreeTM Gels (Bio-Rad). once the gel was run, we used the Trans-Blot® TurboTM Pack (Bio-Rad) with nitrocellulose membrane to transfer proteins from the gel to the nitrocellulose membrane with Trans-Blot® TurboTM Transfer system according to manufacturer's instructions (Bio-Rad). The proteins in the gel was transferred to Amersham Protran supported nitrocellulose membrane (GE Healthcare, Helsinki, Finland) with established protocols and incubated with appropriate primary and

secondary antibodies (See Table 3 for list of antibodies). Pre-stained protein ladder PageRuler PLus (#26619, Thermo Fischer Scientific) was used as a protein size marker in WB. Chemiluminescence reaction by Amersham ECL reagent was detected and quantitated with ChemiDocTM XRS+ using Image LabTM Software (Bio-Rad).

4.6.2 Immunohistochemistry and fluorescence (I-III)

Peyer's patches from the ileum section of the intestine were isolated and washed with cold 1x Phosphate Buffer solution, fixated with 4% parformaldehyde and embedded into paraffin blocks. 5 µm sections were cut from the blocks and rehydrated followed by PBS washes. the sections were incubated with 1% PBS/bovine serum albumin supplemented with 5% normal donkey serum for blocking, antigen retrieval was processed with citrate buffer, pH 6.0 (121°C for 5 min), and stained overnight at 4°C with antibodies (See list of antibodies) This was followed by secondary antibody incubation for an hour. The sections were washed with 1x PBS and examined with a light microscope. For organoid whole-mount immunostaining, crypt organoids were plated in an 8-well chamber and cultured in maintenance and RANKL treated conditions for 4 days, after which organoids were fixed with 4% paraformaldehyde, followed by 0.1% Triton (Gibco) X-100 for permeabilization. The organoids were stained with the necessary primary antibodies which were followed by overnight incubation with secondary antibodies (See table 4). Organoids were washed 3 times with PBS and mounted with ProLong Diamond with 4',6-diamidino-2-phenylindole mounting solution (cat no: 15810083; ThermoFisher). The organoids were analyzed with Nikon (Melville, NY) A1R+ Laser Scanning Confocal Microscope.

4.6.3 Whole-mount Immunofluorescence of Peyer's patches (II)

Peyer's patches from the ileum section of the intestine were cut and transferred to a 10 cm dish containing 30 mL cold 1x phosphate buffer solution. Excess intestinal tissues around the follicle-associated epithelium were removed under a stereomicroscope using blades and forceps. Peyer's Patches were washed thoroughly with the aid of a 1 mL syringe with a 26-gauge needle. Steps were taken to ensure the removal of the mucous layer on the follicle-associated epithelium and flushed out with PBS to prevent background noise detection. Peyer Patches were transferred

to the 1.5 mL tube containing 1 mL 1x Phosphate buffer solution, washed by vortexing after which the supernatant was discarded. After 3 washes, for blocking and permeabilization 300–1000 μL Cytofix/Cytoperm buffer (BD Biosciences, Franklin Lakes, NJ, USA) was added for 25 min at room temperature. Perm/wash buffer (BD Biosciences, Franklin Lakes, NJ, USA) from the kit was used to wash the Peyer's patches after which they were stained with PE-conjugated anti-GP2 antibody (MBL; 1:10 in Perm/Wash buffer) overnight at 4 ◦C. Following the initial staining with primary antibody, PPs were washed 3 times with wash/perm buffer and underwent 30 mins for staining at RT in secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA). The Peyer's patches were washed 3 times with wash/perm buffer and mounted using ProLong Diamond with Dapi mounting solution (Molecular Probes P36962). Slides were examined with a laser scanning confocal microscope (Zeiss LSM 800 LSCM) (Kunimura et al. 2019).

Table 3. List of antibodies used in western blot experiments and stainings.

Target	Dilution	Manufacturer	Cat. number	Purpose
RANK	1:2000	Abcam	ab13918	WB
Spi-B	1:1000	CST	14223	IF
Sox8	1:1000	Abcam	ab 221053	IF
Gp2	1:1000	MBL	D278-3	IF
Esrrg	1:1000	Thermo Fisher	A32731	IHC
Spi-B	1:1500	CST	D4V9S	WB
Gp2	1:200	MBL	D278-5	Whole mount IF
Maf	1:250	CST	PA5-106462	IF
β-actin	1:100 000	Santa-Cruz	sc-1615	WB
H3	1:60 000	Abcam	ab4729	WB
Esrrg	1:1000	Abcam	ab49129	WB
Atoh8	1:1000	Thermo Fisher	PA5-20710	IHC
Gapdh	1:1000	Abcam	ab8245	WB

4.6.4 Flow cytometry analysis (II)

Peyer's patches were isolated from *VilCre* mice and Atoh8 *lox/VilCre* mice were isolated from the ileal section of the intestine. The tissues were washed in 1x PBS buffer 3 times and incubated in spleen dissociation medium 5ml for 15–20 min at 37 ◦C while vigorously shaking at 250 rpm. Single-cell suspension was achieved by

placing the isolated Peyer's patches on a sterile (autoclaved) 70 μm nylon mesh cell strainer and using the base of a plunger from a 1 cc syringe ground into a fine mesh. The suspension was incubated for 5 mins in 1 mm EDTA with a rocker after which the suspension was passed through a 70 μm nylon mesh cell strainer to generate a cleaner population (S. Kimura et al. 2019). After the isolation step, the single-cell suspensions were prepared with FVS510 viability stain (#564406; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and antibodies for identifying lymphocyte subsets from the subepithelial dome. To prevent spectral overlap of emitted fluorescence, cells were divided into replicates, and two separate antibody panels were used in the staining procedure. Flow cytometry was analyzed with FACSAria™ Fusion (Becton, Dickinson, and Company), and data were quantified with FlowJo (v. 10.6.1, Tree Star, OR, USA).

Table 4. List of antibodies used in flow cytometry.

Marker/ Protein	Conjugate/ fluorochrome	Clone	Source / Isotype	Vendor	Product code	Concentration
Viability dye	FVS510			Becton Dickinson	564406	0.2μg/μl
CD3e	FITC	145-2C11	Armenian hamster / IgG	eBioscience	11-0031-85	0.5μg/μl
CD8a	PerCP-Cy5.5	53-6.7	Rat / IgG2a, kappa	eBioscience	45-0081-82	0.2μg/μl
CD4	APC-eFluor780	GK1.5	Rat IgG2b	eBioscience	47-0041-80	0.2μg/μl
PD1	Super Bright 780	J43	Armenian hamster / IgG	eBioscience	78-9985-80	0.2μg/μl
Fas (CD95)	PE-Cy7	Jo2	Armenian Hamster IgG2, λ2	Becton Dickinson	557653	0.2μg/μl
B220	eFluor450	RA3-6B2	Rat / IgG2a, kappa	eBioscience	48-0452-80	0.2μg/μl
CXCR5	SuperBright 600	SPRCL5	Rat / IgG2a, kappa	eBioscience	63-7185-82	0.2μg/μl
GL7	PE	GL-7 (GL7)	Rat / IgM	eBioscience	12-5902-82	0.2μg/μl
IgA	APC	mA-6E1	Rat / IgG1, kappa	eBioscience	17-4204-80	0.2μg/μl

RANKL	PE	MIH24	Mouse / IgG2b, kappa	eBioscience	12-6619-82	0.2µg/µl

4.6.5 β-galactosidase staining of Peyer's patches and organoids (III)

Peyer's patches from the ileum section of the intestine were cut and transferred to a 10 cm dish with 30ml of cold 1x PBS. Excess fat was removed from tissues and tissues were embedded into paraffin blocks. Organoids grown in RANKL and ENR were washed with 1x phosphate buffer solutions and made into paraffin blocks. Sections were cut at 10um from the block and mounted on a slide. X-gal (5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside, Goldbio) was dissolved in dimethylformamide at 50 mg/ml and paraffin blocks were fixed for 10 minutes in 4% paraformaldehyde. The slides were washed 3 times with 1x PBS for 5 minutes wash and a final wash with distilled water. Slides were dried and incubated in X-gal working solution for 24 hours at 37 C in a moist chamber. The sections were washed in 1x PBS solution 2 times for 5 minutes each which was followed by a distilled water rinse, the sections were counter-stained with nuclear fast red for 3-5 minutes after which they were dried and rinsed in distilled water for 2 minutes. For the final steps, sections were dehydrated serially for 3 minutes each in 70%, 95%, and twice with 100% ethanol and thrice with xylene. The sections were mounted with mounting media and covered with a coverslip and imaged with a digital slide scanner Hamamatsu Nanozoomer.

4.7 Functional assay to measure transcytosis by M cells (II)

6 *VilCre* mice and Atoh8 *lox*/*VilCre* mice were fasted for 3 hours and 1000ul of 200 nm diameter polystyrene nanoparticles (09834-10, Fluoresbrite YG; Polysciences Warrington, PA, USA) were administered via oral gavage. After 4 hours, 2 Peyer's patches were collected from the ileum and jejunum section of the intestine and fixed for 2 hours with a solution of 3.7% formalin/PBS. Fixed tissues were kept for 16 hours overnight with 30% sucrose in phosphate buffer solution and finally embedded in the OCT compound (Sakura Fintech, Torrance, CA, USA). 10 sequential 15 µm sections were cut from the block and examined by fluorescence microscopy. The uptake of fluorescent beads into the Peyer's patches was counted manually (Shunsuke Kimura, Kobayashi, et al. 2019; Kunimura et al. 2019) .

4.8 Gene expression and data analysis (I)

Three different data sets based on organoids grown in stem cell condition (Wnt, Epidermal growth factor, noggin, R-spondin, Chir-99021,), enterocyte condition (IWP2-Wnt inhibitor), and M cell condition (Epidermal growth factor, noggin, R-spondin, and RANKL) were utilized to study PRC2 regulated differential expression in the 3 different conditions. Analyses were performed as described previously (Core, Waterfall, and Lis 2008; Kaikkonen et al. 2014; George et al. 2021). The data have been deposited in the NCBI Gene Expression Omnibus database (GSE157629). Gro-seq and ChIP-seq data for the individual genes are shown in Supplementary Tables 1 and 2, respectively of paper (I).

4.9 Statistical analysis (I-III)

For studies I, II, and III results of qPCR and protein staining counts were benchmarked and analyzed by utilizing the unpaired two-tailed Student's t-test. Statistical significance was standardized at $p < 0.05$ throughout all experiments. All statistical analyses were measured by using GraphPad Prism 6 software. No statistical methods were used to determine the sample size. These experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment. Statistical analyses of flow cytometry were performed with Prism v. 5.02 (GraphPad Software, San Diego, CA), and calculated using a two-tailed t-test. p values of < 0.05 were considered significant.

4.10 Ethical considerations (I-III)

For the study of Esrrg in Sox8 mice, and Atoh8 characterization with Atoh8 *lox/lox* and *VilCre*, and study of Maf with Maf lacZ animal permits for these animal experiments were approved by the Finnish National Animal Experiment Board (permit ESAVI/5824/2018). Conventional conditions were observed for mice maintenance at the pathogen-free animal facility of the faculty of Medicine and Health Technology.

5 RESULTS

5.1 Polycomb Repressive Complex 2 is required for M cell development and PRC2 regulated Esrrg is needed for M cell differentiation

5.1.1 Members of the PRC2 are present and active in M cell differentiation and do not restrict M cell differentiation.

PRC2 complex plays a major role in maintaining homeostasis between stemness and differentiation in various physiological systems. Several researchers including us validated and identified the presence of PRC2 in intestinal organoids that were grown in the conditions that mimicked stemness and enterocyte differentiation. Before investigating the role of PRC2 in M cell differentiation, we explored to see if PRC2 played a restrictive role like it did in other differentiated cells (Chiacchiera et al. 2016; Oittinen et al. 2017). qPCR analysis observed the expression of Suz12 and Enhancer of Zeste homolog 2 (EZH2) at comparable levels in stem cell conditions and M cell differentiated conditions whereas Suz12 and Ezh2 exhibited a significant reduction in enterocyte condition. This suggests that PRC2 does not restrict M cell differentiation in M cells just as previously observed in stem cell state by Oittinen et al (Oittinen et al. 2017, Benoit et al. 2013) To confirm that PRC2 does not restrict the M cell differentiation, RANKL treated organoids were treated with EZH2 inhibitor. Immunoblot analysis of histone trimethylation of K27 (H3K27me3) exhibited reduced expressions with and without RANKL. Inhibition of EZH2 activity did not ignite M cell differentiation as it did enterocyte differentiation indicating that PRC2 does not restrict M cell differentiation. (See Fig. 1A-C Study I)

5.1.2 PRC2 is critical for regulation of genes during differentiation and development

PRC2 is known to regulate genes for various cell fates such as maintenance and stemness of intestinal stem cells. PRC2 was observed to restrict genes during intestinal differentiation into enterocytes, secretory cell and enteroendocrine cells. To identify PRC2 regulation unique to M cells, we performed a H3K27me3 chromatin immunoprecipitation (ChIP-seq) on intestinal organoids in stem cell conditions (Wnt, Epidermal Growth Factor, Noggin, R-spondin, Chir (WENRC) media), intestinal organoids differentiated with porcupine inhibitor (Epidermal Growth Factor, Noggin, R-spondin, Wnt inhibitor IWP2 (ENRI) media and intestinal organoids grow in in RANKL condition for M cell differentiation (Epidermal Growth Factor, Noggin, R-spondin, recombinant RANKL). In addition to ChIP-seq, we also performed a global run-on nuclear sequencing (Gro-seq) to the M cell differentiated organoids to analyze real time differential gene expression.

From both the sequencing analyses we observed that 38 (9.2%) genes were up regulated but silenced by PRC2 in M cells when compared with stem cell condition and 35 (10.3%) genes were upregulated but silenced by PRC2 in M cell conditions when compared with RANKL. M cell differentiation expressed thirty-two unique PRC2 target genes for M cell differentiation but not enterocyte differentiation however, fifty-two (11.5%) PRC2 target genes were silenced in RANKL conditions but expressed in differentiated conditions and forty-six (27.7%) were silenced in M cell state but active in stem cell conditions (Figure 7).

Gene ontology analysis of the genes revealed many DNA-binding transcription factors during M-cell differentiation. Many of them showed were revealed to be functional for functional capacity such as microvilli development, glycosaminogycan binding and collagen V binding. Several other transcription factors were found to be critical for M cells unique anatomical and morphological features

Final Chip-seq and Gro-seq analysis revealed 12 transcription factors that were expressed differentially and uniquely to M cells Hoxb5, Hoxb9, Sp9, Sp5, Nr4a1, and Atf3 were 6 genes that were silenced by PRC2 in M cells. Sox8, Atoh8, Esrrg, Smad6, Maf, and Zfp819 were genes repressed by PRC2 grown in both WENRC and ENRI conditions but expressed specifically in M cells.

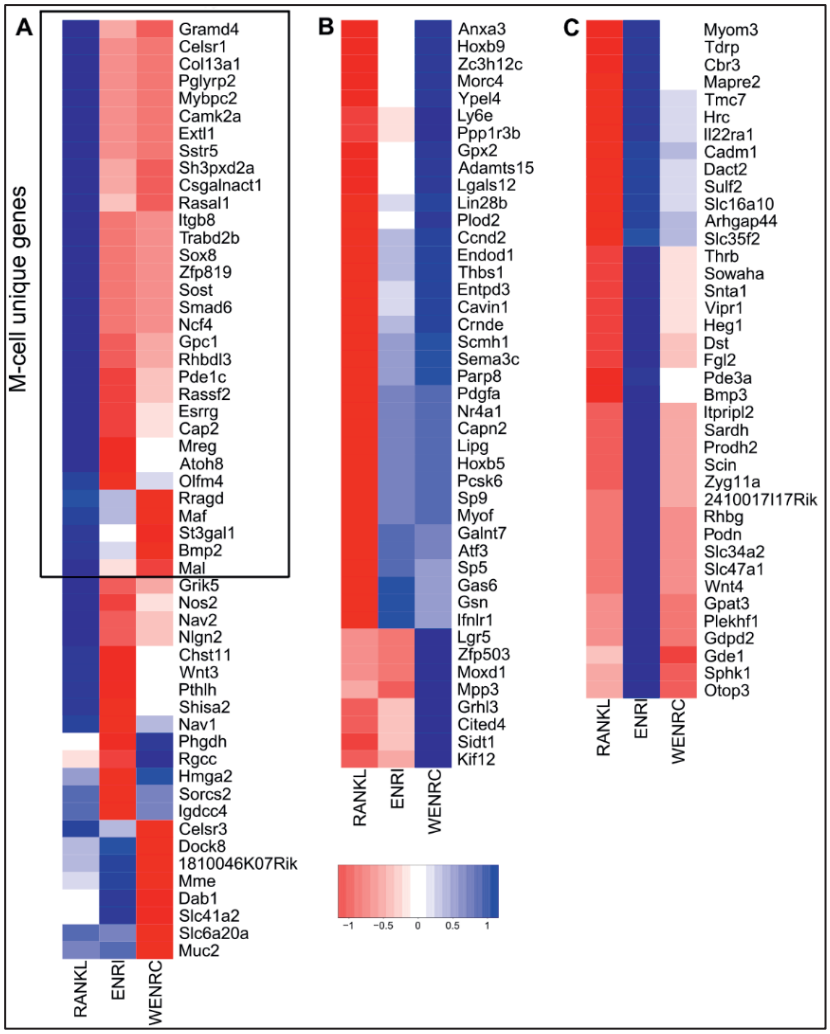

Figure 7. PRC2 regulated genes upregulated in M cell differentiation. Heatmap of differentially expressed (Gro-seq with log2 fc cut-off value at ±2 and *P* value < .0001) and H3K27me3 regulated (log2 fc ±2, *P* < 10-6) genes in M cell (RankL), enterocyte (ENRI), and stem cells (WENRC)-treated organoids showing centered log2 fold change. The genes identified were unique to M cells when compared to crypt stem cell condition and differentiated enterocyte condition. A) Genes upregulated specifically in M cells when compared to crypt stem cells and enterocytes B) Genes downregulated by K27 trimethylation in M cells and enterocytes compared with stem cells C) K27 trimethylation downregulated genes in M cells and stem cells when compared to enterocytes. Modified from Figure 2, George et al. 2021 (Study I).

5.1.3 Expression of Esrrg is localized in M cells and is under the overview of RANK-RANKL signaling

Among the 6 identified transcription factors that were differentially expressed in the differentiation of M cells, Esrrg (log2 fold changes, –6.64 RankL vs ENRI and –4.76 in RankL vs WENRC comparisons) was the most highly expressed PRC2-regulated transcription factor from the list. H3K27me3 occupancy was absent on the promoter of Esrrg in M cell conditions and present in stem cell conditions and enterocyte differentiated state. To validate this result, this observation was explored in a wet lab to understand Esrrg's role in M cell differentiation; immunohistochemical analysis demonstrated Esrrg to be localized among the follicle-associated epithelium in the Peyer's patch of wild type mice. Follicle associated epithelium and villous epithelium were isolated from wildtype mice and RNA was extracted from it and analyzed by qPCR; Gp2 and Esrrg expression were exclusively expressed to the follicle associated epithelium and not the villous epithelium (See Fig 3 B Study I)

Intestinal crypts were isolated from the duodenal tract and cultured with epidermal growth factor, R-spondin and noggin, these organoids were treated with RANKL to induce M cell differentiation. Esrrg was found to be induced by RANKL treatment after 4 days along with GP2 indicating that Esrrg expression was RANKL-mediated. However, to understand if the Esrrg expression was specifically under the overview of RANK-RANKL ligand receptor specific signaling, we checked to see if knocking out RANK would impair Esrrg expression. Employing CRISPR Cas9 gene editing we knocked out RANK receptors in intestinal organoids; these organoids were treated with RANKL for 3 days to observe Esrrg expression. Esrrg expression along with Gp2 expression abrogated in the RANK KO organoids treated with RANKL indicating that the RANKL-RANK signaling were critical for the activation of Esrrg expression. (See Fig 3 B-E Study I)

5.1.4 Esrrg expression is dependent on the activation of noncanonical NF-kB signaling

RANKL-RANK pathways are critical for various inflammatory responses via the Nf-ϰB signaling pathway. RANKL activation is required for the activation of Lymphotoxin-β receptor (LTβR) which is responsible for inducing the classical NK-ϰB (RelA/P50) and non-canonical NK- ϰB heterodimers (Z. B. Yilmaz et al. 2003). Treatment of intestinal mouse organoids with the ligand of LTβR, lymphotoxin alpha1 Beta (LTα1β2) over 3 days activated Esrrg as well as Spi-B. Spi-B was previously exhibited to be activated by LTα1β2 without the presence of RANKL (Takashi Kanaya et al. 2018). Similar to Spi-B activation by LTα1β2 without RANKL, Esrrg can also be activated by just LTα1β2 without RANKL further indicating the significance of either canonical/classical or non-canonical Nf- ϰB pathway (See Fig 4. A Study I).

To identify if classical Nf-ϰB pathway was critical for Esrrg expression, mouse intestinal organoids were treated with RANKL and cultured with and without pharmacological inhibitor of classical pathway (P50/RelA)- SC-514, since it inhibits IϰB kinase-β (inhibitor of nuclear factor kappa B) hence effective at blocking the canonical pathway (Kishore et al. 2003). Our experiments demonstrated that the expression of Esrrg was significantly reduced after 3 days along with Spi-B indicating that RelA/p50 could be necessary for their activation (See Fig 4. B-C Study I). However, p50/RelA (canonical NF-ϰB) directly targets the transcription of RelB indicating the possibility that the inhibition of P50/RelA led to the inhibition of non-canonical signaling which might have impaired Esrrg expression (Bren et al. 2001).

Intestinal organoids were transduced to overexpress RelA/p50 and RelB/p52, both transduced organoids were able to induce Esrrg expression by themselves without RANKL treatment. RelB/p52 overexpressed organoids treated with the pharmacological inhibitor SC-514 inhibiting RelA/p50 signaling exhibited Esrrg expression without RANKL indicating that RelB/p52 activation is necessary and sufficient to induce Esrrg expression (See Fig 4. D Study I). Previous studies of Spi-B revealed similar results of RelB/p52 activation being sufficient for Spi-B expression (Takashi Kanaya et al. 2018).

5.1.5 Esrrg expression is critical for the maturation of M cells and required for Sox8 activation

To understand Esrrg's role in M cell differentiation and development, we wanted to see if deletion of Esrrg led to a significant effect in M cell development, maintenance and function. Guide RNA's with suitable PAM sites were designed to knockout Esrrg expression with LentiCRISPR V2 CRISPR-Cas9 genome editing. Cultured organoids were transduced with the lentiviral vector encoding guide RNA's for knockout. The Esrrg knockout organoids were validated transcriptionally using RT-qPCR and their protein expression via immunoblots to ensure guides that knocked Esrrg over 95% were selected.

Esrrg knockout organoids were treated with and without RANKL for three days along with scrambled organoids. Esrrg knockout organoids exhibited impaired M cell development. Esrrg knockout organoids in RANKL demonstrated significant reduction in mature marker GP2. Other maturation markers such as Sox8 was also significantly reduced when compared to scramble controls. Interestingly, Spi-B an important master regulator of M cell differentiation showed no significant change in M cell in Esrrg null organoids. These results were also validated when protein expression was analyzed using immunofluorescence. Immunofluorescence analysis of Esrrg knockout organoids showed significant reduction in Sox8 and Gp2 expression indicating that Esrrg is critical for the maturation of Gp2+ M cells and required for the expression of Sox8 which has been previously identified to be necessary for maturation for Gp2 as well (see Fig.5 C-E Study I)

Interestingly some early markers of M cell differentiation were significantly affected by the abolition of Esrrg like CCL9 and MarcksL1. TNFAIP2, another early marker of M cell, showed comparable expression to scramble controls indicating that some of the early markers could require the activation of Esrrg. Of follicle associated epithelium associated factors, CCL20 was significantly reduced in Esrrg knockout organoids treated with RANKL (see Fig.5 F Study I).

To ensure that the phenotype observed was not the result of an off-target effect induced by the guide RNA selected. We performed the previous experiment with another guide RNA with efficiency over 85% to knock out Esrrg. The second Esrrg knockout exhibited similar phenotype as the first Esrrg knockout organoid indicating that the knockout of Esrrg played a role the in impairment of M cell differentiation and development.

5.1.6 Esrrg is independent of Spi-B expression and is a candidate for being a co master regulator of M cell differentiation

Given that Spi-B and Sox8 were found to be 2 key transcription factors and previously documented to be master regulators of M cell development and differentiation, we wanted to observe Esrrg's role in relation to Spi-B and Sox8 and how they worked together for M cell development. Spi-B knockout guide RNA's with appropriate PAM sites were designed to knockout Spi-B from intestinal organoids. Spi-B knockout organoids treated with RANKL for 3 days showed slightly reduced expression for Esrrg. These findings were validated transcriptionally by analyzing Rt-qPCR of these Spi-B knockout organoids however, protein expression of Esrrg in Spi-B knockout organoids with RANKL treatment showed comparable levels as that of scramble controls. These finding indicate that Esrrg is independent of Spi-B expression (See Fig.7 D-F Study I)

Using a Sox8 knockout mice we sought out to find how abolition of Sox8 affects Esrrg expression. qPCR analysis of follicle-associated epitheliums isolated from Sox8 controls and Sox8 KO mice showed Esrrg expression to be higher in Sox8 KO. Crypts isolated from Sox8 controls and Sox8 null mice were treated with RANKL; qPCR analysis and immunoblot analysis of these organoids exhibited an increase in Esrrg expression indicating that Sox8 expression was not required for Esrrg expression. These results suggest that Esrrg acts upstream of Sox8 expression during RANKL indication and M cell development and differentiation (See Fig.7 A-C Study I)

5.1.7 Esrrg alone is not sufficient for M cell maturation.

Since we identified Esrrg to be a master regulator we wanted to observe if Esrrg expression was enough to induce M cell differentiation. Intestinal crypts isolated from wildtype mice were transduced with an overexpression vector to induce Esrrg overexpression. Esrrg was cloned into CSII-CMV-MCS-IRES2-Bsd overexpression vector and transduced into mouse intestinal organoids to observe for changes in M cell-associated markers. Rt-qPCR analysis of these transduced organoids showed that there were no comparable changes in Gp2+ expression; Esrrg overexpression

was also observed to be not enough for Spi-B or Sox8 expression either. This data points to the fact Esrrg alone is not sufficient for M cell differentiation.

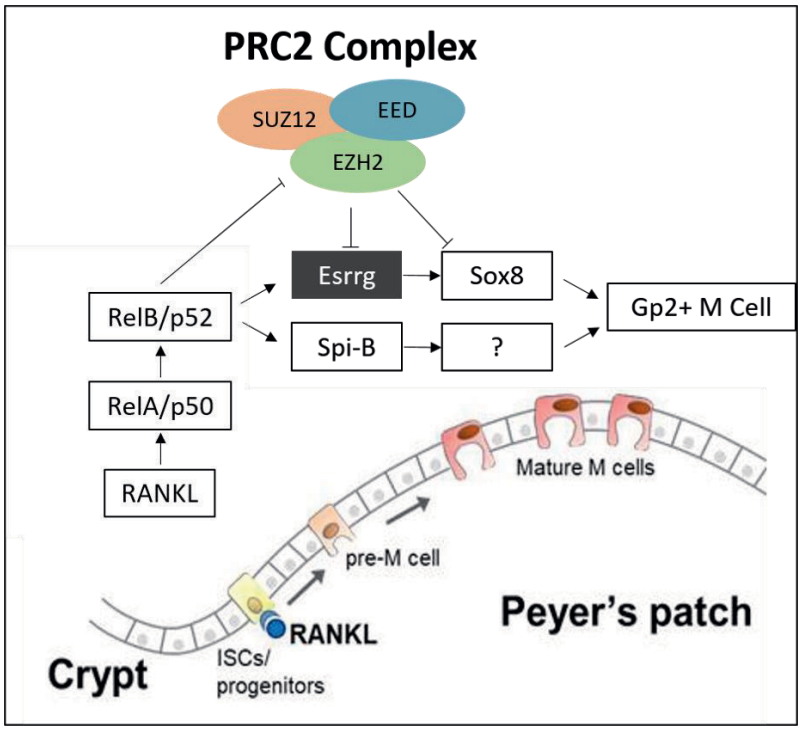

Figure 8. PRC2 regulated Esrrg expression and its role in M cell differentiation. Non-canonical NF-κB was sufficient to express Esrrg, activation of Esrrg is required for Sox8 activity and Sox8 is known to bind to GP2 promoter. Modified from Figure 9, George et al. 2021 (Study I).

Esrrg belongs to the family of ESRR's, members of ESRR's are characteristically known for being orphan receptors. Orphan receptors do not have a known ligand or can specifically activate downstream factors without ligand binding (Giguère 2002). Esrrg does not have a specific agonist or antagonist but 4-hydroxytamoxifen has been demonstrated by previous researchers to bind and inhibit Esrrg activity and phenolic acyl hydrazones GSk4716 was identified as an agonist that enables the activation of Esrrg and its downstream activity (Coward et al. 2001, Zuercher et al. 2005). Intestinal organoids grown in RANKL and OHT exhibited similar results as Esrrg knockout, Spi-B expression was not affected and Sox8 expression was considerably reduced; mature marker Gp2 also showed reduced expression. Organoids treated with RANKL and GSK4716 did not show any changes in expression of Sox8 and Spi-B when Esrrg receptor was activated however, Gp2

expression was slightly elevated in GSK4716 and RANKL treated organoids (See Fig.8 A-C Study I).

5.2 Polycomb regulated Atoh8 regulates the differentiation of M Cells

5.2.1 Atoh8 is regulated by the PRC2 and induced by RANKL-RANK signaling

Among the 6 genes upregulated in our ChIP-seq analysis of differentiated M cells, Atoh8 was regulated by PRC2 significantly (-log2 fold changes −3.15 RankL vs WENRC and -2.58 RankL vs ENRI). To validate Atoh8 as a factor necessary for M cell differentiation, we looked to see If Atoh8 was expressed in cells in the follicle associated epithelium of the Peyer's patches of wildtype mice. Immunohistochemical staining revealed Atoh8 to be expressed in M cells; Atoh8 was found to be localized in the nuclei of M cells in the Peyer's patch. Though we couldn't co-stain Atoh8 with another M cell associated marker, the morphology of the cells expressing Atoh8 was characteristic of M cell morphology as they showed poorly organized microvilli. Villous epithelium and follicle-associated epithelium were isolated from the intestines of wildtype mice; qPCR analysis showed that Atoh8 and GP2 was significantly expressed more in the follicle associated epithelium than the villous epithelium indicating that Atoh8 was localized in the follicle associated epithelium suggesting that it had a role to play in the development of immune epithelial cells (See Fig.1 A-C Study II)

Given that M cell associated factors could fall under both RANKL-RANK signaling and under the purview of S100A4, we sought to identify if Atoh8 expression was dependent on one of these signaling pathways. Using Lenti V2 CRISPR/Cas9 system we knocked out RANK receptor. RANK deficient mouse intestinal organoids were treated with RANKL to demonstrate that Atoh8 and Gp2 expression were significantly reduced suggesting that Atoh8 falls under the RANKL-RANK signaling pathway (See Fig.1 D-E Study II)

5.2.2 Atoh8 expression falls under the RANKL-BMP2/BMP6 signaling

Previous M cell research on factors required for its development and differentiation revealed that the canonical and noncanonical NF-ϰB activation was critical for the expression of several M cell associated markers. Early markers MarcksL1, TNFAIP2 and CCL20 were observed to be under the oversight of canonical NF-ϰB signaling and Spi-B, Esrrg, and Sox8 was dependent on the non-canonical NF-ϰB signaling. We sought to see if Atoh8 expression was dependent on either of the NF-ϰB signaling pathways; interestingly, we observed that neither canonical nor non-canonical NF-kB could not induce Atoh8 expression.

Earlier research studying osteoclast differentiation observed that Atoh8 expression was induced by the activation of BMP2/BMP6 signaling which in turn regulated RANKL/OPG distribution in the system (Yahiro et al. 2020). To understand if BMP2 and BMP6 signaling was active in M cell differentiated state, isolated intestinal mouse organoids were treated with RANKL; after 3 days, BMP2 and BMP6 expression was analyzed and observed to be significantly expressed indicating that BMP2 and BMP6 signaling was RANKL mediated (See Supplementary Fig S3. A Study II)

To understand if BMP2 and BMP6 signaling was sufficient to activate Atoh8 expression without RANKL treatment, intestinal organoids were grown in epidermal growth factor, recombinant BMP2 and R-spondin (Noggin was omitted as noggin acts as an antagonist against BMP signaling). Atoh8 expression was significantly upregulated by BMP2 treatment alone when compared with RANKL treated controls. Similar results were observed when intestinal organoids were grown in Epidermal Growth Factor, recombinant BMP6 and R-spondin. These evidences point to the fact that BMP2 and BMP6 signaling was sufficient to induce Atoh8 expression without the need for RANKL. In addition, we looked to see if expression of other M cell associated factors could be mediated by BMP2 and BMP6 activation; Spi-B showed reduced expression when compared to RANKL treated organoids indicating that Atoh8 activation was specific to BMP2 and BMP6 signaling. (See Supplementary Fig S3. B Study II)

5.2.3 Atoh8 null mice demonstrate an increase in M cell numbers

Considering that Atoh8 was localized in the Peyer's patches and under the overview of RANKL/BMP2/BMP6 signaling, we investigated Atoh8's role in M cell differentiation and development. To ensure Atoh8 knockout was localized to the

gastrointestinal tract, Atoh8 *lox/lox* was crossed with *VilCre* mice (from now on referred to as Atoh8 *lox/VilCre*). Follicle-associated epithelium and villous epithelium were isolated from *VilCre* and Atoh8 *lox/VilCre* mice, RNA isolated from these tissues and analyzed by qPCR's revealed a significant increase in expression of the mature marker GP2+ M cell in Atoh8 *lox/VilCre* mice; other M cell critical transcription factors such as Sox8, Esrrg and Spi-B also demonstrated increased expression when compared to *VilCre* controls (See Fig.2 A Study II) Image analysis of whole mount Peyer's patch isolated from *VilCre* and Atoh8 *lox/VilCre* mice demonstrated an increase in Gp2+ M cells. Immune stained Gp2+ M cells were counted using image J software, the analysis showed almost 1.5-fold increase in M cell numbers counting 22.4 ± 2.85 cells/0.01 mm in Atoh8 *lox/VilCre* mice compared with control *Vil/Cre* mice that had 15± 0.09/0.01 mm (See Figure. 9).

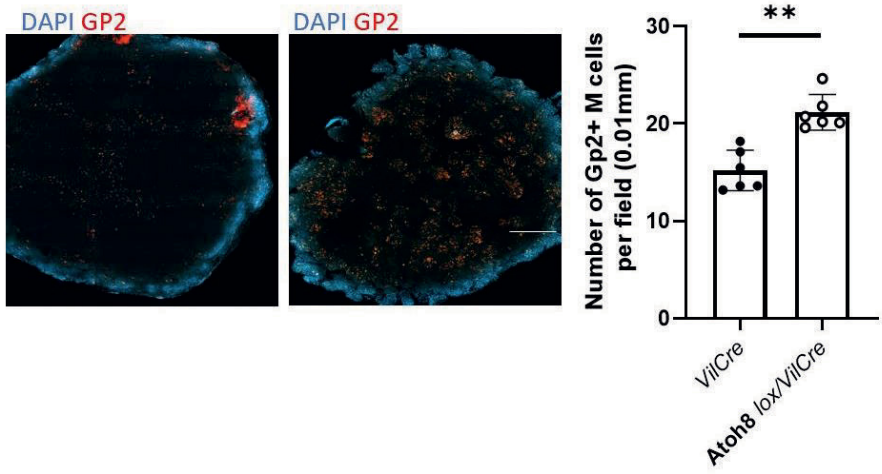

Figure 9. Atoh8 deficient mice exhibited higher number of M cells in the Peyer's Patch compared to its wildtype counterpart. Gp2+ M cells were analyzed and counted using image J software which showed nearly 1.5 times more M cells in Atoh8 *lox/VilCre* than *VilCre* mice.

Early expression markers of M cell differentiation and development such as CCL20, MarcksL1 and TNFAIP2 also exhibited increased upregulation. To understand if OPG, the decoy receptor of RANKL- OPG played a role in the increase in M cell differentiation, we analyzed the expression of OPG. OPG expression was significantly expressed as well but this could be due to increase in M cell population.

5.2.4 Atoh8 deficiency did not alter lymphoid population in the Peyer's patch.

Earlier research studying the effects of Spi-B and Sox8 mice in M cell differentiation observed that in addition to impaired M cell development, they also observed a defect in mucosal immune response (Kimura et al. 2019; Kanaya et al. 2012). Considering that Atoh8 knockout mice exhibited an increase in the number of M cells, we wanted to see if the lack of Atoh8 led to an alteration in the composition of lymphoid cells in Peyer's patches. Lymphoid cells were isolated from Peyer's patch of *VilCre* and Atoh8 *lox/VilCre* mice and subsets of lymphocyte population was identified using appropriate antibodies for IgA+ B cells, T helper cells, GC B cells, B and T cell subpopulations through flow cytometry. We also measured the levels of IgA-producing B cells and RANKL+ T cells to understand if there was an excess of RANKL, which may have led to the increase in M cell population. Flow cytometry analysis of the lymphoid cells showed no significant alteration in either population levels. Atoh8 *lox/VilCre* mice exhibited similar levels of lymphocyte population as that of *VilCre* mice (See Fig.3 A-C Study II).

5.2.5 Epithelium intrinsic Atoh8 is responsible for the increase in M cell population

The subepithelial dome is home to various lymphocytes and stromal cells. These cells are responsible for various signaling pathways with potential effects on the M cell population kinetics. To ensure that the increase in M cell population was solely related to epithelial intrinsic deficiency of Atoh8 in the follicle-associated epithelium we used organoid cultures composed solely of epithelial cells. Mouse intestinal organoids were isolated from *VilCre* and Atoh8 *lox/VilCre* mice and cultured in 100ng/ml of RANKL for 4 days to induce M cell differentiation. Critical transcription factors Spi-B, Sox8 and Esrrg were significantly upregulated along with the mature marker Gp2 indicating that the epithelial cells mimicked the in vivo expression in mice.

Early-stage markers of M cell differentiation CCL9, MarcksL1 and TNFAIP2 showed upregulated expression when treated with RANKL as well. Immunostaining images for epithelial organoids from both *VilCre* and Atoh8 *lox/VilCre* mice showed higher expression of Gp2+ M cells in the Atoh8 *lox/VilCre*. Taken together, these data indicate that epithelial intrinsic Atoh8 is responsible for the regulation in M cell population. (See Fig.4 A-B Study II)

5.2.6 Deletion of Atoh8 leads to increased functional transcytosis capacity

Atoh8 null mice exhibited increased Gp2+ M cell population. Mature Gp2+ M cells in the Peyer's patches are required for the uptake of antigens and foreign pathogens to initiate a mucosal immune response. To ensure that these newly differentiated M cells with Gp2 receptors are functional, *VilCre* and Atoh8 *lox/VilCre* mice were subject to a functional assay. Both sets of mice were administered orally equal volumes of fluorescent nanoparticles of 200nm, and 4 hours after administration Peyer's patch of both these mice were isolated and fixed for immunostaining. The number of particles transcytosed by Peyer's patch in these mice was counted with a fluorescence microscope. Atoh8 *lox/VilCre* mice exhibited a 2-fold increase in uptake of nanobeads when compared to the control *VilCre* indicating a functional phenotype (See Figure. 10).

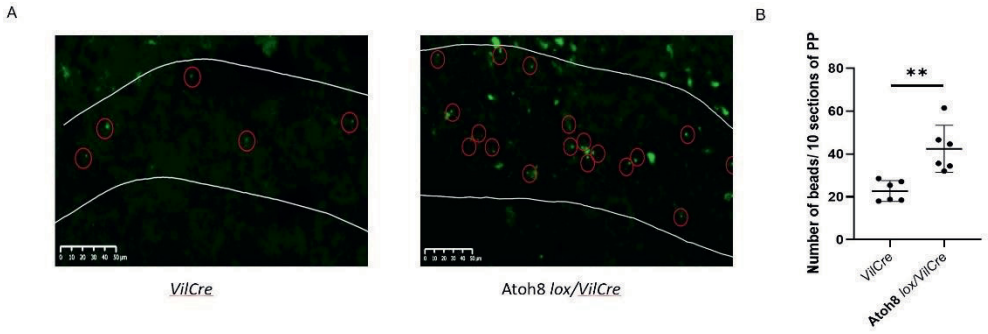

Figure 10. Atoh8 null mice demonstrated an increase in transcytosis capacity. Green florescent latex nanobeads were administered orally in equal volumes. Peyer's patches isolated 3 hours later exhibited higher antigen uptake in Atoh8 *lox/VilCre* mice than in *VilCre* mice. Adapted from Figure 5, George et al. 2021 (Study II).

5.3 Polycomb regulated Maf is critical for maturation of Microfold Cells

5.3.1 Maf is regulated by the PRC2 and localized in the Peyer's patch.

Another gene identified by our ChIP-seq and GRO-seq analysis of differentiated M cells is the expression of Maf. H3K27me3 occupancy at CpG islands spanning the promoter and first exon of the Maf gene was significantly reduced in organoids

treated with RANKL (M cells) than organoids treated with Wnt3a and Chir99021 to mimic crypt intestinal stem cell. This indicates that Maf was regulated by the PRC2 complex and significantly upregulated in our analysis (- log2 fold change -2.66 RANKL vs WENRC crypt/ISCs).

To understand the role of Maf we leveraged the Maf LacZ mouse model where the Maf gene is disrupted by positioning LacZ gene in the Maf locus. To investigate the localization of Maf in the Peyer's patch, we performed a β-galactosidase activity (Ring et al. 2000) staining of the GALT of Maf heterozygous mice. Maf expression was found to be localized among the follicle-associated epithelium in the Peyer's patch of Maf heterozygous mice (See Figure. 11). Immunofluorescence of organoids treated with RANKL revealed Maf to be localized with the mature marker expression of M cell, Gp2. (See Fig.1 A-C Study III)

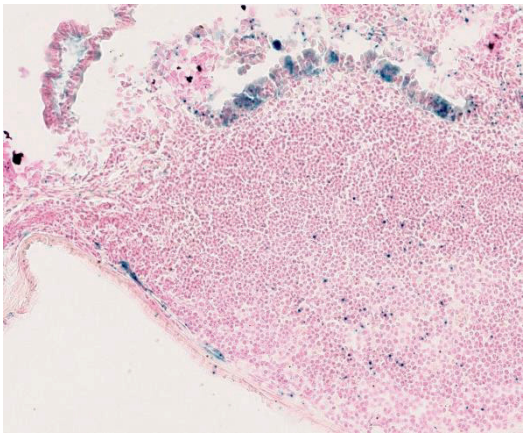

Figure 11. β-galactosidase staining of Maf in Peyer's patches from Maf lacZ heterozygous mice demonstrated Maf expression among the follicle-associated epithelial cells. Modified from Figure 1, George et al. 2021 (Study III).

5.3.2 Maf expression is RANKL mediated and dependent on RANK-RANKL signaling

The subepithelial dome comprises of several lymphocytes and is an affront to multiple signaling pathway. Maf expression has been previously studied for its role in T helper 2 cells (Th2), development and differentiation of innate immune cell types B lymphocytes and T- cell subsets in Peyer's patches (Parker et al. 2020; Pokrovskii et al. 2019; Zuberbuehler et al. 2019.; Kusakabe et al. 2018.). To understand if Maf expression is epithelial intrinsic and RANKL mediated or induced

by lymphocytes in the sub-epithelial dome. Mouse intestinal organoids were isolated from wildtype mice and treated with RANKL, Maf expression along with Gp2 was observed to be significantly upregulated. β-galactosidase staining of RANKL treated organoids exhibited cells that stained for Maf expression in RANKL conditions (See Fig.2 A-B Study III).

To further confirm RANKL's role in M cell differentiation, RANK receptors were knocked out of intestinal organoids using Lenti V2 CRISPR/Cas9 viral vector. Suitable guide RNA's were designed for RANK and transduced to intestinal organoids. RANK knockout organoids treated with RANKL exhibited significant decrease in expression of Maf and mature M ell marker Gp2 expression indicating that Maf expression in M cells is RANKL induced (See Fig.2 C Study III).

5.3.3 Maf expression is critical for M cell differentiation

To understands Maf's role in M cell differentiation we utilized a Maf LacZ mouse model. Unfortunately complete knockout of the Maf gene leads to a lethal phenotype and mice pups were born stillborn or only survived for up to 4 hours (Kawauchi et al. 1999; J. I. Kim et al. 1999). We isolated the pups in their gestational period closer to their date of delivery and genotyped them to affirm the complete knockout of Maf. Organoids were isolated from Maf wildtype mice and Maf KO mice and cultured in media conditioned with epidermal growth factor, noggin, and R-spondin, Wnt, and Chir99021 for 2 weeks till the respective crypts were well propagated. These organoids were grown with and without the presence of RANKL. RNA was isolated and qPCRs were analyzed for these organoids; critical transcription factors of M cell differentiation Spi-B, Sox8 and Esrrg exhibited significantly reduced expression in Maf KO organoids compared to Maf wildtype indicating an impaired maturation of M cells (See Fig.3 A-B Study III).

Early developmental markers of M cell such as MarcksL1 and TNFAIP2 also exhibited reduced expression suggesting Maf was critical for M cell differentiation to initiate. Protein expression analysis using immunofluorescence of Maf knockout organoids exhibited a complete lack of Gp2 expression in Maf KO organoids treated with RANKL indicating that PRC2 regulated MAF is absolutely critical for Gp2+ M cells (Figure 12).

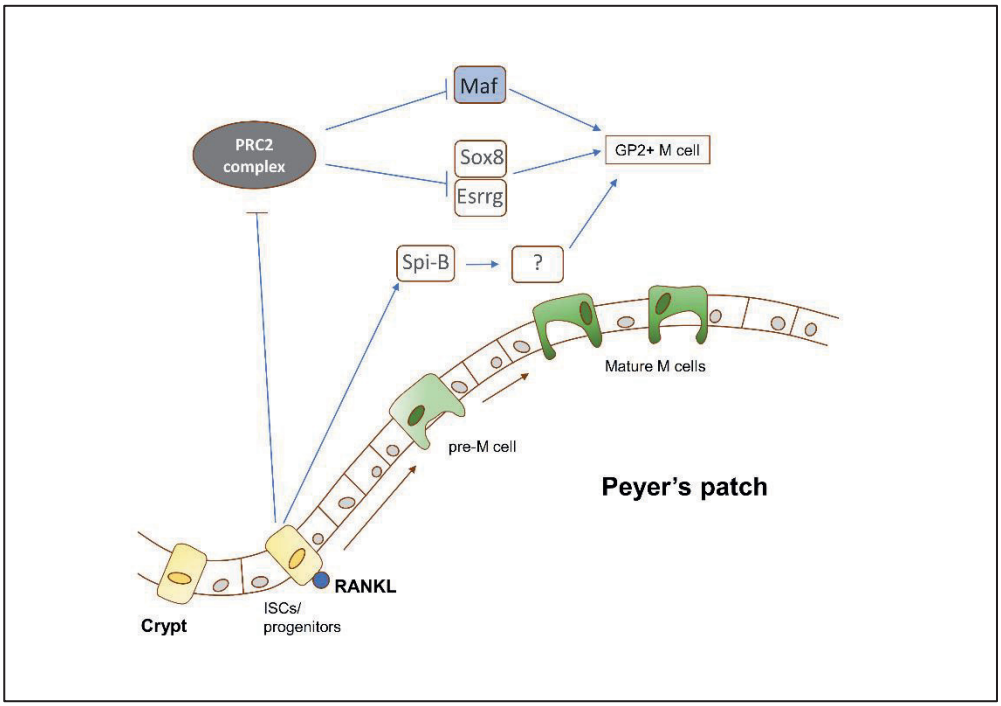

Figure 12. Maf is critical to initiate M cell differentiation. Maf deficiency exhibited significant reduction in early-stage M cell markers suggesting that it could be needed to commence M cell differentiation.

6 DISCUSSION

6.1 Polycomb Repressive Complex 2 role in M cell development

The Polycomb repressive complex (PRC2) is pivotal for stemness, differentiation, and maintenance of various physiological processes (Vizán, Beringer, and Di Croce 2016; Benoit et al. 2013). In the gastrointestinal tract, PRC2 has demonstrated its ability to regulate of Achaete-Scute Family BHLH Transcription Factor 2 (Ascl2) and Atonal BHLH Transcription factor1 (Atoh1) (Oittinen et al. 2017; Chiacchiera et al. 2016; Schuijers et al. 2015). Since PRC2 regulates several genes in stem conditions in the crypt and differentiated state in the villous epithelium, we reasoned that PRC2 regulates genes involved in M cell development and signaling and furthermore reveals the network of genes necessary for the proper functioning of this specialized epithelial cell type involved in immunity.

Even though all the different cell types in the intestinal epithelium differentiate from Lgr5+ stem cells in the crypt, PRC2 plays a distinct role for each of these subtypes. Previous work has demonstrated that disrupting PRC2 activity leads to a precocious expression of terminal differentiation markers of the intestinal epithelium and PRC2 was essential to preserve intestinal progenitors, stem cells and restrict secretory cell progenitors (Oittinen et al. 2017; Chiacchiera et al. 2016; YD Benoit et al. 2012). Expression levels of PRC2 members Enhancer of Zeste homolog 2 (Ezh2) and Suppressor of Zeste homolog 12 (Suz12) in RANKL treated organoids were significantly high and comparable to organoids grown in crypt/stem cell conditions, indicating that PRC2 did not play a restrictive role in M cell differentiation and development.

Considering that M cells are a rare cell type and only account for 8% of epithelial cells in Peyer's patches, it would be impossible to isolate enough M cells for a ChIP-seq experiment which requires around 1 million cells for deep sequencing. Given these circumstances, we isolated intestinal organoids and cultured them in epidermal growth factor, noggin and R-spondin until we were able to reach the appropriate cell count. After which these cells were grown in 3 different conditions before collecting

them for ChIP-seq protocol - M cell differentiated conditions (RANKL), stem-like conditions (Wnt/Chir), and organoids in enterocyte differentiated state (Wnt inhibitor IWP2). Comparison of ChIP-seq of the three different cell fates revealed a unique set of genes that were specifically regulated by the PRC2 for M cell differentiation and development. In addition to ChIP-seq, we also performed GRO-seq with RANKL treated organoids to collect data set of all transcriptionally active genes in M cell differentiation. Gene ontology analysis exhibited several genes critical for the development of the distinct anatomy and morphology of M cells and also genes required for the functional transcytosis capacity of M cells. ChIP-seq revealed a total of 12 transcription factors that were expressed differentially; 6 of them were expressed specifically in M cells but repressed by PRC2 in crypt stem cell organoids and enterocyte differentiated organoids. These 6 comprise of Sox8, Atoh8, Esrrg, Smad6, Maf, and Zfp819. PRC2 also silences 6 different M cell unique genes- these are Hoxb5, Hoxb9, Sp9, Sp5, Nr4a1, and Atf3.

The PRC2 complex regulation of the 6 genes (Sox8, Atoh8, Esrrg, Smad6, Maf, and Zfp819) expressed in M cells could mean that they play critical roles related to differentiation and development. Among the 6, Sox8 has been characterized by Kimura et al. 2019 and we characterized Esrrg, Atoh8, and Maf role in M cell development and we discuss them in-depth in the following sections.

Smad6 is a member of the Smads family that exhibits structurally similar proteins which are the main transducers for receptors of the TGF-B superfamily (Derynck, Zhang, and Feng 1998). The Smads consists of 9 family members; Smad1, Smad2, Smad3, Smad5, and Smad8/9 comprise the R-smads and are involved in direct signaling from the TGF-B receptor whereas Smad6 and Smad7's are I-smads that work to repress the activity of R-smads. Smad6 is predominantly found in the nucleus and acts as a direct transcriptional regulator, Smad6 activity is more associated with BMP signaling in cartilage development (Estrada et al. 2011). In our work, we identified the existence of BMP signaling in M cells and its critical role in regulating M cell differentiation. Smad6 has also been known to inhibit TRAF6 activity AML-12 mouse liver cells and primary hepatocytes (Jung et al. 2013); in M cells Traf6 has been documented to be required for canonical NF-κB activation which is needed for the expression of early M cell markers such as MarcksL1. It is possible that Smad6 plays a role in inhibiting Traf6 activity along with the activation of BMP signaling and Atoh8, which we documented to be essential for controlling

M cell population numbers, however, more work is required to characterize Smad6 role in M cell differentiation.

Zfp819 is a member of the Krüppel-associated box (KRAB) family, they have been documented to encode a spermatogenic cell-specific transcription factor. The KRAB and C2H2-type zinc finger motifs (KRAB-ZF) family are one of the largest transcription factor groups in vertebrates, they exhibit a transcriptional repressive activity by binding to DNA in a sequence-specific manner (Urrutia 2003). They are usually found to be expressed in various adult tissues and play roles in cell proliferation, tumorigenesis, and apoptosis (Urrutia 2003). Researchers have found that overexpression of Zfp819 affected cell proliferation and induced apoptosis in somatic cell lines specifically the overexpression of Zfp819 led to alterations in the expression of B cell lymphoma protein-2 (BCL-2) and poly (ADP-ribose) polymerase (PARP) (Jin et al. 2015). The role of Zfp819 in M cell development and differentiation is unknown and requires further exploration.

6.2 Esrrg is a candidate for being a co-master regulator of M cell differentiation

Our ChIP-seq analysis revealed Esrrg to be one of the highly expressed genes in M cell differentiation especially when the M cell dataset was compared to crypt stem cell state with Wnt and Chir or compared to differentiated state with IWP WNT inhibitor (log2 fold changes, –6.64 RankL vs ENRI and –4.76 in RankL vs WENRC comparisons). Given this significant level of expression, it was reasonable to validate this analysis in wet lab settings.

Estrogen-related–receptor γ (Esrrg)is a member of the Estrogen related receptor (ERR) nuclear-receptor family. Other members of the family are Estrogen related receptor alpha (ESRRA) and Estrogen related receptor beta (ESRRB) (Giguère 2002), all these members share an identical DNA binding domain, which is composed of two C4-type zinc finger motifs. The members of this family share a characteristic feature of being orphan nuclear receptors, they bind to estrogen response element and steroidogenic factor 1 response element and can activate genes downstream of their activity without binding to a ligand. They are called orphan receptors for two reasons; the identity of their ligand is unknown or they are able to be activated without the binding of a ligand. The ERR family has demonstrated its

role in sharing target genes, coregulatory ligands, and common sites of action. Esrrg in particular has been identified to control macrophage function indirectly through regulation of intracellular iron (M.-H. Kang et al. 2018). Mice that are responding to Salmonella typhimurium infection exhibit an upregulation in hormone hepcidin by the direct activity of interleukin 6's activation of Esrrg (D. K. Kim et al. 2014).

We found Esrrg expression to be localized in the follicle-associated epithelial cells in the Peyer's patches exclusively when compared to the crypt cells and villous epithelium indicating that Esrrg could have a significant role in the development, organization, and function of M cells. Esrrg was found to be RANKL-mediated and under the overview of RANKL-RANK signaling. Previous work on RANKL signaling has also found that RANKL binds to Lgr4 receptors in bone development signifying the importance of receptors needed for M cell differentiation (Luo et al. 2016). RANKL treatment of RANK receptors knockout organoids revealed a significant reduction in expression of Esrrg and Gp2 indicating that Esrrg expression was dependent on RANKL-RANK signaling. The NF-$\varkappa$B pathways were demonstrated to be critical for the expression of early-stage M cell markers and critical transcription factors such as Spi-B and Sox8; more specifically the non-canonical RelB/p52 pathway was demonstrated to be sufficient induce Spi-B and Sox8 expression (Takashi Kanaya et al. 2018). In our experiments involving organoids overexpressed with RelB/p52, we observed non-canonical NF-$\varkappa$B signaling sufficient to induce Esrrg expression. Inhibition of canonical NF-$\varkappa$B signaling with pharmacological inhibitor SC-514 on RelB/p52 overexpressing organoids did not suppress Esrrg expression.

To understand the role of Esrrg, we knocked out Esrrg in intestinal organoids using lentiviral CRISPR/Cas9. We observed a drastic reduction in expression of Gp2 the mature marker of M cells indicating that M cells were not able to differentiate without Esrrg. In addition, we also observed a significant reduction in Sox8 expression. This suggested that perhaps Esrrg played a role upstream to that of Sox8. However, Spi-B, another transcription factor documented to be a co-master regulator for M cell maturation was found to be in comparable levels with the control indicating that Spi-B expression was not dependent on Esrrg. Early-stage markers of M cell differentiation such as MarcksL1 and CCL20 also showed reduced expression but TNFAIP2 levels remained unchanged. Further experiments with an Esrrg null mouse model would paint a better picture in understanding Esrrg's role in early developmental markers of M cells. Our protein analysis experiments with

immunofluorescence corroborated our gene profiling experiments. To ensure that the phenotype observed was not due to off-target effects of the guide RNA, these experiments were repeated with a second guide RNA knocking out Esrrg, we observed similar results to our first Esrrg knockouts isolating the phenotype unique to the absence of Esrrg.

To confirm that Esrrg plays an upstream role to Sox8 in M cell differentiation, we looked at Esrrg expression in our Sox8 knockout mice. Follicle-associated epithelium in Sox8 wildtype and Sox8 knockout mice revealed Esrrg expression present and active in both sets of mice. This data combined with the absence of Sox8 in Esrrg null organoids indicates that Sox8 is dependent on Esrrg expression. To understand Spi-B's relationship to Esrrg in M cell development, we knocked out Spi-B using Lenti V2 CRISPR/Cas9 and observed a reduction in Esrrg expression in our qPCR analysis, but protein expression studies showed that Esrrg was still active in Spi-B knockout organoids. This data and the presence of Spi-B in Esrrg null organoids indicate that Esrrg and Spi-B expression are independent of each other.

We observed that overexpression of Esrrg was not sufficient for the differentiation of M cells suggesting that M cell differentiation requires the activation of multiple factors. Sox8 expression could not increase Gp2 expression by itself since SOX proteins require DNA binding partners specific for each member of the SOX family. These DNA binding partners aid in stabilizing the SOX family to their target regions (Kamachi, Cheah, and Kondoh 1999). Also, considering Sox8 was revealed to bind to the Gp2 promoter, they could require another DNA binding partner probably downstream of Spi-B or another pathway to bind to Gp2 to induce its expression. Given that SpiB is a master regulator needed for M cell maturation and Esrrg activation is required for induction of Sox8 and Gp2, it is reasonable to infer that Spi-B and Esrrg act as co-master regulators in M cell differentiation.

6.3 PRC2 regulated Atoh8 expression is critical to maintaining M cell population

Atoh8 was revealed to be one of the 6 transcription factors upregulated by the PRC2 in M cell development. When comparing M cell differentiated organoids with crypt stem cell organoids and differentiated organoids Atoh8 turned out to be significantly

expressed in M cell differentiated state (log2 fold changes −3.15 RankL vs WENRC and -2.58 RankL vs ENRI).

Atoh8 (Atonal BHLH Transcription Factor 8) is a member of the basic Helix-Loop-Helix bHLH transcription factor family. All the members of this family have 2 highly conserved binding domains that together makeup 60 amino acid residues. bHLH transcription factors have been documented to be involved in the regulation of the cell cycle, cardiovascular development, hematopoiesis, stem cell maintenance, and skeletal muscle development. Atoh8 is a member of the group A of the bHLH transcription factor family. Atonal superfamily members control numerous aspects of differentiation for vertebrate organ development (R. T. Yan 1998; Tomita et al. 2000; Hutcheson and Vetter 2001; Durand et al. 2012). In the intestinal Atonal BHLH Transcription Factor 1 or Atoh1 plays a critical role in regulating Paneth cell, these cells provide stem cell niche to Lgr5+ cells in the intestinal crypt and is also required for the differentiation of functional secretory lineages through lateral inhibition (Durand et al. 2012). Atoh8 being the sole mammalian member of the bHLH factor that is a part of the NET family (Rawnsley et al. 2013), its role in M cell differentiation was unknown.

Atoh8 expression was found to be localized in the follicle associate epithelium of the Peyer's patches in wildtype mice. To investigate Atoh8's role in M cell differentiation and development, we used an Atoh8 intestinal-specific knockout to characterize its role. We observed a higher number of Gp2+ mature M cell expression in the isolated follicle-associated epithelium when compared to its *VilCre* counterpart. Our whole-mount immunofluorescence of Peyer's patch isolated from Atoh8 *lox/VilCre* and *VilCre* confirmed our expression studies as we observed a 1.5-fold increase of M cells in Atoh8 knockout mice. Early developmental markers such as MarcksL1 and TNFAIP2 were observed to be significantly upregulated indicating that Atoh8's activity could be upstream of the canonical RelA/p50. Critical transcription factors that act as master regulators of M cell development, Spi-B, Esrrg and Sox8 were revealed to be upregulated as well including the expression of Sox8. To understand if this phenotype observed was related to epithelial deletion of Atoh8 or lymphocytes in the Peyer's patches, we isolated organoids from the Atoh8 *lox/VilCre* mice and *VilCre* mice. RANKL treated Atoh8 null organoids exhibited a significant increase in Gp2 expression along with Spi-B, Esrrg, and Sox8 indicating that epithelial intrinsic Atoh8 was responsible for the increase in M cell numbers. Next, we checked to see if the increase in M cells in Atoh8 knockout mice meant an increase in

functional transcytosis capacity. Atoh8 *lox/VilCre* and Atoh8 *VilCre* mice were orally administered with equal volumes of 200nm fluorescent latex beads, Atoh8 *lox/VilCre* mice were able to uptake these beads significantly higher than *VilCre* mice indicating that M cells in the Atoh8 null mice were functional.

OPG is a receptor that binds to RANKL and acts as a decoy receptor to RANK receptor, lack of OPG increased RANKL expression leading to an increase in M cells higher transcytosis capabilities, and enhanced immune response (Shunsuke Kimura et al. 2020). We explored the possibility if the lack of Atoh8 led to an increase in RankL production in our mice however, our flow cytometry analysis showed no increase in RankL+ T cells, indicating that Atoh8 acts independent of RankL signaling in the GALT. Interestingly, even though OPG deficient mice exhibited a higher number of M cells and enhanced immune responses, they were highly susceptible to infection by pathogenic bacteria. Botulinum toxins and scrape prion protein have been known to exploit M cells to gain entry and induce inflammation and infection (Matsumura et al. 2015). Therefore, given that Atoh8 null mice exhibited an increase in M cells, it is logical to reason that Atoh8 plays a role in regulating M cell numbers to limit the transcytosis of pathogenic agents. This delicate equilibrium of maintaining M cell density in the Peyer's patch via Atoh8 and OPG may have been established over time as an evolutionary mechanism to control intestinal immune homeostasis with context to invasive antigens and mucosal immune responses. Atoh1, a family member of Atoh8, has been documented to regulate Paneth cell differentiation in the intestinal crypt via notch signaling and lateral inhibition (T. H. Kim et al. 2014). Atoh1 and notch signaling prevents the adjacent cell next to the Paneth cell from expressing Atoh1 and thereby differentiating into another Paneth cells; it can be speculated that Atoh8 employs a similar mechanism in maintaining the population. However, further investigative studies are needed to understand the mechanistic pathway with which the epithelial intrinsic Atoh8 regulates the population of M cells in the Peyer's patches.

Our attempts to identify if canonical (RelA/p50) or non-canonical (RelB/p52) NF-ϰB signaling was responsible for Atoh8 expression revealed no changes in Atoh8 expression. Overexpression of RelA/p50 and RelB/p52 failed to induce or significantly upregulate Atoh8 expression without RANKL indicating the existence of another RANKL mediated pathway in M cell differentiation. Previous research studied Atoh8's role in the differentiation of osteoblasts; in this context, Atoh8 was revealed to be induced by BMP signaling and was essential to regulate the

RANKL/OPG ratio indirectly via Runx2 to regulate osteoclast number and to maintain appropriate bone volume in mice (Yahiro et al. 2020). To confirm the presence of BMP signaling in M cells, Intestinal organoids isolated from wildtype mice and grown in RANKL exhibited significant upregulation of BMP2 and BMP6 signaling compared to organoids grown without RANKL. Next, we explored if Atoh8 expression was induced by BMP2 or BMP6, we grew intestinal organoids in maintenance condition (Egf, noggin, and R-spondin), M cell conditions (RANKL), in the presence of BMP2 and BMP6 (noggin was removed as they act an antagonist against BMP signaling) and organoids in EGF and R-spondin for control purposes. We observed a significant increase in Atoh8 expression in both the organoid cultures that were grown with just BMP2 recombinant protein and BMP6 recombinant protein. The expression of Atoh8 in these organoids was significantly higher than organoids grown in RANKL alone. These combinations of experiments prove that besides RANKL induced NF-$\varkappa$B signaling in M cell differentiation, there also exists a RANKL-induced BMP signaling which is essential for activation of Atoh8. We further investigated to see if Runx2 played a role in BMP induced Atoh8 signaling, we did not observe any significant changes to Runx2 expression indicating that further investigation is needed to find if RANKL induced BMP signaling directly activates Atoh8 expression.

6.4 PRC2 regulated Maf is required for the differentiation of M cells

Our ChIP-seq analysis revealed Maf to be among genes that were regulated by the PRC2 and significantly upregulated in M cells. Our ChIP-seq analysis comparing M cells and intestinal stem cells revealed Maf to be highly expressed in M cells (log2 fold change -2.66 RANKL vs WENRC (crypt/ISCs). Musculoaponeurotic fibrosarcoma (Maf) encodes for the transcription factor Maf (c-Maf). Maf family is highly conserved and possesses a unique leucine zipper structure (bZIP). The Maf transcription family is composed of 7 family members which are broadly divided based on their size into 2 subclasses- MAFA/L-MAF, MAFB, MAF/c-Maf, and NLR (neural retina leucine zipper comprise the large Maf proteins while MAFK, MAFG, and MAFF comprise the smaller Maf proteins. A distinct feature of the small Maf proteins is the lack of amino-terminal transactivation domain. Previous research has found c-Maf to be required for the development of cornea and lens development, differentiation of chondrocytes, and mechanoreceptors involved in

touch sensations (Kawauchi et al. 199; J. I. Kim et al. 1999; Wende, Lechner, and Birchmeier 2012; Wende et al. 2012.). In the intestine, Maf has been identified as an immune regulator and a transcription factor for T helper 2 cells (Th2), more importantly, Maf plays a pivotal role in the development of innate immune cell types B lymphocytes, and T- cell subsets (Parker et al. 2019.; Pokrovskii et al. 2020.; Zuberbuehler et al. 2019.). Further evidence of Maf's critical role in signaling and the physiological process is exhibited by the lethal phenotype of complete Maf knockout in Mice. Maf deficient mice with C57BL/6 background are lethal embryonically or perinatally, Maf KO pups are often born stillborn or only survive upto 4 hours after birth (Kawauchi et al. 1999. J. I. Kim et al. 1999). However, Maf knockout with BALB/c background lives to adulthood.

Through β-galactosidase staining we traced the gene expression of Maf in the GALT of Maf LacZ heterozygous mice. Maf expression co-localized with Gp2 implies a potential role of Maf in M cell development. Maf expressing cells were identified in organoids isolated from Maf heterozygous mice and grown in RANKL using β-galactosidase staining indicating that Maf was induced by RANKL signaling.

Given that complete knockout of Maf resulted in a lethal phenotype, we were unable to characterize the role of Maf in M cell differentiation in a conventional manner. To circumvent this situation, we surgically isolated pups close to their due date in their late gestational period and isolated crypts from the intestinal tract. These crypts were cultured and propagated for 2 weeks to organoids prior to growing them in the presence and absence of RANKL. Maf null organoids showed a significant decrease in M cell mature marker Gp2 expression signifying impaired development of M cells. Reduction in expression was also observed in co-master regulators of M cell differentiation Spi-B, Esrrg and Sox8, early markers of M cells such as MarcksL1 and Tnfaip2 also exhibited a significant reduction in expression indicating that perhaps Maf is probably needed to initiate M cell differentiation. Given that Maf plays a developmental role in B cells, T cells, and other lymphocytes in the Peyer's patches, it can be assumed that lymphocytes lacking Maf could play a role in the impaired development of M cells. However, organoids isolated from Maf KO and grown in the presence of RANKL exhibited impaired M cell phenotype indicating that that epithelial intrinsic Maf was responsible. In ocular lens differentiation and osteogenic differentiation Maf expression was found to be regulated by BMP signaling ((Zhu et al. 2019; Xie et al. 2016)). Since our earlier work on the characterization of Atoh8 revealed BMP signaling in M cells, it is reasonable to infer that RANKL induced

BMP signaling could be required for inducing the expression of Maf in M cells. However, further experiments with Maf deficiency in mice with BALB/c background are critical to understanding the physiological significance and the mucosal immune response in Maf knockout mice in vivo.

6.5 Esrrg, Atoh8 and Maf play a crucial role in regulating M cell differentiation and the future potential of M cell research

From characterizing Esrrg, Atoh8 and Maf, we came to the conclusion that all three transcription factors are critical for differentiation of a fully mature M cell. Esrrg was found to be under the overview of non-canonical RelB/P52 signaling and essential for Sox8 activation which is required for Gp2+ M cell. Atoh8 was discovered to be under the overview of RANKL-BMP2/BMP6 signaling played a regulatory role in controlling M cell population. Atoh8's role is necessary to maintain M cell population as commensal viral particles such as oral prions can exploit it as a portal for entry. RANKL induced Maf was necessary for all M cell associated transcription factors to be activated suggesting that MAF is probably needed for activating canonical and non-canonical NFKB signaling (Figure 13). Although these transcription factors have roles in human physiology that are comparable to those in mouse in several organs and conditions, further experiment about their role in human M cell biology is required to fully comprehend for future therapeutic purposes.

Though we identified key previously unknown transcription factors and characterized their roles in M cell differentiation, there are some drawbacks of our research to be considered. The characterization of Esrrg was conducted in in vivo settings, organoids isolated from the duodenum were genetically modified to knockout Esrrg in order to understand its role. A definitive picture of Esrrg's role could perhaps be understood with an Esrrg knockout mouse model. To understand the loss of Esrrg in regard to the functionality of M cells, numerous bacterial assays and latex nano bead assays could be carried out to test the transcytosis capacity and the associated immune response. Though Atoh8's role in maintaining M cell population is critical to control the rate of transcytosis of bacterial and viral particles, the mechanism as to its mode of regulating the population remains to be elucidated. The identification of BMP signaling in M cells is novel and perhaps the role of BMP signaling is to activate other regulatory

elements that are directly or indirectly associated with Atoh8 to control M cell population in the gut. Atoh8 has been previously indicted in pathogenesis of osteoclastogenesis which cause bones to lose their integrity, This BMP induced Atoh8 leads to disruptions in the RANKL/OPG balance which leads to the formation of bone-resorbing cells, called osteoclasts, from precursor cells of myeloid origin causing excessive bone breakdown (Yahiro et al.2020). Our experimental observations revealed that a loss of Atoh8 in the gut led to increase in population of M cells and transcytosis. Though we didn't have the animal license to perform bacterial experiment on the Atoh8 knockout mice, frequently scheduled helicobacter testing by the facility showed increased Helicobacter infections when compared to other mice caged in the facility. Though this data is anecdotal, it perhaps indicates that an increase in M cell population leads to a higher rates of infection in Atoh8 knockout mice. Additionally, a better understanding of the role of Atoh8 could potentially establish it as a biomarker to identify Crohn's disease, irritable bowel syndrome and ulcerative colitis. OPG as discussed earlier is an indication for increase in M cell population and constitutes as a biomarker for Crohn's disease, ulcerative colitis and irritable bowel disorder (Kimura et al.2020). It is possible that (1) An increase in M cell in the human gut could possibly lead to an increase in symptoms of irritable bowel syndrome as IBS can develop after a severe bout of diarrhea (gastroenteritis) caused by bacteria or a virus. IBS has been revealed to be associated with a surplus of bacteria in the intestines (bacterial overgrowth). 2) increase in M cell population and constitutes as a biomarker for Crohn's disease, ulcerative colitis and irritable bowel disorder. (Kimura et al.2020). 3) abnormal Atoh8 expression could be an indication of these gastrointestinal diseases. However, further research is required to ascertain if Atoh8 has any direct or indirect effect on RANKL/OPG imbalance. Even though we utilized a Maf knockout mice to explore MAF's role in M cell differentiation, we were limited by the absence of in vivo Peyer's patch, follicle associated epithelium and functional studies on tissues due to the lethal phenotype of MAF homozygous knockout. To further comprehend MAF's role in the development of M cells, future studies should utilize MAF knockout mice with BALB/C background.

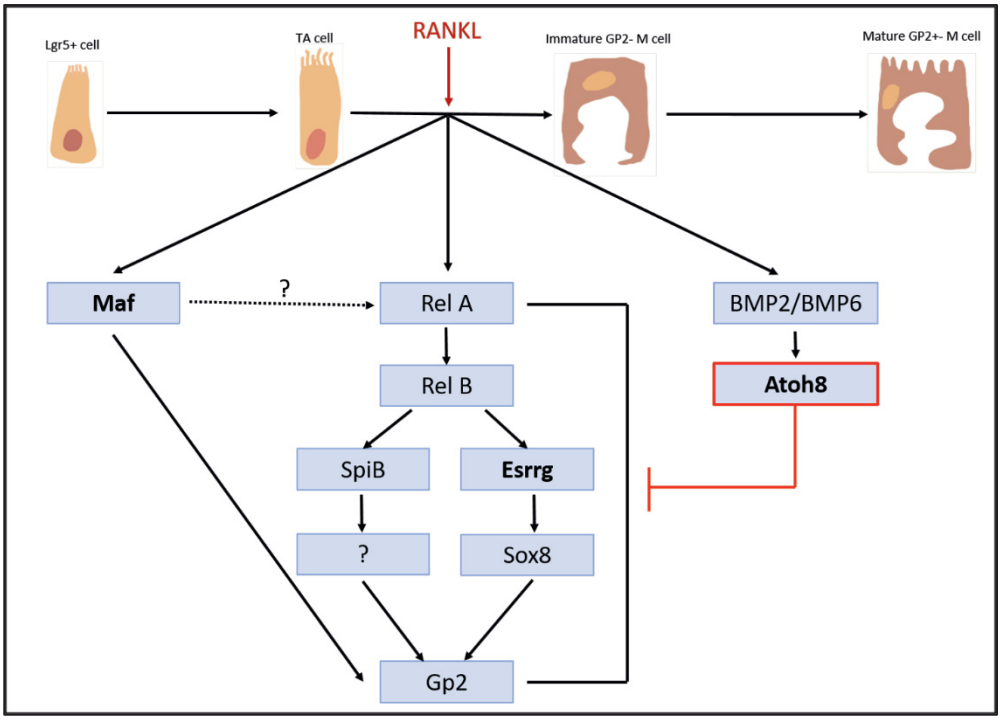

Figure 13. M cell differentiation pathway with novel PRC2 regulated genes; Esrrg, Atoh8, and Maf fit in the M cell differentiation network with previously identified M cell transcription factors.

7 CONCLUSIONS

In this thesis, we sought to uncover the role of Polycomb repressive complex 2 in M cell differentiation. We also identified several transcriptional factors of which Esrrg, Atoh8 and Maf were characterized to be critical for M cell development.

1. We were able to demonstrate that the PRC2 does not restrict the differentiation of M cells as it does for enterocyte differentiation. From our ChIP-seq analysis we identified a total of 12 transcription factors that were expressed differentially by the PRC2: 6 were silenced by PRC2 in M cells and 6 were expressed specifically in M cells. The 6 PRC2 target genes expressed specifically in M cells were Sox8, Atoh8, Esrrg, Smad6, Maf, and Zfp819. The 6 genes silenced were Hoxb5, Hoxb9, Sp9, Sp5, Nr4a1, and Atf3.

2. We characterized the novel PRC2-regulated Esrrg and found it to be critical for M cell maturation. Esrrg null organoids were unable to differentiate into fully mature M cells with Gp2+ expression. We identified Esrrg to be essential for critical transcription factor Sox8 activation and is upstream to Sox8 activity. Esrrg was independent of Spi-B expression and lack of Spi-B did not affect Esrrg expression either

3. Our ChIP-seq analysis revealed PRC2 regulated Atoh8 to be active in M cells in the follicle associated epithelium. Atoh8 was critical as it regulated the population of M cells in the Peyer's patches. Atoh8 null mice demonstrated a significant increase in M cells with an increased transcytosis capacity which is detrimental to staving off infections as pathogens can exploit M cells as an entry portal.

4. PRC2-regulated Maf was identified to be critical for M cell differentiation. Maf null organoids grown in RANKL were unable to activate early-stage M cell markers indicating that Maf might be one of the first transcription factors activated after RANKL-RANK binding.

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PUBLICATIONS

PUBLICATION I

Polycomb Repressive Complex 2 Regulates Genes Necessary for Intestinal Microfold Cell (M Cell) Development

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ORIGINAL RESEARCH

Polycomb Repressive Complex 2 Regulates Genes Necessary for Intestinal Microfold Cell (M Cell) Development

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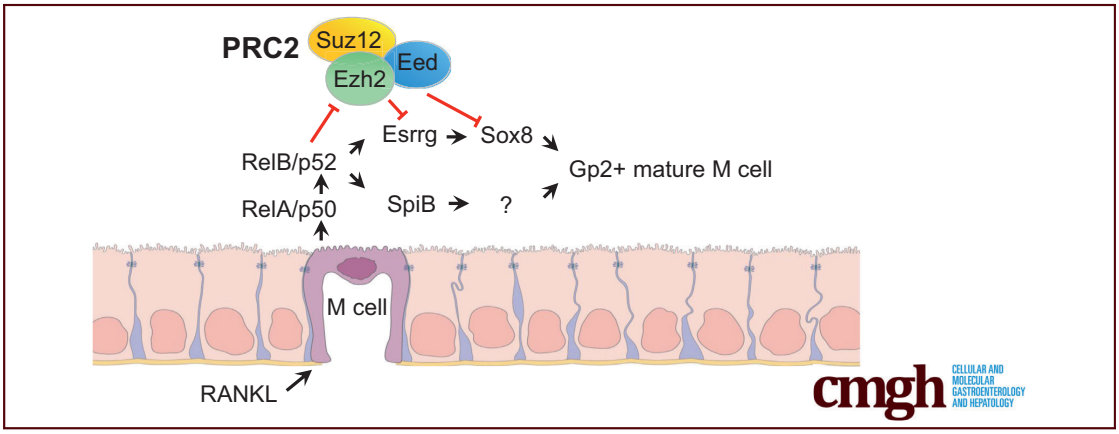

SUMMARY

Chromatin immunoprecipitation and global run-on sequencing analysis of gut Microfold cells (M cells) showed 12 previously unknown novel transcription factors and, one of them, estrogen-related–receptor γ, plays a critical role in M-cell differentiation. Lack of estrogen-related–receptor γ showed an immature and nonfunctional M-cell phenotype.

BACKGROUND & AIMS: Microfold cells (M cells) are immunosurveillance epithelial cells located in the Peyer's patches (PPs) in the intestine and are responsible for monitoring and transcytosis of antigens, microorganisms, and pathogens. Mature M cells use the receptor glycoprotein 2 (GP2) to aid in transcytosis. Recent studies have shown transcription factors, Spi-B and SRY-Box Transcription Factor 8 (Sox8). are necessary for M-cell differentiation, but not sufficient. An exhaustive set of factors sufficient for differentiation and development of a mature GP2+ M cell remains elusive. Our aim was to understand the role of polycomb repressive complex 2 (PRC2) as an epigenetic regulator of M-cell development. Estrogen-related–receptor γ (Esrrg), identified as a PRC2-regulated gene, was studied in depth, in addition to its relationship with Spi-B and Sox8.

METHODS: Comparative chromatin immunoprecipitation and global run-on sequencing analysis of mouse intestinal organoids were performed in stem condition, enterocyte conditions, and receptor activator of nuclear factor κ B ligand–induced M-cell condition. Esrrg, which was identified as one of the PRC2-regulated transcription factors, was studied in wild-type mice and knocked out in intestinal organoids using guide RNA's. Sox8 null mice were used to study Esrrg and its relation to Sox8.

RESULTS: chromatin immunoprecipitation and global run-on sequencing analysis showed 12 novel PRC2 regulated transcription factors, PRC2-regulated Esrrg is a novel M-cell–specific transcription factor acting on a receptor activator of nuclear factor κB ligand–receptor activator of nuclear factor κB–induced nuclear factor-κB pathway, upstream of Sox8, and necessary but not sufficient for a mature M-cell marker of Gp2 expression.

CONCLUSIONS: PRC2 regulates a significant set of genes in M cells including Esrrg, which is critical for M-cell development and differentiation. Loss of Esrrg led to an immature M-cell phenotype lacking in Sox8 and Gp2 expression. Transcript

profiling: the data have been deposited in the NCBI Gene Expression Omnibus database (GSE157629). *(Cell Mol Gastroenterol Hepatol 2021;12:873–889; https://doi.org/10.1016/j.jcmgh.2021.05.014)*

Keywords: PRC2; Microfold Cells; Esrrg; RankL; Gut Immunity.

The gut-associated lymphoid tissue (GALT) is involved in immune surveillance of antigens, microorganisms, and foreign pathogens that constantly thrive on the mucosal surface of the intestinal tract. The GALT is the immune initiation site against mucosal antigens and houses specialized gut immune epithelial cells known as microfold cells (M cells). These cells occur on the epithelial surface of lymphoid nodules of the GALT, which includes Peyer's patches (PPs) in the terminal ileum, solitary lymphoid nodules scattered throughout the small intestine, the appendix, and rectal patches in the terminal colon.[1–3] The principal role of M cells is the uptake and transcytosis of luminal antigens into the GALT because they have a high phagocytic and transcytosis capacity, which is responsible for the rapid transport of bacterial antigens to antigen-presenting immature dendritic cells.[4,5] Subsequently, these dendritic cells undergo maturation and activate antigen-specific naive T cells, which support B-cell activation, ultimately resulting in the generation of IgA-producing plasma cells.[6] It has been shown previously that the absence of M cells or their antigen uptake receptor glycoprotein 2 (GP2) impairs the mucosal immune responses by T cells in mice infected with *Salmonella enterica* serovar Typhimurium. This is predominantly owing to a lack of bacterial transcytosis by the mature GP2 receptor into the GALT.[7,8] Correspondingly, perturbances in transcytosis of *Yersinia enterocolitica* in PPs were observed in Allograft inflammatory factor 1 (Aif1) mutant mice.[9] Recently, it was shown that M cells self-regulate their differentiation by expressing osteoprotegerin, a soluble inhibitor of receptor activator of nuclear factor-κB ligand (RANKL), which suppresses the differentiation of adjacent follicle-associated epithelium (FAE) cells into M cells. This self-regulatory machinery of M-cell density is necessary because $Opg^{-/-}$ mice are highly susceptible to mucosal infection by pathogenic bacteria because of the augmentation of bacterial translocation via M cells.[10] Overall, defects in M-cell–dependent antigen uptake led to a decrease in production of antigen-specific secretory IgA in the gut.[5,9,11]

M-cell differentiation from cycling intestinal crypt cells that express Leucine Rich Repeat Containing G Protein-Coupled Receptor 5 (Lgr5) and receptor activator of nuclear factor κB (RANK) receptors is induced by the RankL. RankL is secreted by stromal cells, also known as M-cell inducer cells or immune cells in the subepithelial dome.[1,12] RankL-deficient mice have very few M cells, but exogenous administration of recombinant RankL was able to mitigate that loss.[13] RankL binding to Rank receptor leads to the activation of the intracellular adaptor molecule of RANK; TNF Receptor Associated Factor 6 (TRAF6), which in turn leads to activation of both canonical (RelA/p50 heterodimer) and noncanonical nuclear factor-κB (NF-κB) (RelB/p52 heterodimer) activation.[14–16] The canonical RelA/p50 activation led to expression of early M-cell markers such as Marcks like 1 (MarcksL1) and Chemokine (C-C motif) ligand 9 (CCL9), whereas noncanonical RelB/p52 activation led to expression of Spi-B and Sox8 transcription factors, both deemed essential to maturation of M cells.[8,16] Along with RankL, expression of Spi-B and Sox8 are essential for the development of GP2-positive M cells. Both Spi-B and Sox8 mutant mice showed the absence of mature M cells with GP2, whereas $Marcksl1^{+}AnnexinV^{+}$ immature M cells were intact.[7,8,17,18] Spi-B-/- still showed Sox8 expression and Sox8-/- mice expressed Spi-B, and even though both mice had activation of both NF-κB transcription pathway, p50/RelA, and p52/RelB, they still showed an immature M-cell phenotype lacking the expression of Gp2.[16] Taken together, despite their critical role in the onset of mucosal immune responses, M-cell development and their differentiation into maturity have not yet been fully characterized, partly because of their rarity in the gastrointestinal tract.[19] Importantly, the sole overexpression of Spi-B and Sox8 are not sufficient for the induction of GP2 receptor (ie, M-cell maturation), suggesting that additional M-cell–specific transcription factors are needed.[8]

Intestinal cell differentiation, development, and functionality are regulated by several factors, one of the indispensable ones being polycomb group proteins. Polycomb group proteins are essential for embryonic stem cell self-renewal and pluripotency, but they also are necessary for the maintenance of cell identity and cell differentiation throughout life.[20] They broadly form 3 groups of polycomb-repressive complexes (PRCs) known as PRC1, PRC2, and polycomb repressive DeUBiquitinase, each of these complexes reassemble chromatin by explicitly defined mechanisms that involve variable configurations of core and accessory subunits. This configuration is shown by the way PRC2 catalyzes trimethylation of histone H3 lysine 27 (H3K27me3) and presents a binding site for PRC1 in embryonic stem cells.[21] Previously, it has been shown that PRC2 played a repressive role of expression of developmental regulators necessary for cell differentiation.[22] Interestingly, genes critical for cell identity lose their methylation on H3 lysine K27, whereas genes that regulate

Abbreviations used in this paper: **ChIP-seq, chromatin immunoprecipitation sequencing; FAE, follicle-associated epithelium; ENR500, epidermal growth factor, Noggin, R-spondin 500 ng/mL media; ENRI media, epidermal growth factor, Noggin, R-spondin, Wnt inhibitor IWP2 media; Esrrg, estrogen-related receptor γ; GALT, gut-associated lymphoid tissue; GP2, glycoprotein 2 receptor; Gro-seq, global run-on sequencing; KO, knockout; LTβR, lymphotoxin-β receptor; M cell, Microfold cell; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PP, Peyer's patch; PRC2, polycomb repressive complex 2; Rank, receptor activator of nuclear factor κB; RankL, receptor activator of nuclear factor κB ligand; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; WENRC media, Wnt, epidermal growth factor, Noggin, R-spondin, Chir media.**

Most current article

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alternate cell types keep their methylation and remain repressed.[23] In human embryonic stem cells, Wingless/integrated (Wnt)-signaling genes are bound by PRC2, analogously this also is shown in adult tissues (eg, in adipogenesis).[24] The integrity and homeostasis of healthy intestine is regulated partly by canonical Wnt signaling and it also has been shown that secretory and absorptive progenitor cells show comparable levels of histone modifications at most of the same cis elements in the genome.[25] Our study and others have found out how PRC2 regulates a substantial subset of genes that were involved in canonical Wnt signaling and contributed to the differentiation of Lgr5-expressing stem cells to secretory and absorptive cell types in the intestine.[26–28]

To further understand the complexity of M-cell differentiation, we asked if PRC2 regulates M-cell differentiation. We used high-throughput tools such as chromatin immunoprecipitation sequencing (ChIP-seq) and global run-on sequencing (Gro-seq) to identify factors that contribute to the function and development of M cells in the intestine. We identified a total of 12 transcription factors that are regulated by PRC2 exclusively during M-cell differentiation, of which 6 were down-regulated and 6 were up-regulated. One of the M-cell–specific transcription factors, estrogen-related receptor γ (Esrrg), was found to be essential for the differentiation of mature GP2+ M cells in vitro. We found that Esrrg was expressed exclusively in M cells in Peyer's patches and was shown to be critical for the activation of Sox8 transcription factor. Esrrg expression was intact even in Sox8-deficient mice, and was dependent on the activation of noncanonical NF-κB signaling. These observations show that Esrrg is a crucial player in the differentiation and functionality of a mature GP2+ M cell.

Results

PRC2 Does Not Restrict M-Cell Differentiation

We and others have previously shown that disrupting PRC2 activity leads to a precocious expression of terminal differentiation markers of intestinal epithelium.[27–29] Moreover, PRC2 has been shown to preserve intestinal progenitors and restrict secretory cell differentiation.[26] Contrary to absorptive cell differentiation, when organoids were treated with RANKL the level of expression of PRC2 members Enhancer of Zeste homolog 2 (Ezh2) and Supressor of Zeste homolog 12 (Suz12) were comparable with the levels in organoids grown in standard organoid culture media with ENR500 (epidermal growth factor, Noggin, R-spondin 500 ng/mL) (Figure 1*A*). Next, we asked if PRC2 inhibition (Figure 1*B*) can augment M-cell differentiation and we saw that, contrary to enterocyte differentiation, expression of all M-cell markers are down-regulated when the activity of PRC2 is inhibited pharmacologically during the RANKL-induced, M-cell differentiation (Figure 1*C*).[27,29]

PRC2 Regulated Genes During M-Cell Differentiation

It has been shown previously that PRC2 regulates transcription factors that are necessary to intestinal stem cell maintenance and differentiation (eg, Achaete-Scute Family BHLH Transcription Factor 2 [Ascl2][27,30] and Atonal BHLH Transcription Factor 1 [Atoh1][26]). Because PRC2 mainly regulates genes involved in development or signaling,[31] we reasoned that identifying genes regulated by PRC2 during M-cell differentiation might show the gene network necessary to this cell type in the intestine. M-cell differentiation was induced in mouse intestinal organoids with recombinant RankL, gene expression was analyzed with Gro-Seq, and PRC2 target genes were identified with ChIP-seq by using H3K27me3 antibody. Genes expressed differentially after RankL treatment are shown in Figure 2*A* (Supplementary Table 1). ChIP-seq performed with H3K27me3 antibody showed a significant number of genes regulated by PRC2 for M-cell differentiation. When comparing our 3 different Chip-seqs (organoids in Wnt, epidermal growth factor, Noggin, R-spondin, Chir [WENRC] media [stem cell conditions]; epidermal growth factor, Noggin, R-spondin, Wnt inhibitor IWP2 [ENRI] media [enterocyte conditions]; and RankL media [M-cell differentiation]), we observed that, in M cells, 38 (9.2%) and 35 (10.3%) genes were up-regulated but silenced by PRC2 in WENRC and ENRI, respectively. Thirty-two PRC2 target genes were uniquely up-regulated during M-cell differentiation but not in enterocyte differentiation. Forty-six (27.7%) and 52 (11.5%) PRC2 target genes were silenced in organoids treated with RANKL, but expressed in stem cell and differentiation conditions, respectively. Forty-two genes were uniquely silenced in M-cell, but not in enterocyte, differentiation. PRC2 target genes are shown in Figure 2*B–D*, and the M-cell–specific accumulation and ablation of H3K27me3 signal in the gene promoters are shown in Figure 2*E*. Gene ontology analyses indicated that PRC2 regulates many DNA-binding transcription factors during M-cell differentiation (Figure 2*G*). A total of 12 transcription factors were expressed differentially: 6 were silenced by PRC2 in M cells and 6 were expressed specifically in M cells but repressed by PRC2 in organoids grown in both stemness and enterocyte conditions. The 6 PRC2 target genes expressed specifically in M cells were *Sox8, Atoh8, Esrrg, Smad6, Maf,* and *Zfp819*. The 6 genes silenced were *Hoxb5, Hoxb9, Sp9, Sp5, Nr4a1,* and *Atf3*.

Esrrg Is Expressed in M Cells and Induced by Rank–RankL Signaling

Of the 6 transcription factors that were expressed specifically in M cells in a PRC2-dependent manner (Figure 2*A* and *B*), Esrrg turned up as one of the most highly expressed PRC2-regulated transcription factors during M-cell differentiation ($\log_2$ fold changes, –6.64 RankL vs ENRI and –4.76 in RankL vs WENRC comparisons) (Figure 3*A*). Immunohistochemistry analysis for Esrrg in PP showed that Esrrg was localized in the FAE cells (Figure 3*B*). RNA was isolated from FAE isolated from PP and villus epithelium and the reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis confirmed that the Esrrg was enriched significantly in FAE (Gp2 as a marker) when compared with villus epithelium (Figure 3*C*). To ascertain

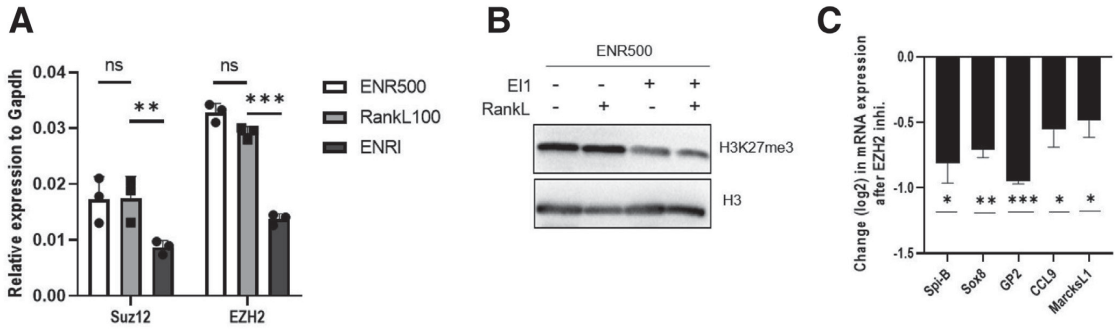


Figure 1. PRC2 members are expressed in M cells. (*A*) RT-qPCR analyses of the expression of Suz12 and Ezh2 in mouse intestinal organoids grown in ENR, ENR+RankL, and ENRI conditions. (*B*) Immunoblot of H3K27me3 and H3 in organoids treated with Ezh2 inhibitor (EI1) inhibitor. Data are representative of 2 independent experiments. (*C*) RT-qPCR analyses of the expression of M-cell marker genes in RankL-treated organoids with or without EZH2 inhibitor EI1. An unpaired 2-tailed Student *t* test was performed. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .005$, N = 3 independent experiments. Values are presented as means ± SD. Gapdh, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA.

that Esrrg is a novel RankL-induced M-cell gene, RT-qPCR analyses were performed for organoids before and after 4 days of RankL treatment. The expression of Esrrg, together with M-cell marker Gp2, was induced significantly with RankL treatment (Figure 3*D*). To confirm that Esrrg expression was regulated specifically by Rank-Rankl signaling, we generated Rank-deficient mouse intestinal organoids using the Lenti-V2 CRISPR/Cas9 plasmid and found that in Rank-deficient organoids, RankL treatment did not induce the expression of Esrrg (Figure 3*E*). The Rank knockout (KO) organoids were validated by immunoblot analysis (Figure 3*F*).

Noncanonical NF-κB Activation Is Necessary and Sufficient for Esrrg Expression

Rank–RankL signaling was shown previously to activate canonical as well as noncanonical NF-κB pathways. Lymphotoxin-β receptor (LTβR) signaling was implicated in inducing both classic p50–RelA and noncanonical p52–RelB heterodimers in PP.[32] Mouse intestinal organoids were grown for 3 days in the presence of LTα1β2, the ligand of LTβR, and we observed that, similar to Spi-B, Esrrg expression also was increased significantly (Figure 4*A*). To identify if canonical NF-κB had a role in Esrrg expression specifically, mouse organoids were grown in the presence and absence of RankL and SC-514, a specific inhibitor of IκB kinase-β (inhibitor of nuclear factor kappa B).[33] We found that inhibiting canonical NF-κB with SC-514 completely abrogated the expression of Esrrg (Figure 4*B*), as was reported similarly for Spi-B.[16] Both p50-RelA and p52-RelB overexpression led to increased expression of Esrrg (Figure 4*C*). It has been shown previously that p50/RelA (canonical NF-κB) directly targets the transcription of RelB.[34] The treatment of p52/RelB overexpression organoids with SC-514 could not suppress the activation of Esrrg (Figure 4*D*). To conclude, these data indicate that noncanonical NF-κB is necessary and sufficient to induce Esrrg.

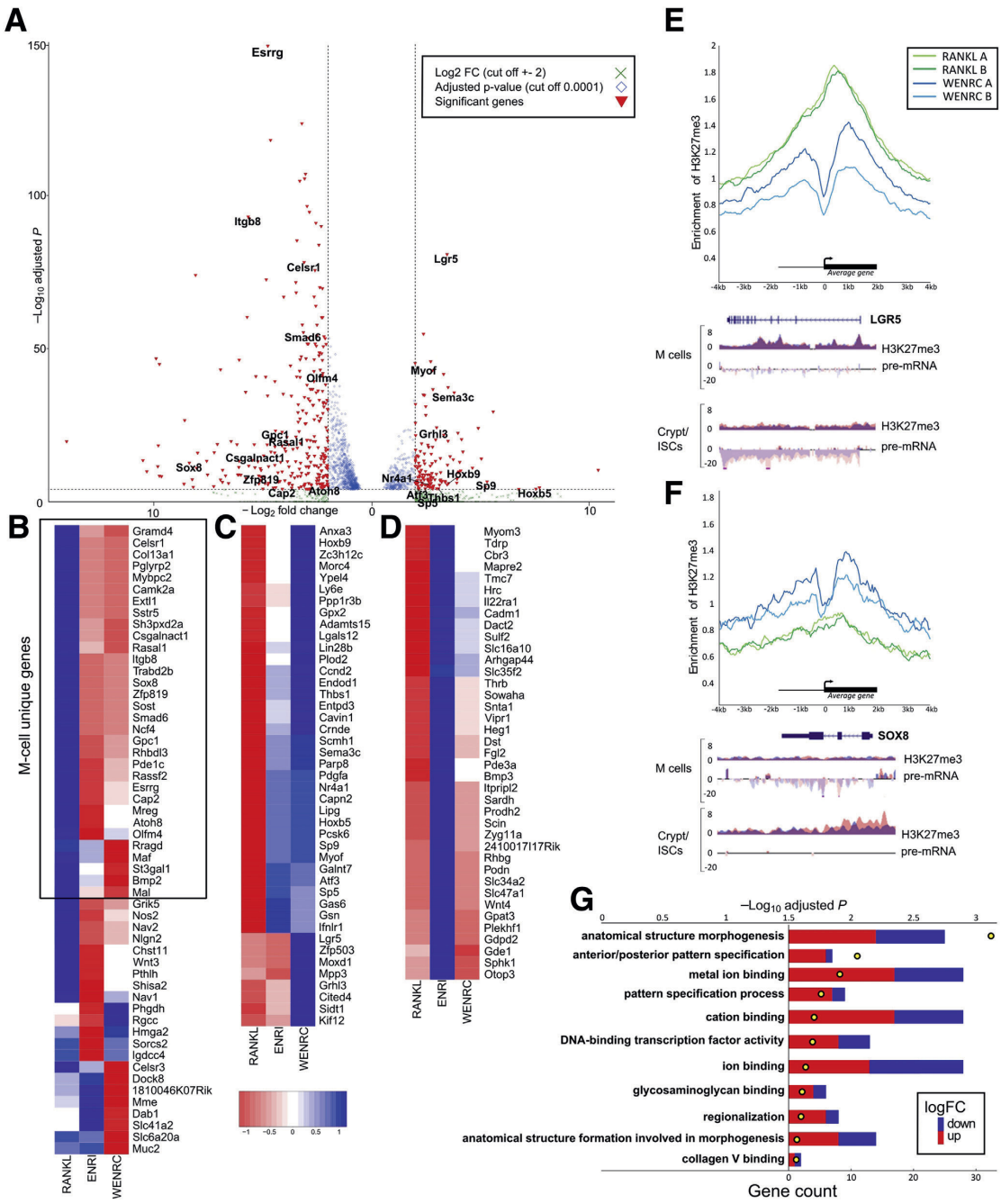
Esrrg Expression Is Required for Sox8 Activation and Maturation of M Cells

Given that Esrrg was expressed prominently in PP and regulated by noncanonical NF-κb expression, we sought to see if abolition of Esrrg had any effect on M-cell differentiation and development. To investigate this, mouse intestinal organoids deficient in Esrrg were generated by LentiCRISPR V2 CRISPR-Cas9 genome editing. Targeting of Esrrg by the guide RNA selected (Figure 5*A*) resulted in a significant reduction in expression of Esrrg protein (Figure 5*B*). Esrrg-deficient organoids were grown in the presence and absence of RankL for 3 days. RT-qPCR analysis showed that Gp2 was nearly absent and Sox8 expression was reduced significantly in Esrrg-targeted organoids (Figure 5*C*). Gp2 and Sox8 immunostaining in Esrrg-targeted organoids showed an absent and reduced expression, respectively (Figure 5*D* and *E*). However, expression of Spi-B and TNF Alpha Induced Protein 2 (Tnfaip2) remained unaffected in the Esrrg-deficient organoids (Figure 5*C* and *E*). Early M-cell differentiation markers such as CCL20, CCL9, and MarcksL1 were affected significantly by the lack of Esrrg as well (Figure 5*F*). Aif1, which is a regulatory gene for transcytosis in M cells, also was found to be affected severely by lack of Esrrg expression (Figure 5*F*). Our observations showed that Esrrg is required for the expression of Sox8 and for the early markers as well as late maturation steps in M-cell differentiation. To validate for off-target effects, we knocked out Esrrg with a second guide RNA; comparable results were observed (Figure 6*A* and *B*).

Esrrg Acts Upstream of Sox8 Expression

Spi-B and Sox8 were found to be 2 key transcription factors involved and essential for M-cell differentiation and regulation of expression of other M-cell–associated genes.[7,8,17,18] Spi-B and Sox8 mutant mice lacked GP2+ mature M cells and were unable to transcytose antigens. However, it was observed that Spi-B was dispensable to

the expression of Sox8 even though Spi-B expression was reduced moderately in the Sox8 mutant mice.[8] To investigate if Esrrg is affected by knockout of Sox8, RNA was isolated from FAE of PPs from Sox8+/+ and Sox8-/- mice. RT-qPCR analysis of FAEs from PPs of Sox8+/+ and Sox8-/- showed that the expression of Esrrg was higher in Sox8-/- mice (Figure 7*A*). Organoids isolated from Sox8+/+ and Sox8-/- mice were treated with RankL and expression of Esrrg expression was analyzed by RT-qPCR and Western blot. The analysis



showed Esrrg expression was intact and similar to the in vivo data (Figure 7*B* and *C*). This suggests that Esrrg acts upstream of Sox8 and could play a role in the activation of Sox8. Next, we explored how Esrrg was affected by Spi-B. Spi-B–deficient organoids were generated by LentiCRISPR V2 genome editing and grown in the presence and absence of RankL. qPCR analysis showed that Esrrg was affected moderately by the lack of Spi-B (Figure 7*E*). Western blot analysis also indicated that Esrrg was expressed less in Spi-B–deficient organoids (Figure 7*F*). Spi-B KO organoids were validated by immunoblot analysis (Figure 7*D*).

Overexpression of Esrrg Is Not Sufficient for Gp2+ M Cells but Esrrg Agonist Augmented Gp2 Expression

Spi-B expression in Sox8 KO mice and Sox8 expression in Spi-B KO mice did not lead to a mature GP2+ M-cell phenotype in either of these mice.[7,8] Because we found that the expression of Gp2 was dependent on Esrrg (Figure 5*C*), we sought to investigate if the overexpression of Esrrg alone could lead to up-regulation of Sox8 or Gp2 expression. Esrrg cloned into CSII-CMV-MCS-IRES2-Bsd overexpression vector was transduced into mouse intestinal organoids. RT-qPCR analysis showed that Esrrg alone was not adequate enough to induce Gp2 or other M-cell–specific transcription factor such as Spi-B or Sox8 (Figure 8*A*). Esrrg is an orphan nuclear receptor without known natural ligands. However, 4-hydroxytamoxifen has been shown to bind and inhibit Esrrg activity and phenolic acyl hydrazones; GSk4716 was identified as an agonist that enables the activation of other co-activators and other downstream targets.[35,36] The antagonist 4-hydroxytamoxifen along with RankL induced a similar result to the lack of Esrrg protein, Spi-B remained unaffected, although Sox8 and Gp2 were affected significantly (Figure 8*B*). Organoids treated with agonist GSK4716 and RankL did not show a significant increase in Sox8 or Spi-B expression, however, Gp2 expression was augmented slightly with GSK4716 (Figure 8*C*).

Discussion

Our data show the PRC2-regulated genes during the differentiation of intestinal microfold cells. Because PRC2 is the master regulator of development, it is very likely that many of the identified PRC2 targets that are induced specifically in M cells contribute to the maturation of this cell type. Transcription factors usually are expedient to development, and we identified 6 PRC2-regulated transcription factors up-regulated specifically after the RankL-induced M-cell differentiation. Among those was previously identified transcription factor Sox8, which we also here showed to be necessary for M-cell differentiation.[8] Because Esrrg was clearly the most highly induced PRC2 target gene during M-cell differentiation, we studied it in more detail in intestinal organoids and showed that it is indispensable to the maturation of intestinal stem cells into GP2+ M cells. Esrrg is a member of the ESRR nuclear-receptor family, which also includes ESRRA and ESRRB.[37] This subfamily of orphan nuclear receptors has been shown to share target genes, coregulatory ligands, and sites of action with ERs. Esrrg was implicated to control macrophage function indirectly through regulation of intracellular iron. In response to *Salmonella typhimurium* infection, hepatic expression of the hormone hepcidin is up-regulated by ERRγ downstream on interleukin 6 signaling.[34,38] However, no prior data about the expression and function of Esrrg in M cells and M-cell induced transcytosis of antigens exist. We defined the specific expression of Esrrg by M cells in mouse FAEs and how its expression was up-regulated by induction of RankL and was under the influence of the RankL–Rank pathway. The loss of Esrrg led to a lack of expression of the GP2 receptor in Esrrg KO organoids, which is characteristic of a mature M cell, and a lack of GP2 in M cells has been shown to result in attenuation of antigen sampling and transcytosis. This distribution of Esrrg along with the phenotype of Esrrg KO organoid highlights a critical role for Esrrg in the maturation of functional M cells.

RelB/p52 activation was shown to up-regulate Spi-B.[16] Similarly, here we show that Esrrg also is regulated by the activation of the noncanonical NF-κB pathway. We believe that the expression of Esrrg and Spi-B is regulated downstream in parallel by RankL-Rank-RelB/p52 signaling (Figure 9). Esrrg has been shown to behave as a constitutive activator of transcription.[39] Here, we show that Esrrg is needed for the activation of Sox8. Sox8 was discovered to be indispensable for the expression of GP2, and Sox8 KO mice showed a decrease in uptake of antigens and a significant

Figure 2. (***See previous page***). **PRC2-regulated genes during M-cell differentiation.** (*A*) Differentially expressed genes during M-cell differentiation detected with Gro-Seq. Signal is depicted by volcano plot comparing organoids before and after RankL treatment. X-axis and Y-axis indicate the log2 fold change and –log10 adjusted *P* value. Differentially expressed genes are marked (Gro-seq with log2 fold change cut-off value at ± 2, $P < .0001$). Up-regulated genes from ENRI conditions were removed to show only RankL-specific regulation. (*B*) Genes up-regulated in M cells compared with stem cells and enterocytes. Heatmap of differentially expressed (Gro-seq with log2 fc cut-off value at ± 2 and *P* value $< .0001$) and H3K27me3 regulated (log2 fc ± 2, $P < 10^{-6}$) genes in RankL, ENRI, and WENRC-treated organoids showing centered log2 fold change. (*C*) Genes down-regulated by H3K27me3 in M cells and enterocytes compared with stem cells. (*D*) Genes down-regulated by H3K27me3 in stem cells and M cells compared with enterocytes. Composite enrichment analysis of H3K27me3 signal density +-4000 bases around transcription start sites in genes (*E*) specifically silenced by PRC2 in M cells and (*F*) specifically expressed in M cells. (*E*) Example genes Lgr5 (*below*) and (*F*) Sox8 (*below*). (*G*) Gene ontologies enriched ($P < .05$) in differentially expressed PRC2-target genes between M cells and stem cells (Gro-seq log2 fc ± 2, $P < .0001$, and H3K27me3 log2 fc ± 2, $P <$ 10-6) in molecular function and biological process. LogFC shows the direction of gene expression between M cells and stem cells. ISC, intestinal stem cells; mRNA, messenger RNA.

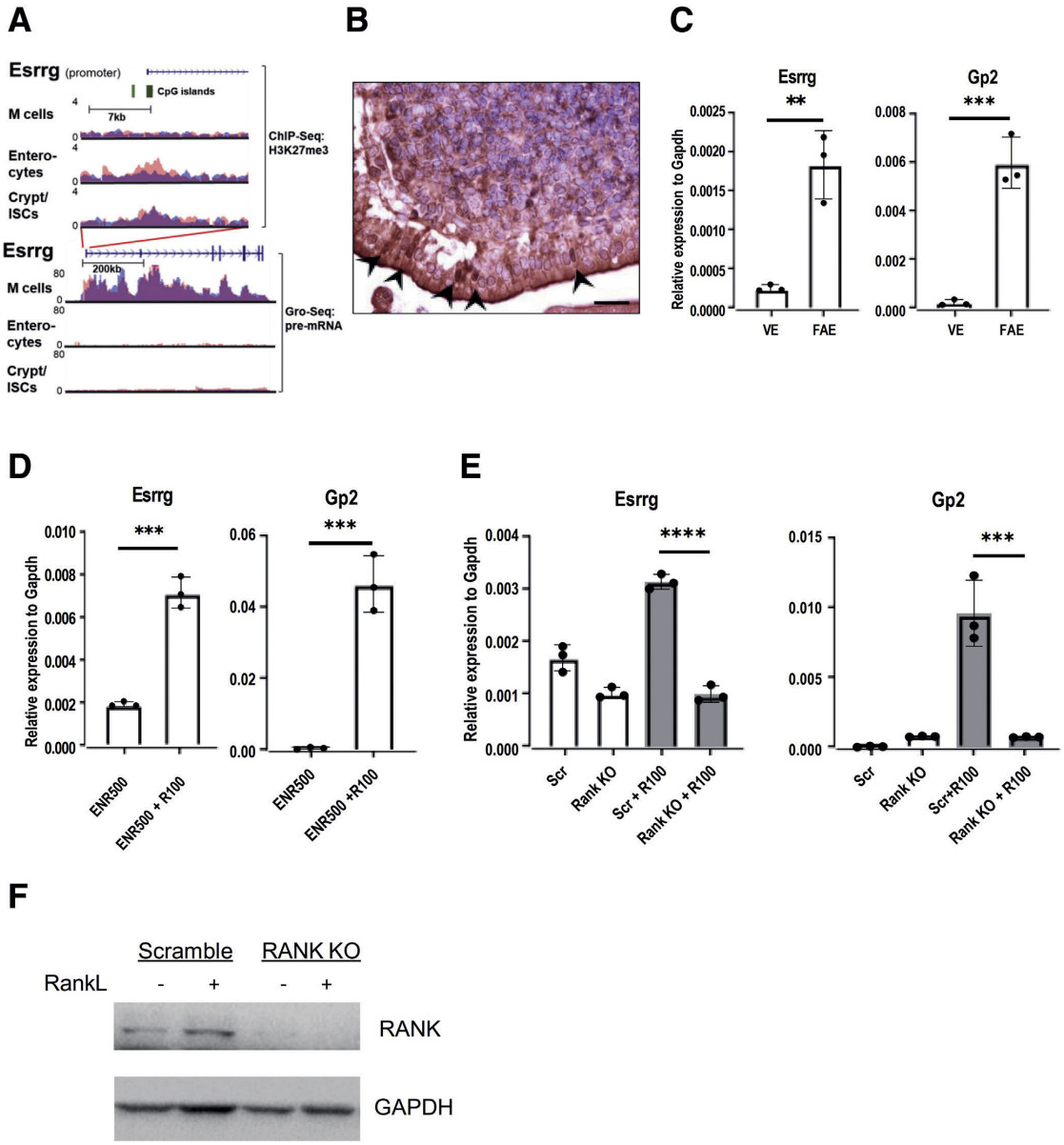


Figure 3. Esrrg is expressed in FAE in PPs and is dependent on Rank–RankL signaling. (*A*) H3K27me3 occupancy at CpG islands spanning the promoter and first exon of the Esrrg gene in organoids treated with RankL (M cells) or inhibited with IWP2 (enterocytes) or treated with Wnt3a and Chir99021 (crypt/intestinal stem cells). Below, pre–messenger RNA (mRNA) expression of Esrrg in organoids treated as described earlier (y-axis: normalized tag count, ENR500 = R-spondin 500 ng/mL, R100 = Rankl 100 ng/mL). (*B*) Section of PP from wild-type mice stained with Esrrg antibody. *Arrowheads* indicating Esrrg expression in the nuclei of M cells in FAE. (*C*) RT-qPCR analysis of Esrrg and Gp2 in the FAE and villous epithelium (VE) from C57BL/6JRj mice (N = 3 from wild-type mice). (*D*) Organoids generated from wild-type mice were stimulated with 100 ng RankL for 4 days. Esrrg and Gp2 expression was examined by qPCR analysis. (*E*) Rank KO organoids and Scrambled organoids generated by lentiCRISPR *v*2 were incubated with RankL for 4 days, Esrrg and Gp2 expression was analyzed by quantitative RT-qPCR. (*C–E*) An unpaired 2-tailed Student *t* test was performed for 3 independent experiments. $^{****}P < .0005$, $^{***}P < .005$, and $^{**}P < .01$. Values are presented as means ± SD. (*F*) RANK protein expression in RANK KO and Scrambled cells generated by lentiCRISPR v2 genome editing in C57BL/6JRj intestinal organoids. Organoid lysates were analyzed by Western blot. Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

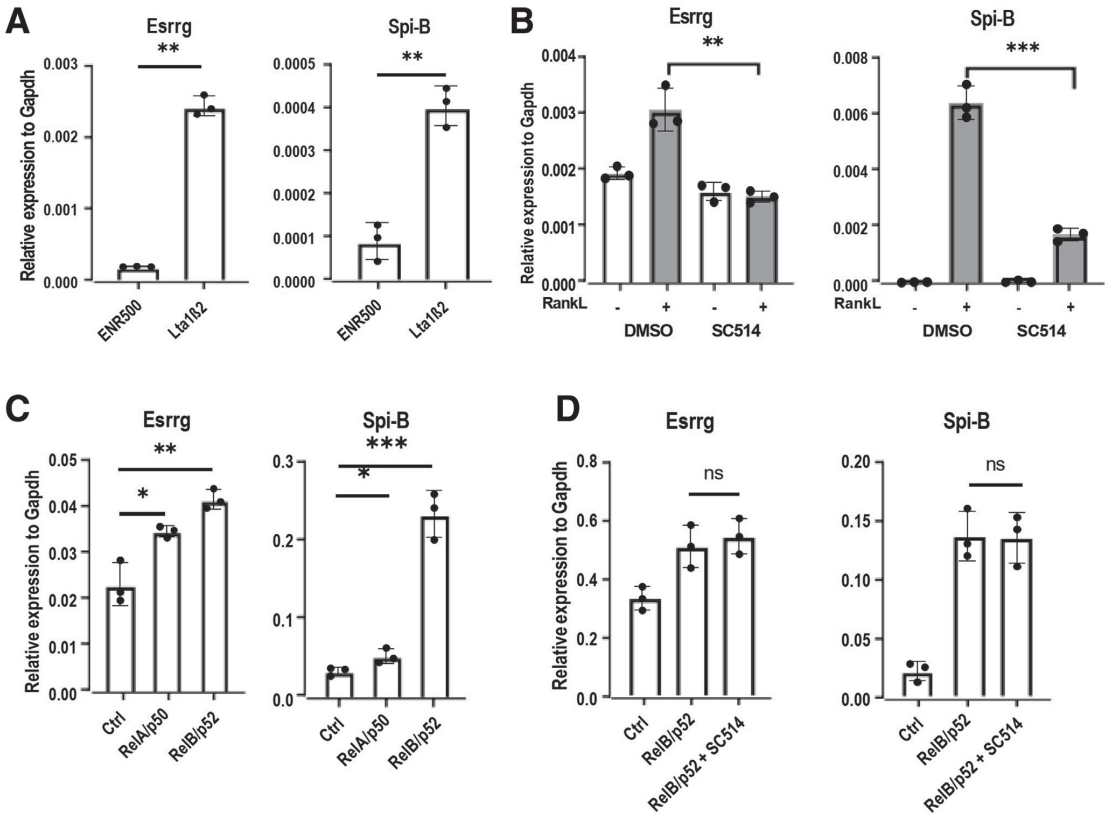
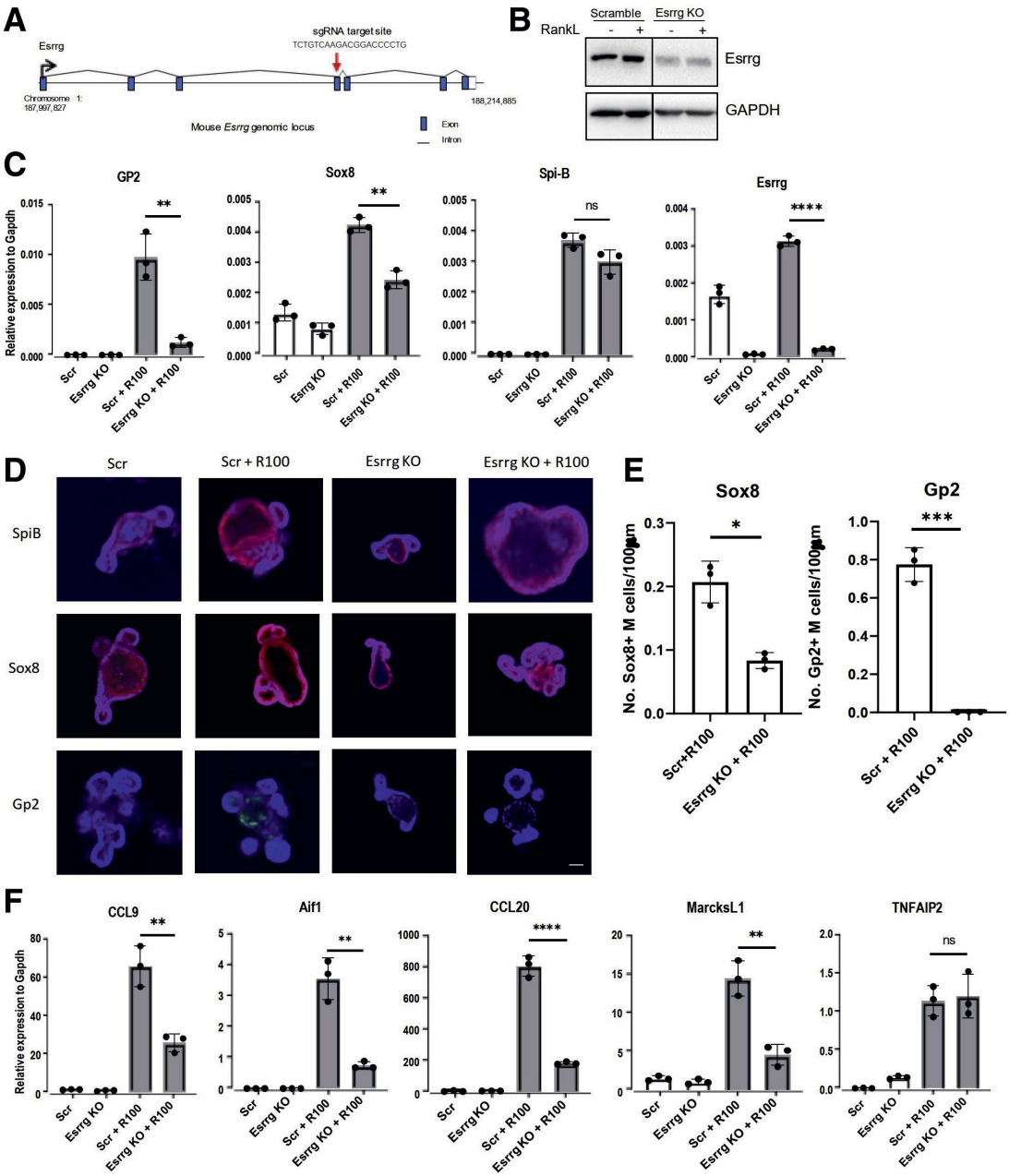


Figure 4. Esrrg expression is induced by RelB/p52 activation. (*A*) Ltα1β2 prominently up-regulated the expression of Esrrg in organoids. Organoids from C57BL/6JRj mice were stimulated with LTα1β2 for 3 days, and the gene expression was analyzed by qPCR. (*B*) Organoids from wild-type mice were stimulated with RankL for 3 days in the absence or presence of 125 μmol/L SC-514. Gene expression was analyzed by qPCR. (*C*) Organoids were transduced to express classic and noncanonical NF-κB and the expression of Esrrg and Spi-B (control) are represented relative to glyceraldehyde-3-phosphate dehydrogenase (Gapdh). (*D*) Organoids expressing p52 and RelB in the presence of SC-514 for 3 days, and the expression of Esrrg and Spi-B was analyzed by qPCR. Values in all are presented as the means ± SD and an unpaired 2-tailed Student *t* test was performed, N = 3 independent experiments. **P* < .05, ***P* < .01, ****P* < .005. Ctrl, control; DMSO, dimethyl sulfoxide.

decrease of IgA+ immunoglobulins. Sox8 also was shown to bind directly to the Gp2 promoter along with SpiB.[8] The significant decrease in Sox8 expression could explain why Esrrg KO organoids were not able to activate Gp2. Sox8 expression alone cannot lead to an increase in Gp2 expression because enhancer activation by SOX proteins require DNA binding partners specific for each member of the SOX family. These DNA binding partners aid in stabilizing the SOX family to their target regions.[40] This suggests that Esrrg-activated Sox8 requires another molecule downstream of Spi-B or through another pathway to bind to Gp2 to induce its expression, however, further exploration is required to confirm this. Esrrg overexpression alone did not lead to expression of Sox8, confirming the need of a ligand and/or other factors to activate downstream targets.

A major portion of our investigation of Esrrg in M cells was conducted in vitro and Esrrg ablation in mouse models is required to further ascertain its role. Interestingly, Sox8 deficiency did not affect early M-cell markers, however, Esrrg KO organoids showed a drastic decrease in expression of early M-cell markers such as CCL9 and MarcksL1. Mature marker Gp2-receptor expression was decreased significantly as well. The loss of Aif1 expression in Esrrg KO means that transcytotic capacity of the M cells would be affected as well. Esrrg KO organoids also impaired the expression of Sox8, and in the Sox8 KO mice we observed that Esrrg expression still was intact and even observed to have a higher expression in vivo and in vitro, suggesting that Esrrg was not affected by the absence of Sox8 and possibly could be acting upstream of it. The significance of Esrrg was confirmed further with our antagonist and agonist experimental study. Although tamoxifen is known to trigger multiple signaling pathways in the cell, it has been identified as an antagonist for Esrrg

receptor; tamoxifen was able to significantly decrease the expression of Gp2 and Sox8 similar to the Esrrg KO organoids. The treatment of intestinal organoids with RankL and Esrrg agonist GSK4716 augmented the expression of Gp2 when compared with organoids with just RankL treatment. This could be because GSK4716 binds to Esrrg and activates several other unknown downstream targets that combine to attenuate Gp2 expression. Esrr family members are known to be orphan receptors, meaning they might not need a ligand for its function, or the ligand remains unknown. Overexpression of Esrrg did not lead to an increase in Sox8 expression or



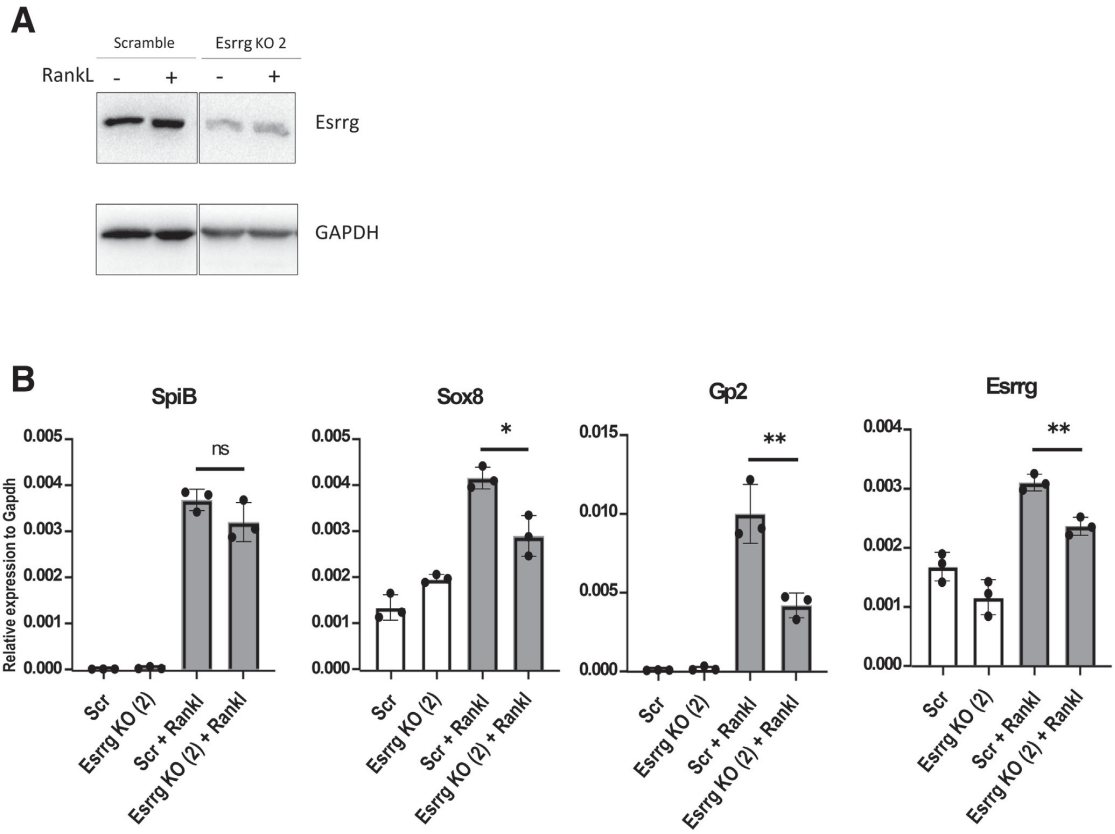


Figure 6. Esrrg abolition by genomic RNA (gRNA) 2 impairs Sox8 activation and the functional maturation of M cells. (*A*) Esrrg protein expression in Esrrg KO2 with gRNA 2 and Scrambled cells generated by lentiCRISPR v2 genome editing in C57BL/6JRj intestinal organoids. Organoid lysates were analyzed by Western blot. (*B*) qPCR analysis of M-cell–associated genes expressed in Esrrg KO2 and Scrambled intestinal organoids stimulated by RankL for 4 days. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Values in all are presented as the means ± SD. Unpaired 2-tailed Student t test, N = 3 independent experiments $^{*}P < .05$, $^{**}P < .01$.

other transcription factors necessary for mature M-cell differentiation, presumably because a specific ligand might be necessary. However, further studies are needed to prove this.

In conclusion, we identified several previously unknown PRC2-regulated genes implicated in M-cell differentiation. One of the genes we identified, Esrrg, is a key transcription factor, and therefore is required for the functional development and M-cell differentiation that is pertinent for constant surveillance of the mucosal lining of the gastrointestinal tract. We believe that the further exploration of other activators of Gp2 will lead to better elucidation of M-cell maturation and antigen transcytosis. This will create the potential to

Figure 5. (***See previous page***). **Abolition of Esrrg impairs Sox8 activation and the functional maturation of M cells.** (*A*) Schematic representation of Esrrg KO design by CRISPR-Cas9 genome editing in mouse intestinal organoids. Exon, intron, and genomic position are indicated. (*B*) Esrrg protein expression in Esrrg KO and Scrambled cells generated by lentiCRISPR v2 genome editing in C57BL/6JRj intestinal organoids. Organoid lysates were analyzed by Western blot. (*C*) qPCR analysis of M-cell–associated genes expressed in Scrambled and Esrrg KO stimulated by RankL for 4 days. (*D*) Immunostaining images for Spi-B (red), Sox8 (red), and GP2 (green) in Scrambled organoids with and without 100 ng RankL for 4 days and Esrrg KO with and without 100 ng RankL. *Scale bars*: 100 μm. (*E*) The number of GP2+ M cells and Sox8+ M cells per length of epithelium of organoids was compared between Scr+R100– and Esrrg KO+R100–treated organoids (n = 3). Images are representative of 3 independent experiments. (*F*) qPCR analysis of early markers of M-cell–associated genes expressed in vitro in the presence of RankL for 4 days. Values in all are presented as the means ± SD. Unpaired 2-tailed Student t test, N = 3 independent experiments. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .005$, $^{****}P < .0005$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sgRNA, single guide RNA.

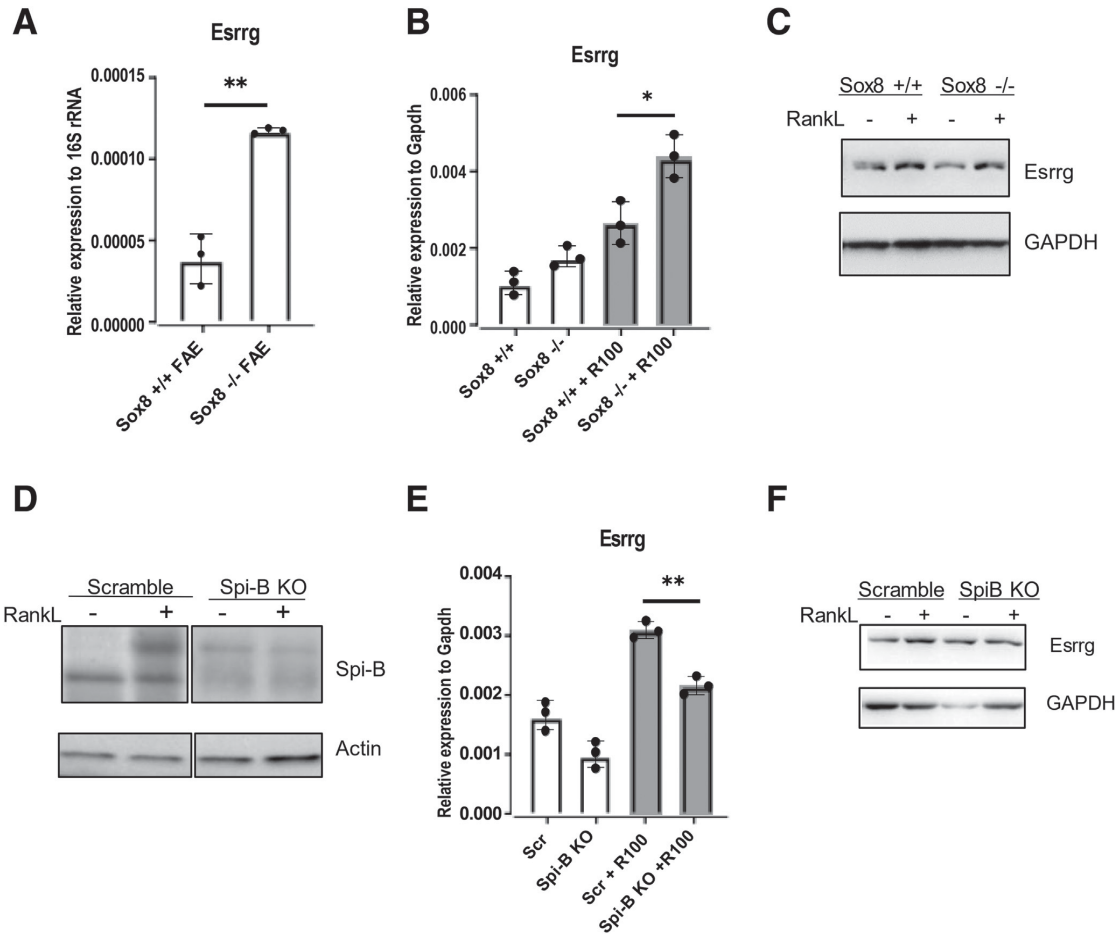


Figure 7. Esrrg expression and its relation to other M-cell developmental markers Spi-B and Sox8. (*A*) Esrrg expression was unaffected by lack of Sox8 expression. qPCR analysis of Esrrg and Gp2 in the FAE and villous epithelium from Sox8+/+ and Sox8-/- mice. (*B*) Organoids generated from Sox8+/+ and Sox8-/- mice were stimulated with and without RankL for 4 days. Esrrg expression was examined by qPCR analysis. (*C*) Organoids isolated from Sox8 wild-type and Sox8 KO mice were lysed and analyzed by Western blot for Esrrg expression. (*D*) Spi-B protein expression in Spi-B KO and Scrambled cells generated by lentiCRISPR v2 genome editing in C57BL/6JRj intestinal organoids. Organoid lysates were analyzed by Western blot. (*E*) qPCR analysis of Esrrg in a Spi-B KO intestinal organoid by lentiCRISPR v2. (*F*) Organoid lysates for Esrrg in Scrambled and SpiB KO organoids were analyzed by Western blot. Values in all are presented as the means ± SD. Unpaired 2-tailed Student t test, N = 3. $^{*}P < .05$, $^{**}P < .01$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; rRNA, ribosomal RNA.

provide strategic innovation in support of mucosal/oral vaccine advancement.

Materials and Methods

Animals

C57BL/6JRj mice were purchased from Janvier labs (Le Genest-Saint-Isle, France) and were maintained with constant breeding. The *Bac-Cre-ERT2;Sox9f/f;Sox8-/-;Rosa26Eyfp* mice were a gift from Raphael Jimenez (University of Granada, Granada, Spain). These mice were backcrossed with C57BL/6JRj to isolate the Sox8-/- allele. Sox8+/- were bred to obtain Sox8-/- and littermate controls: Sox8+/- and Sox8+/+. F1–4 mice were used for gene or protein expressions. Genotyping of the wild-type, heterozygous, and deleted alleles was performed by PCR with the following primers: F1, 5'-GTCCTGCGTGGCAACCTTGG-3'; R1, 5'-GCCCACACCATGAAGGCATTC-3'; and F3, 5'-TAAAAA TGCGCTCAGGTCAA-3'. Conventional conditions were observed for the maintenance of these mice at the pathogen-free animal facility of the faculty of Medicine and Health Technology. All animal experiments were approved by the Finnish National Animal Experiment Board (permit ESAVI/5824/2018).

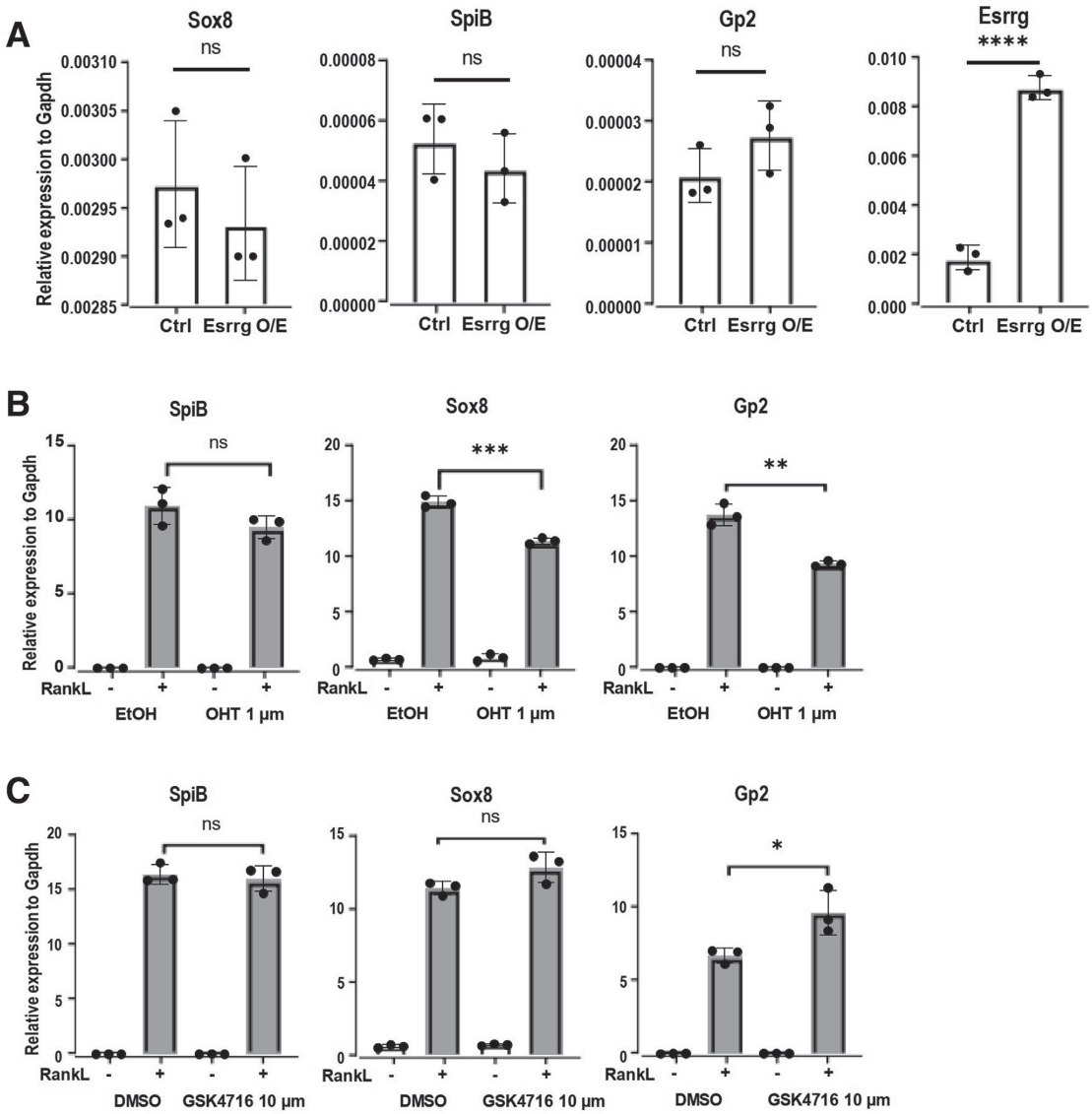


Figure 8. Esrrg alone is not sufficient for maturation of Gp2+ M cells. (*A*) Intestinal organoids were dissociated and transduced by lentivirus encoding Esrrg. qPCR analysis of Spi-B, Sox8, and GP2 showed no significant changes. (*B*) Organoids were grown in the presence and absence of 100 ng/mL Rankl and 1 um tamoxifen-antagonist of Esrrg. Spi-B, Gp2, and Sox8 expression were analyzed with RT-qPCR. (*C*) Intestinal organoids from mice were grown in the presence and absence of 100 ng/mL Rankl and 10 um GSK 4718-agonist of Esrrg for 3 days. Spi-B, Gp2, and Sox8 were analyzed with RT-qPCR. Values are presented as the means ± SD. Unpaired 2-tailed Student t test, N = 3. *$P < .05$, **$P < .01$, ***$P < .005$, ****$P < .0005$. Ctrl, control; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; O/E, overexpression.

Intestinal Organoid Culture

Intestinal crypt isolation and culture techniques were observed as previously established by the protocols of Sato and Clevers[41] and de Lau et al.[17] Mouse duodenum were cut longitudinally, and the villi were gently scraped off with 2 glass slides. After a couple of phosphate-buffered saline (PBS) washes, they were cut into 2-mm pieces and pipetted up and down 5 times in 15 mL PBS with a 10-mL pipette, this step was repeated 3 times with fresh PBS. The pieces were incubated in 10 mmol/L EDTA in PBS for 20 minutes, rocking at room temperature. The pieces were vigorously suspended in cold PBS and the mixture was strained

through a 70-μm cell strainer (cat no: 22363548; Fisher Scientific, Waltham, MA). This mixture was enriched to crypt fraction through centrifugation at 150 $\times$ g for 5 minutes. The enriched crypts were embedded in Matrigel (cat no: 356255, lot 9119006; Corning, Corning, NY), and 30 uL were plated on a 24-well plate. Crypts were cultured in an optimal medium consisting of advanced Dulbecco's modified Eagle medium/F12 (cat no: 12634010; Thermo Fisher Scientific, Waltham, MA) that contained HEPES (10 mmol/L, cat no: 15630-080; Sigma-Aldrich, St. Louis, MO), Glutamax (2 mmol/L, cat no: 35050-038; Thermo Fisher Scientific), penicillin-streptomycin (100 U/mL, cat no: 11659990; Sigma-Aldrich), B-27 supplement minus vitamin A (cat no: 17504-044; Thermo Fisher Scientific), N-2 supplement (cat no: 17502-001; Thermo Fisher Scientific), *N*-acetylcysteine (1 mmol/L, cat no: A9165, lot SLCB3719; Sigma-Aldrich), recombinant murine epidermal growth factor (50 ng/mL, cat no: PMG8043, lot 2135273; Gibco, Waltham, MA), recombinant murine Noggin (100 ng/mL, cat no: 250-38; PeproTech), and recombinant mouse R-spondin 1 (1 μg/mL, cat no: 120-38; R&D Systems, Minneapolis, MN). Media were changed every 2 days. For M-cell differentiation, recombinant mouse RankL (100 ng/mL, cat no: 315-11; PeproTech, Cranbury, NJ) was added to the media and incubated for 4 days. PRC2 was inhibited by addition of 5 μm of Ezh2 inhibitor (cat no: CAS 1418308-27-6 C; Calbiochem Chemicals, San Diego, CA). For activation of NF-κB, human LTα1β2 (1 μg/mL, cat no: 8884-LY; R&D Systems) was added into organoid cultures. Restriction of IκB kinase-β activity was achieved by adding SC-514 (125 μmol/L, cat no: 354812-17-2; Selleckchem, Houston, TX) for 3 days.[42] (Z)-4-Hydroxytamoxifen (1 μm, cat no: 68392-35-8; Sigma-Adrich) was used as an antagonist of Esrrg, and GSK 4716 (10 μm, cat no: 101574-65-6; Sigma-Aldrich) was used as an agonist of Esrrg.

ChIP-Seq Analysis

Intestinal organoids (in ENR500, ENRI, and RankL culturing conditions) were isolated from Matrigel with Cell Recovery Solution (Corning). This was followed by washes with cold PBS and dissociation into single-cell suspension using TrypLE Express (cat no: 12604013; Thermo Fisher Scientific) and counted. Cells (10×10^6) of each condition were cross-linked with formaldehyde, after which nuclei were isolated with lysis buffers 1, 2, and 3 as described,[43] and sonicated with a Covaris S220 ultrasonicator (Woburn, MA). The resulting nuclear extract was incubated with Dynal protein G beads, which were preincubated with 5 μg H3K27me3 (cat no: ab6002; Abcam, Cambridge, UK) or H3 antibody (cat no: ab1791; Abcam), respectively, at 4°C overnight. After washing and elution of bound complexes from the beads, cross-links were reversed by heating to 65°C. Immunoprecipitation and input DNA then were purified by treatment with RNase A, proteinase K, and phenol:chloroform extraction. The NEBnext UltraDNA-library preparation kit for Illumina (cat no: E7805; NEB, Ipswich, MA) was used to construct libraries from immunoprecipitation and input DNA and subjected to 50-bp, single-end, read sequencing with an Illumina (Ipswich, MA) Hiseq 2000 at EMBL Genecore (Heidelberg, Germany).

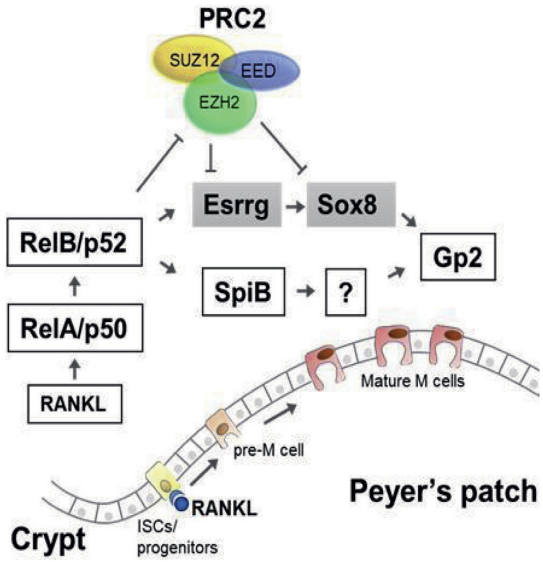


Figure 9. PRC2 regulates the differentiation of M cells. In the absence of Rank–RankL signaling, Esrrg and Sox8 genes are repressed by PRC2 in the intestinal epithelium. When migrating progenitors with Rank receptors bind to RankL in PPs, this induces NF-κB signaling, leading to loss of H3K27me3 from the gene promoters and activation of Esrrg and Sox8. Sustained expression of Sox8 and GP2 and differentiation of M cells is dependent on Esrrg. ISC, intestinal stem cells.

Gro-Seq Analysis

ENR500 and RankL-treated organoids were harvested (as in ChIP-Seq) and GRO-Seq was performed for an equal number of isolated nuclei. Nuclei extraction and a run-on reaction were performed as previously established.[44] For each replicate, 3 million cells were suspended to a final volume of 80–200 μL of freezing buffer. TRIzol LS (cat no: 10296028; Life Technologies, Carlsbad, CA) was used to extract RNA and fragmented for 13 minutes in 70°C using RNA fragmentation reagents (cat no: AM8740; Life Technologies), and later purified by running through a RNase-free P-30 column (cat no: 7326250; Bio-Rad, Hercules, CA). T4 Polynucleotide Kinase was used to dephosphorylate RNA for 2 hours (cat no: M02014; New England Biolabs, Ipswich, MA), followed by heat-inactivation. A total of 65 μL of blocking solution was added (5$\times$ volume of 0.25 $\times$ SSPE, 1 mmol/L EDTA, 37.5 mmol/L NaCl, 0.05% Tween-20, 0.1% Polyvinylpyrrolidone, and 0.1% ultrapure bovine serum albumin for 1 hour in room temperature), the anti-bromodeoxyuridine bead slurry (cat no: sc-500780; Santa Cruz Biotech, Dallas, TX) suspended in 500 μL binding buffer (0.25 $\times$ Sodium Chloride-Sodium Phosphate-EDTA [SSPE], 1 mmol/L EDTA, 37.5 mmol/L NaCl, 0.05%

Tween-20) was used to purify the dephosphorylated reaction. After binding for an hour in room temperature, the beads were washed 2× with binding buffer, 2× with low-salt buffer (0.2 × SSPE, 1 mmol/L EDTA, 0.05% Tween-20), 1× with high-salt buffer (0.2 × SSPE, 1 mmol/L EDTA, 135 mmol/L NaCl, 0.05% Tween-20), and, lastly, 2× with Tris elution buffer (1 × TE, 0.05% Tween-20). The elution of the RNA was completed with 130 μL elution buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 1 mmol/L EDTA, and 20 mmol/L dithiothreitol, followed by ethanol precipitation overnight. All buffers were supplemented with SUPERase ln (2 μL/10 mL, cat no: AM2694; Life Technologies). Library preparations were performed the next day as previously described.[45] The library was amplified with 14 cycles and the final product of 190–135 bp was extracted from a 10% Tris/Borate/EDTA gel. The DNA was purified from the gel using the Gel Extraction Kit (cat no: KO961, ThermoFisher) and eluted in TE buffer (TE 0.1% Tween + 150 mmol/L NaCl). ChIP DNA Clean and Concentrator Kit (cat no: D5205; Zymo Research Corporation, Irvine, CA) was used to purify the library, the DNA was quantified with the Qubit fluorometer (Waltham, MA), and sequenced with an Illumina HiSeq 2000 at EMBL Genecore.

ChIP- and Gro-Seq Data Analyses

Analyses were performed as described previously.[27,44,45] The data have been deposited in the NCBI Gene Expression Omnibus database (GSE157629). Gro-seq and ChIP-seq data for the individual genes are shown in Supplementary Tables 1 and 2, respectively.

Immunohistochemistry and Immunofluorescence

PPs from the ileum were isolated and washed with cold PBS and embedded into paraffin blocks. Sections from the blocks were rehydrated and washed with PBS. After incubation with 1% PBS/bovine serum albumin supplemented with 5% normal donkey serum for blocking, antigen retrieval was processed with citrate buffer, pH 6.0 (121°C for 5 min), and stained overnight at 4°C for Esrrg (cat no: ab49129; Abcam) and GP2 (cat no: D278-3; MBL, Woburn, MA) antibodies. This was followed by anti-rabbit secondary for Esrrg (cat no: A32731; ThermoFisher) and anti-rat secondary for GP2 (cat no: A48261; ThermoFisher). The sections were examined with a light microscope. For whole-mount immunostaining, crypt organoids were plated in an 8-well chamber and cultured for 4 days, after which they were fixed with 4% paraformaldehyde, followed by permeabilization with 0.1% Triton (Gibco) X-100. The organoids were stained with the following primary antibodies overnight at 4°C: rabbit anti–Spi-B (Spi-B [D3C5E], cat no: 14223; CST, Danvers, MA), rabbit anti-Sox8 (cat no: ab221053; Abcam), and rat anti-GP2 (cat no: D278-3; MBL). This was followed by incubation with secondary antibody anti-rabbit Alexa Fluor 568 (cat no: A-11011; ThermoFisher) for Spi-B and Sox8 and anti-rat Alexa Fluor 488 (cat no: A-11006; ThermoFisher) for GP2. Cells were analyzed with a Nikon (Melville, NY) A1R+ Laser Scanning Confocal Microscope after mounting with ProLong Diamond with 4′,6-diamidino-2-phenylindole mounting solution (cat no: 15810083; ThermoFisher).

Table 1. List of Oligonucleotides Primers Used for RT-qPCR

Oligonucleotide	Sequence, 5' to 3'
Gapdh_fwd	TGTGTCCGTCGTGGATCTG
Gapdh_rev	CCTGCTTCACCACCTTCTTGA
Suz 12_fwd	GATGAGAAAGATCCAGAATGGC
Suz12_rev	ATAATTTTCTACAAACAGCATACAGGC
Ezh2_fwd	GTCTGATGTGGCAGGCTGG
Ezh2_rev	GCCCTTTCGGGTTGCATC
Spi-B_fwd	GGAGTCTTCTACGACCTGGACAG
Spi-B_rev	GCAGGATCGAAGGCTTCATAGG
Sox8_fwd	GGACCAGTACCCGCATCTCC
Sox8_rev	TTCTTGTGCTGCACACGGAGC
GP2_fwd	GTGTACAAGTTACAGGGTACCCC
GP2_rev	GACAAGTAATCTCACAATTCTTGG
CCL9_fwd	GCCCAGATCACACATGCAAC
CCL9_rev	AGGACAGGCAGCAATCTGA
MarcksL1_fwd	CCCGTGAACGGAACAGATGA
MarcksL1_rev	CCCACCCTCCTTCCGATTTC
Esrrg_fwd	GTGTCTCAAAGTGGGCATGC
Esrrg_rev	GCTGTTCTCAGCATCTATTCTGC
Aif1_fwd	GGATTTGCAGGGAGGAAAA
Aif1_rev	TGGGATCATCGAGGAATTG
CCL20_fwd	TGTACGAGAGGCAACAGTCG
CCL20_rev	TCTGCTCTTCCTTGCTTTGG
TNFAIP2_fwd	GTGCAGAACCTCTACCCCAATG
TNFAIP2_rev	TGGAGAATGTCGATGGCCA
18s rRNA_fwd	GTAACCCGTTGAACCCCATT
18s rRNA_rev	CCATCCAATCGGTAGTAGCG

fwd, forward; rev, reverse.

Isolation of Villous Epithelium and FAE Cells

Villous epithelium and FAE were prepared by isolating ileal PPs and small pieces of ileum from the intestine. These pieces were washed in cold PBS and later incubated in 30 mmol/L EDTA (cat no: 1557-038; Gibco), 5 mmol/L dithiothreitol (cat number: R0861; ThermoFisher) in PBS, and gently shaken in ice on a rocker for 20 minutes. After which, surrounding epithelial cells were peeled off from lamina propria and PPs. FAE was carefully cleaned off from surrounding villous epithelium tissues with a 26-gauge needle under a stereo microscope.

CRISPR–Cas9 Gene Editing of Intestinal Organoids

Guide RNAs for Rank, Spi-B, and Esrrg were designed with the CRISPR design tool (http://crispr.mit.edu).[46] The guides were cloned into lentiCRISPR v2 vector (52961; Addgene, Watertown, MA). The cloned vector was

transfected into 293FT cells (cat no: R7007; ThermoFisher) and the supernatant was collected at 48 hours and concentrated with Lenti-X concentrator (cat number: 631231; Clontech, Mountain View, CA). The 293FT cell line was found to be negative for mycoplasma. Cultured intestinal organoids were grown in EGF, Noggin, Chir, Y-27632; epidermal growth factor, Noggin, Chir-99021 (cat no: S1263; Selleckchem) and Y-27632 (cat no: 72304; Selleckchem) 2 days before transduction. Organoids were dissociated into single cells mechanically along with TrypLE Express (Thermo Fisher Scientific) supplemented with 1000 U/mL DNase I for 5 minutes at 32°C. The single-cell suspension was washed once with Advanced Dulbecco's modified Eagle medium and resuspended in transduction medium (ENR media supplemented with 1 mmol/L nicotinamide, Y-27632, Chir99021, 8 μg/mL polybrene, cat no: 28728-55-4; Sigma-Aldrich) and mixed with concentrated virus. The cell–virus mixture was spinoculated for 1 hour at 600 $\times$ g at 32°C followed by a 2- to 4-hour incubation at 37°C, after which they were collected and plated on 60% Matrigel overlaid with transduction medium without polybrene. Transduced organoids were selected with 2 rounds of 2 μg/mL of puromycin (cat no: P8833, 10 mg; Sigma-Aldrich) on day 2 and day 4, after which clones were expanded in maintenance ENR medium. KO was confirmed by Western blot to check for the expression of deleted gene.

Oligonucleotides used for generation of genomic RNAs were as follows: Esrrg (1) CACCGTCTGTCAAGACGGACCCCTG, AACCAGGGGTCCGTCTTGACAGAC, Esrrg (2) CACCGTGGCGTCGGAAGACCCACCA; AAACCAGGGGTCCGTCTTGACAGAC. Spi-B (1) CACCGAGACTCCTTCTGGGTACTGG, AAACCCAGTACCCAGAAGGAGTCTC; and Rank (1) CACCGAAAGCTAGAAGCACACCAG, AAACCTGGTGTGCTTCTAGCTTTC.

Lentivirus Infection for Overexpression

RelB, p52, RelA, and p50 plasmids were a gift from Hiroshi Ohno's laboratory (RCIMS, Kangawa, Japan) and Esrrg complementary DNA was cloned by Twist Bioscience (San Francisco, CA). These were cloned into CSII-CMV-MCS-IRES2-Bsd vector, which was kindly provided by the RIKEN Bioresource Center (Ibaraki, Japan) and Hiroyuki Miyoshi. The same protocol for Crispr-Cas9 lentiviral generation and transduction was followed and cells were embedded into Matrigel and incubated for 2–3 days.

Immunoblotting

Organoids were recovered from Matrigel with Cell Recovery media (cat no: 354253; Corning). Organoids were washed with PBS and the cells were lysed with 2$\times$ Laemmli solution and boiled at 98ºC. Protein concentrations were measured by a Pierce (Waltham, MA) 660 nm Protein Assay Reagent and IDCR (cat no: 22660; ThermoFisher Scientific). Samples were loaded equally in terms of protein concentration into 10% Bis-Tris protein gels (cat no: 4561033; Bio-Rad) and blotted on nitrocellulose membranes. Membranes were incubated with primary antibodies: anti-Esrrg (cat no: ab49129; Abcam); anti–Spi-B (Spi-B D4V9S, cat no: 14337; CST); anti-H3K27me3 (cat no: ab192985; Abcam); anti-H3 (cat no: ab1791; Abcam), anti-Rank (cat no: MBS9133424; MyBioSource, San Diego, CA), and anti-glyceraldehyde-3-phosphate dehydrogenase (cat no: ab8245; Abcam) at 4°C overnight, and horseradish-peroxidase–conjugated anti-rabbit (1:5000, cat no: RABHRP1-10UL; Sigma-Aldrich) or anti-mouse (1:1000, cat no: 7076; CST) for 1 hour at room temperature. Signal was detected using ECL reagent (cat no: 2232; Amersham, Amersham, UK).

Real-Time RT-qPCR

Total RNA was prepared using TRIzol (cat no: 15596018; Life Technologies) from intestinal organoids and epithelium isolated from mice. Isolated RNA was transcribed to first-strand complementary DNA using the iScript complementary DNA synthesis kit (1708891; Bio-Rad). qPCR amplification was detected using Ssofast evergreen supermixes (172-5203; Bio-Rad). The specific primers used are listed in Table 1.

Data Availability

The ChIP-seq and Gro-seq data have been deposited in the NCBI Gene Expression Omnibus database (GSE157629).

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The authors disclose no conflicts.

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PUBLICATION II

PRC2 Regulated Atoh8 Is a Regulator of Intestinal Microfold Cell (M Cell) Differentiation

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PRC2 Regulated Atoh8 Is a Regulator of Intestinal Microfold Cell (M Cell) Differentiation

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Abstract: Intestinal microfold cells (M cells) are a dynamic lineage of epithelial cells that initiate mucosal immunity in the intestine. They are responsible for the uptake and transcytosis of microorganisms, pathogens, and other antigens in the gastrointestinal tract. A mature M cell expresses a receptor Gp2 which binds to pathogens and aids in the uptake. Due to the rarity of these cells in the intestine, their development and differentiation remain yet to be fully understood. We recently demonstrated that polycomb repressive complex 2 (PRC2) is an epigenetic regulator of M cell development, and 12 novel transcription factors including Atoh8 were revealed to be regulated by the PRC2. Here, we show that Atoh8 acts as a regulator of M cell differentiation; the absence of Atoh8 led to a significant increase in the number of Gp2+ mature M cells and other M cell-associated markers such as Spi-B and Sox8. In vitro organoid analysis of RankL treated organoid showed an increase of mature marker GP2 expression and other M cell-associated markers. Atoh8 null mice showed an increase in transcytosis capacity of luminal antigens. An increase in M cell population has been previously reported to be detrimental to mucosal immunity because some pathogens like orally acquired prions have been able to exploit the transcytosis capacity of M cells to infect the host; mice with an increased population of M cells are also susceptible to *Salmonella* infections. Our study here demonstrates that PRC2 regulated Atoh8 is one of the factors that regulate the population density of intestinal M cell in the Peyer's patch.

Keywords: gut immunity; M cells; Peyer's patch; RankL; transcytosis

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1. Introduction

The gastrointestinal tract is subject to constant exposure to antigens, microorganisms, and foreign pathogens. The mucosal lining of the intestinal tract employs multiple mechanisms for immunosurveillance, which include epithelial tight junctions, production of antimicrobial peptides by Paneth cells, mucins from goblet cells, innate antigen receptors, and acquired immunity in the form of secretory IgA [1]. Phagocytic and transcytosis capabilities are critical to inducing an antigen-specific immune response. For this, the mucosal immune system is organized into inductive tissues such as the gut-associated lymphoid tissue (GALT), these include a specialized region known as Peyer's patches (PPs) in the intestine. PPs are covered by dome-shaped follicle-associated epithelium (FAE) which are composed of specialized intestinal epithelial cells (IECs) known as Microfold cells (M cells) [2,3].

M cells are phagocytic epithelial cells that enable the uptake and transcytosis of luminal antigens into the GALT, they are responsible for the rapid transport of bacterial antigens to antigen-presenting immature dendritic cells [4,5]. M cells display characteristically

different morphology from neighboring epithelial cells. On their apical side, they have irregular, short microvilli, while on their basolateral side they have an M-shaped pocket structure that houses antigen-presenting cells such as macrophages, B cells, and dendritic cells [6–9]. Mature M cells express the receptor Glycoprotein 2 (Gp2), critical for the uptake of *Salmonella typhimurium* and several other pathogenic antigens [10]. Consequently, mice lacking M cells or Gp2 receptors exhibit profound deficiencies in immune response as demonstrated by a decline in the production of antigen-specific secretory IgA (SIgA) in the gut and impaired antigen-specific T cell responses in mice infected with *Salmonella typhimurium* [5,10,11].

M cells arise from cycling intestinal stem cells in the crypts (which express Lgr5), but they are predominantly localized in the FAE due to the stimulation by nuclear factor κ B ligand (RankL) [12,13]. RankL is secreted from specialized stromal cells under the FAE which are known as M cell inducer cells [14]. RankL binds to Rank receptors on the Lgr5+ cells to activate TRAF6, which in turn leads to a signaling cascade of NFκB signaling, both classical and non-canonical [15]. Spi-B and Sox8 were identified as important transcription factors necessary for differentiation and functionality of M cells; Spi-B null and Sox8 null mice showed a lack of M cells and severely impaired transcytosis capabilities [16,17]. However, neither Spi-B nor Sox8 was sufficient for Gp2 expression as seen in both Spi-B null mice with intact Sox8 activation and Sox8 null mice with intact Spi-B activation. Although the progenitor cells approaching the FAE are constantly stimulated by activating signals, only ~10–20% of the cells in FAE are M cells. This suggests the presence of a regulatory mechanism in the FAE that prevents all the cells from differentiating into M cells. Osteoprotegerin (OPG) is a soluble decoy receptor for RankL, and it plays a regulatory role in maintaining the M cell density in the intestine by competing with Rank for binding to RankL. OPG null mice exhibited an increase in functionally mature M cell [18]. Despite the important role that M cells play in initiating mucosal responses, the mechanism for M cell development has yet to be fully characterized.

To further understand the differentiation and development of M cells, we had previously performed Chip-seq and Gro-seq for M cells and discovered 12 transcription factors that were epigenetically regulated (six upregulated and six silenced) [19]. One of the genes upregulated in our analysis was *Atoh8*. Here, we find that Atoh8 plays a critical role in regulating the differentiation of M cells; Atoh8 was expressed exclusively in M cells in the Peyer's patches and was critical to maintaining the density of M cells in the FAE. Moreover, intestinal-specific Atoh8 deletion showed an increased capacity for transcytosis. Overall, our findings show that Atoh8 plays a homeostatic role in maintaining the M cell population and that it limits the translocation of invasive antigens from the luminal side into Peyer's patches.

2. Results

2.1. Atoh8 Is Expressed in M Cells and Induced by RankL-Rank Signaling

In our previous study, we sought to identify how PRC2 regulates M cell differentiation and discovered 12 transcription factors that were PRC2-regulated specifically in M cell differentiation (six upregulated and six silenced) [19]. *Atoh8* turned up as one of the RankL-induced PRC2 regulated genes (log2 fold changes −3.15 RankL vs WENRC and -2.58 RankL vs ENRI) (Figure 1A). Here, immunohistochemistry analysis of murine PPs revealed that Atoh8 is localized in the nuclei of cells in the Peyer's patches (Figure 1B). RNA was isolated from the FAE and villous epithelium, and RT-qPCR analysis confirmed that Atoh8 was significantly enriched in the Peyer's patches (Gp2 as a marker) when compared to the villi (Figure 1C). Mouse intestinal organoids isolated from the crypts were treated with RankL for 4 days, *Atoh8* was observed to be significantly upregulated along with GP2, confirming that Atoh8 is induced by RankL signaling (Figure 1D). Rank-deficient mouse intestinal organoids were generated using the Lenti V2 CRISPR/Cas9 system; RankL treatment did not induce *Atoh8* expression, suggesting that *Atoh8* expression falls under the purview of

Rank-RankL signaling (Figure 1E). Validation of RANK KO was confirmed via immunoblot analysis (Supplementary Figure S1A).

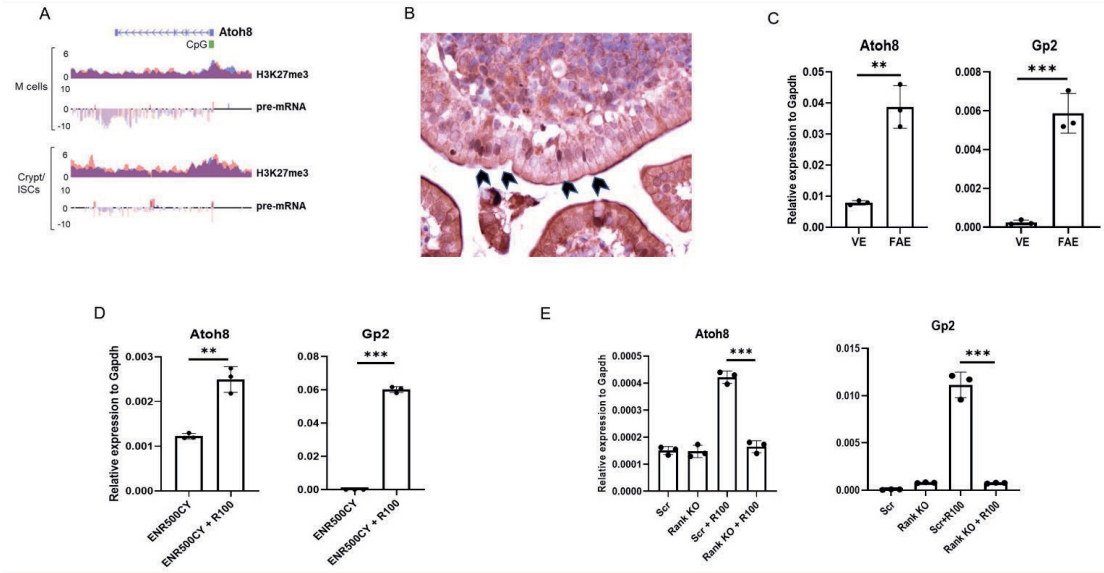

Figure 1. Atoh8 is expressed in FAE in Peyer's patches and is dependent on Rank-RankL signaling. (**A**) H3K27me3 occupancy at CpG islands spanning the promoter and first exon of the *Atoh8* gene in organoids treated with RankL (M cells) or treated with Wnt3a and Chir99021 (Crypt/ISCs). Below, pre-mRNA expression of *Atoh8* in organoids treated as above (y-axis: normalized tag count, ENR500 = R-spondin 500 ng/mL, R100 = Rankl 100 ng/mL). (**B**) Section of PP from wild type mice stained with Atoh8 antibody. Arrowheads indicating Atoh8 expression in the nuclei of M cells in FAE. (**C**) RT-qPCR analysis of *Atoh8* and *Gp2* in the FAE and VE from C57BL/6JRj mice ($n = 3$). (**D**) Organoids generated from wild type mice were stimulated with 100 ng of RankL for 4 d. *Atoh8* and *Gp2* expression was examined by RT-qPCR analysis. (**E**) Rank KO organoids and Scrambled organoids generated by lentiCRISPR v2 were incubated with RankL for 4 days, *Atoh8* and *GP2* expression was analyzed by RT-qPCR. In panels (**C**–**E**), unpaired two-tailed Student's *t*-test was performed for three independent experiments, *** $p < 0.005$; ** $p < 0.01$.

Previously in osteoclast, it was shown that BMP-induced Atoh8 regulated the RankL/OPG ratio indirectly via Runx2 to regulate osteoclast number and maintain bone volume in mice [20]. Our Gro-seq analysis of RankL organoids from our previous paper revealed that RankL signaling did lead to an upregulation of *BMP2* and *BMP6* but not *Runx2* [19]. The *BMP6* and *BMP2* activation by RankL was confirmed by qPCR analysis (Supplementary Figure S3A). Furthermore, treating intestinal organoids in EGF, R-spondin and 100 ng/mL of BMP2 and BMP6 showed that *Atoh8* could be induced by BMP2 and BMP6 alone (Supplementary Figure S3C,D).

2.2. Atoh8 Deficiency Augments M Cell Differentiation along with Other M Cell-Associated Transcription Factors

Since our previous and current data demonstrate that *Atoh8* is a PRC2-regulated gene in M cell differentiation, located in PPs and induced by RankL, we hypothesized that Atoh8 may contribute to M cell differentiation. Intestine-specific Atoh8 knockouts were generated by crossing Atoh8 *lox/lox* with *VilCre* mice (from now on referred to as Atoh8 *lox/VilCre*). M cells were analyzed from the GALT of Atoh8 *lox/VilCre* mice. RT-qPCR analysis of RNA isolated from the FAE of Atoh8 *lox/VilCre* mice showed an increase in the expression of mature GP2+ M cells when compared to the control *VilCre* mice (Figure 2A). Spi-B and Sox8, which are transcriptional factors critical for functional M cell development,

were markedly higher in the Atoh8 *lox/VilCre* mice (Figure 2A). Esrrg which is previously known to be essential for the development of M cells was also shown to be upregulated (Figure 2A). Early expression markers of M cells such as *CCL20*, *MarcksL1*, and *TNFAIP2* were also upregulated (Figure 2A). *OPG*, the decoy receptor for RankL was also showed higher expression in the Atoh8 *lox/VilCre* mice. The increase in Gp2+ M cells was also corroborated with whole-mount immunostaining (Figure 2B). We observed increased Gp2+ cells in the Atoh8 *lox/VilCre* mice, counting 22.4 ± 2.85 cells/0.01 mm in comparison with control *Vil/Cre* mice that had 15± 0.09 cells/0.01 mm.

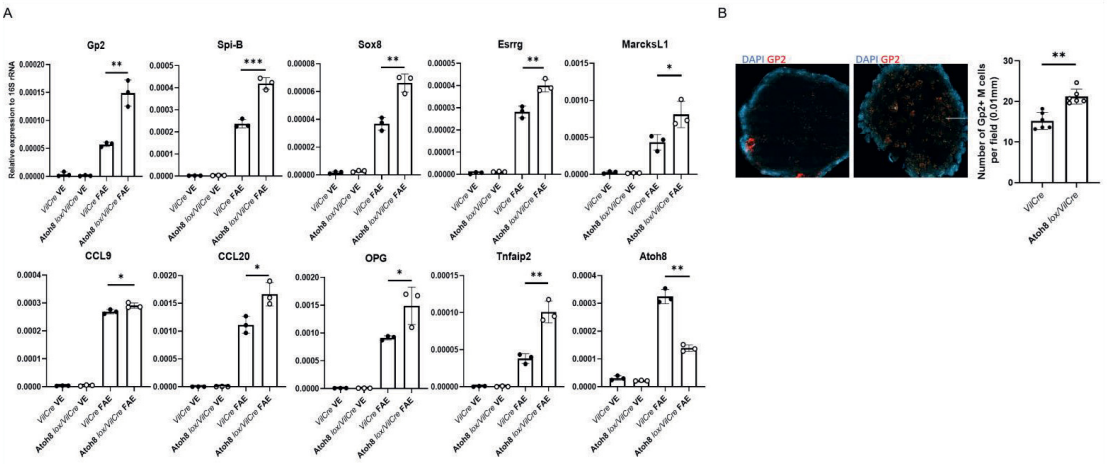

Figure 2. The loss of Atoh8 increases the number of mature M cells in the FAE. (**A**) qPCR analysis of M cell-associated genes in the FAE and VE of Atoh8 *lox/VilCre* and *VilCre* mice. ***, $p < 0.005$, *, $p < 0.05$ **, $p < 0.01$; n.s., not significant; unpaired two-tailed Student's *t*-test, $n = 3$. (**B**) Whole-mount immunostaining of PPs from *VilCre* and Atoh8 *lox/VilCre* mice. The number of GP2+ M cells per field of FAE (0.01 mm2) was compared between them ($n = 8$). Scale bars, 200 μm.

2.3. Atoh8 Deficiency Does Not Affect the B and T Cell Composition of Peyer's Patches

Germinal centers (GC) in the GALT are important structures in which mature B cells proliferate and undergo somatic hypermutation and class switch recombination mediated by the follicular T helper cells (Tfh cells) [21,22]. Knockdown of transcription factors necessary for M cell differentiation like Spi-b and Sox8 leads to a lack of Gp2+ cells as well as causes a reduction in B and T cell populations in the Peyer's patch [16,17]. OPG is another gene upregulated in M cell development. The gene transcribes a soluble decoy receptor that binds to RankL. Knockdown of OPG exhibited an increase in the M cell population as well as an increase in lymphoid cells that are involved in mucosal response [18]. Consequently, the increase in functionally mature M cells in Atoh8 *lox/VilCre* mice could affect both B and T cell populations compared to control animals even in steady state. However, similar populations of total B and T cells were observed in the PPs in Atoh8 *lox/VilCre* mice compared to controls, and we did not see any increase in the amount of IgA+ B cells at 4 weeks of age (Figure 3A–C). Furthermore, we also looked at follicular T helper cells (Tfh cells) as they promote germinal center reactions and affinity maturations to produce high affinity IgA [23]. Tfh cells and GC B cells had similar populations in *VilCre* and Atoh8 *lox/VilCre* (Figure 3C), and the amount of RankL producing Th cells was unaltered in both sets of mice. Overall, the lack of Atoh8 does not affect the B and T cell subpopulations or the quantities of IgA-producing B cells and Rankl+ T cells in pathogen-free mice.

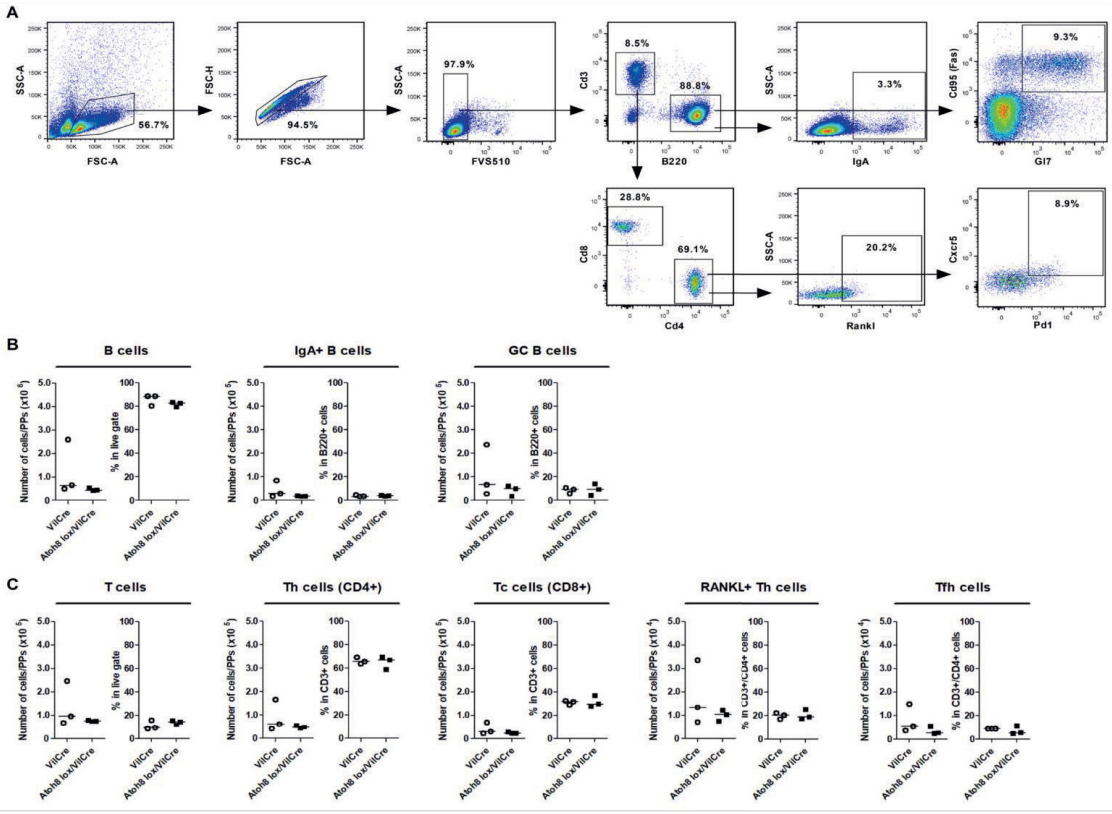

Figure 3. The loss of Atoh8 did not change the composition of lymphoid cells in 4-week-old mice after weaning. (**A**) Gating scheme for the analysis of B and T cell populations in *VilCre* and Atoh8 *lox/VilCre* ileal PPs. B (CD3ε−B220 +) cells were analyzed for IgA+ B and CD95+GL7+ GC B cells. T (CD3ε+B220−) cells were analyzed for total CD4+ Th, CD8α+ cytotoxic T (Tc), and CD4+CD8α−CXCR5+PD-1+ Tfh cells. (**B**,**C**) FlowJo analysis of indicated immune cells in ileal PPs. (**B**) Number of total B cells, GC B cells, and IgA+ B cells in 100,000 recorded cells. (**C**) Number of total T cells, Tc cells, Th cells, and Tfh cells in 100,000 recorded cells; Student's *t*-test, $n = 3$ per group.

2.4. Epithelium Intrinsic Atoh8 Is Responsible for the Increase in M Cell Population

The subepithelial dome is an afront to multiple signaling pathways, therefore it was necessary to validate if Atoh8 regulation of M cell development was epithelium intrinsic. Mouse intestinal organoid culture isolated from crypts of Atoh8 *lox/VilCre* and *control VilCre* mice were treated for 4 days with 100 ng/mL of RankL to induce M cell differentiation. *Spi-B*, *Sox8*, and *Esrrg* were observed to be significantly upregulated when compared to the control. The epithelial cells mimicked the significantly increased Gp2 expression as observed in vivo in the FAE as well as the early markers expressions of *CCL9*, *Marcksl1*, and *TNFAIP2* (Figure 4A). Immunostaining images for Gp2 on intestinal organoids treated with RankL showed higher expression for Atoh8 *lox/VilCre* organoids compared to *VilCre* organoids (Figure 4B). Taken together, these data indicate that epithelial intrinsic Atoh8 is sufficient to regulate M cell differentiation.

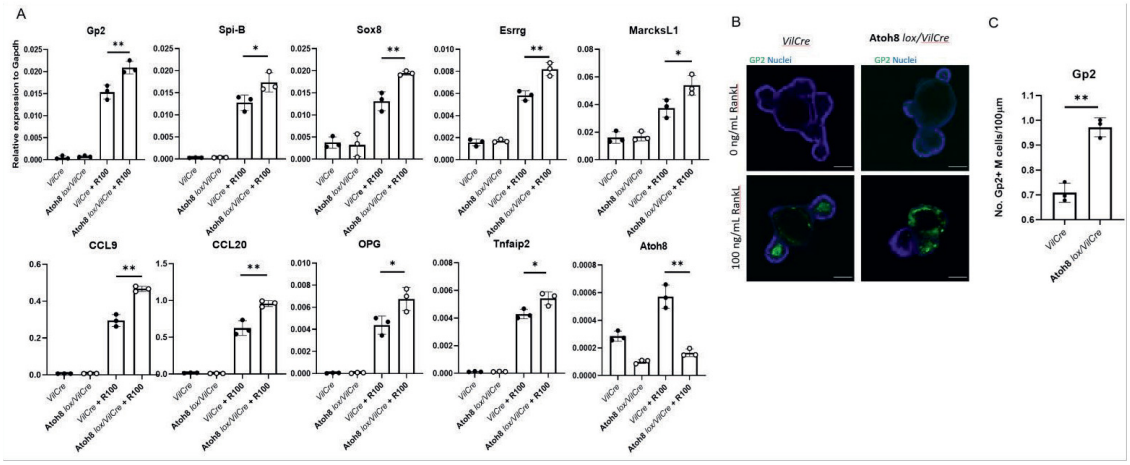

Figure 4. Increase in M cell maturation in Atoh8 *lox/VilCre* results from epithelium-intrinsic defect. (**A**,**B**) Organoids from small intestinal crypts of Atoh8 *lox/VilCre* and *VilCre* mice were cultured with or without RANKL for 3 d. (A) qPCR analysis of M cell-associated genes expressed in the organoid cultures. Values are presented as the mean ± SD; **, $p < 0.01$; *, $p < 0.05$; n.s., not significant; unpaired two-tailed Student's *t*-test, $n = 3$. Data are representative of two independent experiments. (**B**) Immunostaining images for GP2 (green) on organoids. Bars, 100 µm. (**C**) The number of Gp2+ M cells and Sox8+ M cells per length epithelium of organoids were compared between *VilCre* and Atoh8 *lox/VilCre* treated organoids ($n = 3$). Images are representative of three independent experiments.

2.5. Atoh8 Deficiency Leads to Increased Transcytosis Capacity

Gp2+ M cells in the Peyer's patches are essential for transcytosis of antigens and foreign particles to initiate a mucosal immune response. As Atoh8 knockout mice presented more Gp2 cells in the FAE, we investigated if these Gp2 cells are functional. To explore this, we administered orally fluorescent nanoparticles to Atoh8 *lox/VilCre* and control *VilCre* mice. Four hours after the oral administration, PP's from both mice were removed and the number of particles transcytosed in each PP was counted with a fluorescence microscope (Figure 5A). We observed that the uptake of nanoparticles in the Atoh8 *lox/VilCre* Peyer's patch was increased significantly by over 2-fold when compared to the *control* mice (Figure 5B).

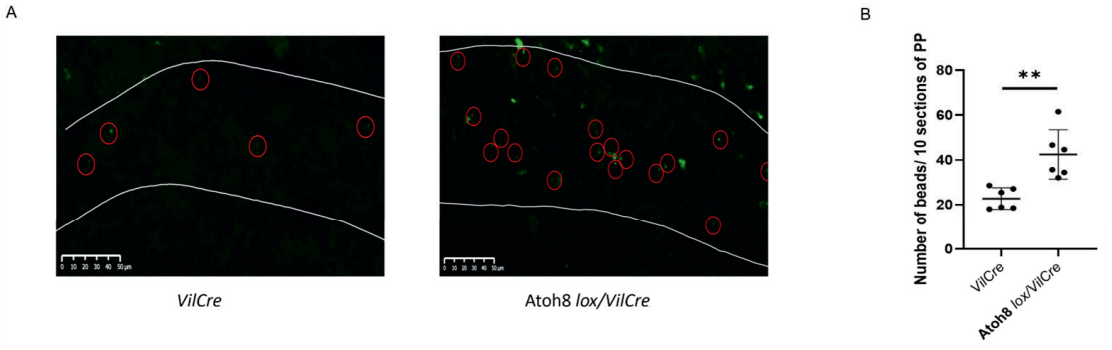

Figure 5. The loss of Atoh8 causes increased uptake of luminal nanoparticles into the follicle. (**A**,**B**) Green fluorescent latex beads (200 nm diameter) were orally administered to *VilCre* or Atoh8 *lox/VilCre* mice. 3 h later, PPs were collected from the jejunum. Ten consecutive cryosections of each PPs were examined by fluorescence microscopy, and the number of particles was counted manually. (**A**) Representative images of cryosections. White lines indicate FAE. Red circles indicate fluorescent particles. Bars, 50µm. (**B**) Quantification of particles in PPs. **, $p < 0.01$; Student's *t*-test; $n = 3$ per genotype.

3. Discussion

M cells are Intestinal epithelial cells specialized in gut immunity and transcytosis of antigens via GP2 to initiate immune responses. Previous research has revealed that M cell differentiation requires activation of Nf-κB signaling, activation of transcription factors Spi-B, Esrrg, and Sox8, membrane-bound RankL from lymphoid cells, and S100A4 from Dock8 cells [15–17,24]. However, other factors controlling M cell developments remain to be elucidated. For our analysis into M cell differentiation, we had previously performed a Chip-seq and Gro-seq of M cells and our data set revealed that PRC2 regulated 12 novel transcription factors (six silenced and six upregulated) in M cell development [19]. Atoh8 was one of the six transcription factors upregulated by the PRC2. Here, we describe that M cells in the FAE express Atoh8, specifically induced by RankL treatment in intestinal organoids differentiating to M cells and under the purview of the RankL-Rank signaling pathway.

Atoh8 belongs to group A of the bHLH transcription factor family. Members of the Atonal superfamily control numerous aspects of differentiation for vertebrae organ development and function [25–27]. Atoh1 is a member of the bHLH family that plays a pivotal role in the differentiation of Paneth cells. Paneth cells are critical for providing stem cell niche to Lgr5+ cells in the intestinal crypts [28]. Atoh8 is the sole mammalian member of the bHLH factor that is a part of the NET family [29]. Atoh8 has been previously studied in the context of the differentiation of osteoblasts. In a recent study, Atoh8 was found to be induced by BMP signaling; BMP-induced Atoh8 regulated the RankL/OPG ratio indirectly via Runx2 to regulate osteoclast number and to maintain bone volume in mice [20]. In our preliminary experiment, RankL-treated intestinal organoids showed a significant increase in expression of BMP2 and BMP6 expression (Supplementary Figure S3A). However, Runx2 expression remained unchanged with RankL treatment. We also observed that Atoh8 expression could be induced by growing organoids in BMP2 and BMP6 alone without RankL treatment (Supplementary Figure S3B). However, as Runx2 was not activated by RankL, further investigation is required to find out if BMP2/BMP6 directly induces Atoh8 to control the density of the M cell population in the Peyer's patch.

Our experiments with Atoh8 intestinal-specific knockout mice exhibited a higher number of Gp2+ M cells when compared to the *VilCre* wildtype. Well-established early markers of M cells such as MarcksL1 and TNFAIP2 were all significantly upregulated. Spi-B, Esrrg, and Sox8, which are critical for the maturation of Gp2+ M cells and fall under the purview of RankL and Nfkb (both classical and non-canonical) signaling, were found to have higher transcriptional expression. This implies that BMP2/BMP6 induced Atoh8 regulates the signaling pathway that leads to Gp2+ M cell development and differentiation (Figure 6). Isolation of intestinal organoids and treatment with RankL indicated that Atoh8 deficiency that led to increased Gp2 was epithelium intrinsic. Previous research involving transcription factors involved in M cell differentiation like Spi-B and Sox8 showed that transcription factor deficient mice also exhibit reduced lymphoid cell population in PP along with reduced GP2 expression [16,17]. Osteoprotegerin (OPG) deficient mice showed higher Gp2+ M cells but also higher numbers for lymphoid cells in terms of population (Kimura et al., 2020). In the current study, the regulation of M cell density by Atoh8 did not affect mucosal lymphoid cell populations that have previously been shown to be mediated by IgA. More specifically, the population of lymphoid cells in the Peyer's patch of Atoh8 *lox/VilCre* remained unchanged after weaning despite the increased Gp2+ M cell quantities. To account for delayed immune response/population changes, we additionally analyzed 10-week-old Atoh8 *lox/VilCre* mice and similarly observed unaltered populations of GC B and Tfh cells when compared to the control (Supplementary Figure S4). Nevertheless, in the older mice, a slight increase in the B and T helper (Th) cell frequencies could be seen. Importantly, we observed a higher transcytosis capacity of nanobeads into the Peyer's patches of Atoh8 intestinal KO animals, twice the amount of uptake when compared to control mice. This indicates that Atoh8 *lox/VilCre* mice with higher Gp2 expression also had functional characteristics during an infection. However, further studies with *Salmonella*

typhimurium infection or other pathogenic antigens would be required to better elucidate the differences in the immune response.

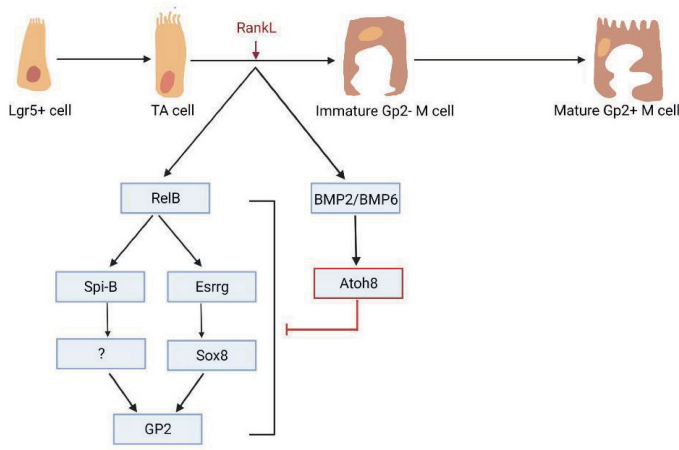

Figure 6. Atoh8 regulates the density of M cell population in the FAE. Loss of Atoh8 significantly increased the expression of M cell-associated transcription factors such as Spi-B, Sox8, Esrrg as well as early M cell expression markers. The population of Gp2+ M cells increased significantly in Atoh8 null mice.

OPG binds to RankL and acts as a decoy receptor instead of Rank. OPG-deficient mice showed higher RankL secretion, more Gp2+ M cells, higher transcytosis capabilities, an increased population of systemic lymphoid tissues, and enhanced immune response [18]. As we looked to see if Atoh8 intestinal KO mice had increased RankL production, our flow cytometric analysis showed no increase in RankL+ T cells, indicating that Atoh8 acts independent of RankL signaling in the GALT. Although the OPG null mice showed higher M cells and enhanced immune responses, they were highly susceptible to infection by pathogenic bacteria. An increase in M cells could also increase the transcytosis of botulinum toxins and scrape prion protein into the body. [30] Therefore, considering that the lack of Atoh8 in the intestine led to an increase in M cells, it is reasonable to conclude that Atoh8 limits transcytosis of pathogenic agents by regulating the number of M cells. The delicate equilibrium of maintaining M cell density in the Peyer's patch via Atoh8 may have been established over time as an evolutionary mechanism to control intestinal homeostasis in relationship to invasive antigens and mucosal immune responses. While Atoh1, a family member of Atoh8, regulates Paneth cell differentiation in the intestinal crypt via notch signaling, simultaneously inhibiting Paneth cell differentiation in the neighboring cell, it can be speculated that Atoh8 employs a similar mechanism in maintaining the population. However, further exploratory studies are needed to understand how the epithelial intrinsic Atoh8 regulates the population of M cells.

4. Materials and Methods

4.1. Mice

All animal experiments were approved by the Finnish National Animal Experiment Board (Permit: ESAVI/5824/2018). Mice were maintained on standard light–dark conditions, with food and water ad libitum at the pathogen-free animal facility of the Faculty of Medicine and Health Technology. B6.Cg-Tg(Vil1-cre)1000 Gum/J mice (Cat No: 021504) were purchased from Jackson Laboratories. Atoh8 lox/lox mice, in which exon 1 is flanked by two loxP sites, were provided by Rosa Gasa (Ejarque, et al. 2016). To generate intestinal deletion of Atoh8, Vil1-cre mice were bred with Atoh8 lox/lox. The F1 generation was backcrossed with Atoh8 lox/lox to generate mice homozygous for floxed Atoh8 allele

carrying the Vil1-cre transgene. Littermates with Vil1-cre allele were used as control. Mice genotypes and Atoh8 deletion were confirmed by PCR with genotyping primers 5′ ATTGGAGGAAGGCTCGGTGAA 3′ and 5′ TTGGCATTCGTCGTGCTGTC 3′. Representative genotyping of PCR genotyping (Supplementary Figure S2).

4.2. Immunohistochemistry and Immunofluorescence

Peyer's patches were isolated from the ileum and washed in cold PBS and embedded into paraffin blocks. Sections were cut from the blocks and rehydrated by washing with PBS. After blocking with 1% PBS/BSA supplemented with 5% normal donkey serum (Sigma -Aldrich, St. Louis, MO, D9663-10ML), antigen retrieval was performed with citrate buffer, pH 6.0 (121 °C for 5 min), and tissue sections stained overnight at 4 °C for Atoh8 (Thermo Fisher Scientific, PA5-20710, Waltham, MA, USA) antibody. Goat Anti-Rabbit was used for the secondary antibody (Thermo Fisher Scientific, A32731). Light microscopy was used for detection and analysis.

Intestinal crypt organoids were analyzed by whole-mount immunostaining, crypt-organoids were grown in an 8-well chamber plate and cultured for 4 days with and without RankL (100 ng/mL) after which they were fixed with 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100. The organoids were stained with Gp2 (MBL, D278-3) antibodies overnight at 4 °C. This was followed by Anti-Rat for the secondary antibody. Gp2 expressing cells were analyzed by Nikon A1R+ Laser Scanning Confocal Microscope after mounting with ProLong Diamond with DAPI mounting solution (Molecular Probes P36962).

4.3. Isolation of Follicle-Associated Epithelial Cells (FAE) and Villous Epithelium Cells (VE)

Illeal PPs along with small pieces of intestine were isolated from the ileum of control mice and Atoh8 *lox/VilCre* mice. After flushing the tissues with cold PBS, they were incubated in 30 mM EDTA, 5 mM DTT in PBS, and gently shaken in ice on a rocker for 20 min. Surrounding epithelial cells were peeled off from lamina propria and PP's. FAE was carefully cleaned off from surrounding VE tissues with a 26-gauge needle under a stereomicroscope. Trizol was added to the cleaned FAE and proceeded by RNA isolation.

4.4. Mouse Intestinal Organoid Culture

Mouse intestinal crypts were isolated and culture techniques were observed as previously described by Sato et al. (2011) and de Lau et al. (2012) [17,28]. Collected duodenum was washed in PBS and cut longitudinally, and villi were gently scraped off using glass slides. After further washes with PBS, the duodenum was cut into 2 mm pieces and pipetted up and down 4–6 times in 10 mL PBS using a 10 mL pipette. Once the suspension was relatively clear, the pieces were suspended in 10 mM EDTA in PBS for 20 min rocking at room temperature. A 70 µm cell strainer (Fisher Scientific, Waltham, MA) was used to strain the crypts from the rest of the epithelium. This mixture was enriched to crypt fraction through centrifugation at 150× *g* for 5 min. The crypts were cultured on a 24-well plate by embedding them in 30 ul of Matrigel (Corning, NY, USA). Organoids were cultured in an optimal medium consisting of advanced DMEM/F12 (Thermo Fisher Scientific) that contained HEPES (10 mM, Sigma-Aldrich, St. Louis, MO, USA), Glutamax (2 mM, Thermo Fisher Scientific), Penicillin–streptomycin (100 U/mL, Sigma-Aldrich-Aldrich, St. Louis, MO, USA), B-27 supplement minus Vitamin A (Thermo Fisher Scientific), *N*-2 supplement (Thermo Fisher Scientific), *N*-acetylcysteine (1 mM; Sigma-Aldrich, St. Louis, MO, USA), recombinant WNT (100 ng/mL R&D Biosystems, Minneapolis, MN, USA), recombinant murine EGF (50 ng/mL; Thermo Fisher Scientific), recombinant murine Noggin (50 ng/mL; PeproTech Cranbury, NJ, USA), Chir99021 (3µm, Selleckchem, Houston, TX, USA) recombinant mouse R-spondin1 (1 µg/mL; R&D BioSystems). Media were changed every 2 days. For M cell differentiation, recombinant mouse RankL (100 ng/mL, Peprotech) was added to the media and incubated for 4 days. To check for Atoh8 expression via BMP2/BMP6, organoids were grown in EGF, R-spondin, and BMP2 (100 ng/mL, R&D Biosystems) and

EGF, R-spondin, and BMP6 (100 ng/mL, R&D Biosystems) according to the protocol of Calpe et al [31]. Noggin was not added for the BMP2/BMP6 experiments as it acts as an antagonist against BMP signaling.

4.5. CRISPR–Cas9 Gene Knockout of Intestinal Organoids

Guide RNAs for the Rank gene were designed using CRISPR design tool (http://crispr.mit.edu, accessed on 7 April 2019) (Shalem, O. et al. Science 343, 84–87 (2014)). The guides were cloned into the lentiCRISPR v2 vector (Addgene, Watertown, MA, USA) and the cloned product was transfected into HEK 293FT cells (ThermoFisher, R7007). After 48 h, the supernatant was collected and concentrated with Lenti-X concentrator (Clontech Mountain View, CA, USA). The 293FT cell line was tested for mycoplasma. Intestinal organoids were grown in ENCY (EGF, Noggin, Chir-99021, and Y-27632) 2 days before transduction. After dissociating organoids into single cells using TrypLE Express (Thermo Fisher Scientific) supplemented with 1000 U/mL DnaseI for 5 min at 32 °C, the cells were washed once with Advanced DMEM and resuspended in transduction medium (ENR media) supplemented with 1 mM nicotinamide, Y-27632, Chir99021, 8 μg/mL polybrene (Sigma-Aldrich) and mixed with the concentrated virus. The mixture was centrifuged for 1 h at 600× *g* 32 °C followed by 3 h incubation at 37 °C, after which they were collected and plated on 60% Matrigel overlaid with enriched transduction medium without polybrene. On day 2 and day 4, transduced organoids were selected with 2 μg/mL of puromycin (Sigma-Aldrich), after which clones were expanded in maintenance ENR medium. Knockout was confirmed by Western blot.

Oligonucleotides used for generation of gRNAs for Rank CACCGAAAGCTAGAAG-CACACCAG, AAACCTGGTGTGCTTCTAGCTTTC.

4.6. Real-Time Quantitative Reverse Transcription PCR

Total RNA was isolated from intestinal organoids, FAE, and VE tissues from mice using TRIzol (Life Technologies Carlsbad, CA, USA). iScript cDNA synthesis Kit (Biorad, Hercules, CA, USA) was used to transcribe the isolated RNA to the first-strand cDNA. qPCR amplification was detected using SsoFast EvaGreen Supermix (Biorad, Hercules, CA172-5203) and a CFX96 detection system (Biorad). The specific primers used, are listed in the supplementary data (Table S1).

4.7. Whole-Mount Immunostaining of M Cells in FAE

Ileal PPs were dissected from the small intestine and transferred to a 10 cm dish containing 30 mL cold PBS. Excess intestinal tissues around the FAE were cut and removed under a stereomicroscope using forceps. PP's were washed sufficiently by using a 1 mL syringe with a 26-gauge needle under a stereomicroscope. The mucous layer on the FAE should be flushed out with a water stream to prevent background noise detection. PPs were transferred to the 1.5 mL tube containing 1 mL PBS, washed by vortexing and the supernatant was discarded. After 3 washes, 300–1000 μL Cytofix/Cytoperm buffer (BD Biosciences, Franklin Lakes, NJ, USA) was used for blocking and permeabilization for 25 min at room temperature. Perm/wash buffer (BD Biosciences, Franklin Lakes, NJ, USA) was used to wash the PPs after which they were stained with PE-conjugated anti-GP2 antibody (MBL; 1:10 in Perm/Wash buffer) overnight at 4 °C. Following the primary antibody staining, PPs were washed 3 times with wash/perm buffer and stained for 30 min at RT in Alexa Fluor 546-conjugated anti-Rat IgG (Thermo Fisher Scientific, Waltham, MA, USA). The PPs were washed again 3 times with wash/perm buffer and mounted with ProLong Diamond with Dapi mounting solution (Molecular Probes P36962). Slides were examined with a laser scanning confocal microscope (Zeiss LSM 800 LSCM).

4.8. Flow Cytometry

Peyer's patches from *VilCre* mice and Atoh8 *lox/VilCre* animals were isolated from the ileal section. The tissues were washed in PBS and incubated in 5 mL Spleen Dissociation

Medium for 15–20 min at 37 °C while vigorously shaking at 250 rpm. To generate a single cell suspension, PPs were placed on a sterile (autoclaved) 70 μm nylon mesh cell strainer and ground into the mesh using the base of a plunger from a 1 cc syringe. The suspension was incubated in 1 mm EDTA for 5 min in a rocker after which the suspension was passed through a 70 μm nylon mesh cell strainer. After the isolation step, the single-cell suspensions were prepared with FVS510 viability stain (#564406; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and CD16/CD32 Monoclonal Antibody (#16-0161-85; Thermo Fisher Scientific,). Surface staining was done using fluorochrome-conjugated anti-mouse antibodies against CD3e, CD8a, CD4, B220, PD1, CXCR5, GL7, FAS, IgA, and RANKL (Thermo Fisher Scientific). To prevent spectral overlap of emitted fluorescence, cells were divided into replicates, and two separate antibody panels were used in the staining procedure. Flow cytometry was done with FACSAria™ Fusion (Becton, Dickinson and Company), and data were analyzed with FlowJo (v. 10.6.1, Tree Star, OR, USA). Statistical analyses were done with Prism v. 5.02 (GraphPad Software, San Diego, CA, USA) calculated using a two-tailed *t*-test. p values of < 0.05 were considered significant.

4.9. Quantification of Transcytosis of Fluorescent Beads by M cells

Atoh8 *lox/VilCre* and *VilCre* mice fasted for 3 h and 1011 of 200 nm diameter polystyrene nanoparticles (09834-10, Fluoresbrite YG; Polysciences Warrington, PA, USA) were orally administered via oral gavage. After 4 h, two PPs were collected from the ileum and jejunum and fixed with 3.7% formalin/PBS for 2 h. Fixed tissues were incubated overnight with 30% sucrose in PBS and finally embedded in the OCT compound (Sakura Fintech, Torrance, CA, USA). Ten sequential 15 μm sections were cut and examined by fluorescence microscopy. The number of fluorescent particles transcytosed was counted manually.

4.10. Statistical Analyses

Results were benchmarked and analyzed by using an unpaired two-tailed Student's *t*-test. Statistical significance was set at $p < 0.05$ throughout all experiments. All statistical analyses were measured by using GraphPad Prism 6 software. No statistical methods were used to determine the sample size. These experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms22179355/s1.

Author Contributions: J.J.G. and K.V.: Study concept and design. R.G.: Generation of Atoh8 flox line. J.J.G. and L.M.-D.: mice dissection and functional assay. J.J.G.: experiments and acquisition of data. M.J.T.O.: FACS and FACS analysis. J.J.G. and K.V.: analysis and interpretation of data. J.J.G. and K.V.: manuscript drafting. M.P. and K.V.: project administration and funding acquisition. J.J.G., L.M.-D., M.J.T.O., M.P. and K.V.: critical revision of the manuscript for important intellectual content. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

M cells	Microfold cells
GALT	Gut-associated lymphoid tissues
PP	Peyer's Patch
FAE	Follicle associated epithelium
VE	villous epithelium
RankL	Receptor activator of nuclear factor kappa B ligand
Rank	Receptor activator of nuclear factor kappa B
PRC2	polycomb repressive complex 2
Esrrg	Estrogen-related receptor gamma

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Supplementary file

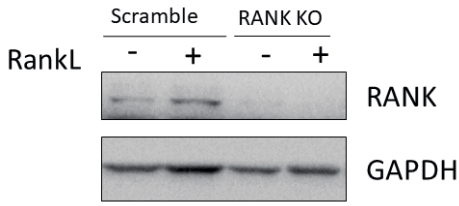

Figure S1. Immunoblot analysis of CRISPR generated RANK KO. RANK KO validation by immunoblot analysis of RANK KO and Scrambled intestinal organoids generated by CRISPR-Cas9 genome editing in C57BL/6rj intestinal organoids. These organoids were grown with and without RankL 100ng for 4 days. Data from Rank KO organoids also published in 10.1016/j.jcmgh.2021.05.014 .

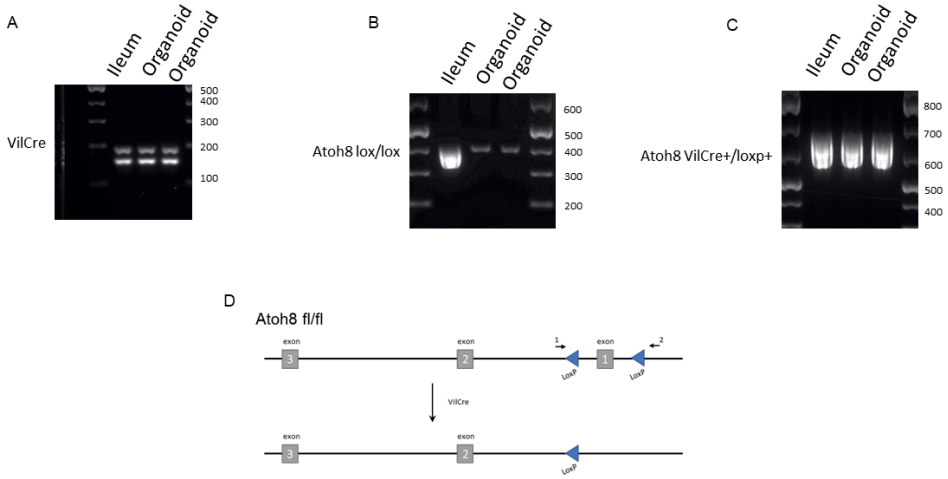

Figure S2. Representative genotyping PCR amplification of intestinal *VilCre,* Atoh8 *flox/flox* wild-type and Atoh8 *VilCre/loxp*. **A**) PCR for detection of VilCre recombinase is shown. Upper band: internal control (182 bp). Lower band: VilCre transgene (150 bp). 100 bp molecular marker brightest line corresponds to 500 bp. **B**) Floxed allele 400 bp. **C**) Genotyping of Atoh8 *lox/VilCre* 600bp. **D**) Scheme and representative PCR amplification verifying Cre-mediated recombination of the Atoh8 floxed allele in the intestine.

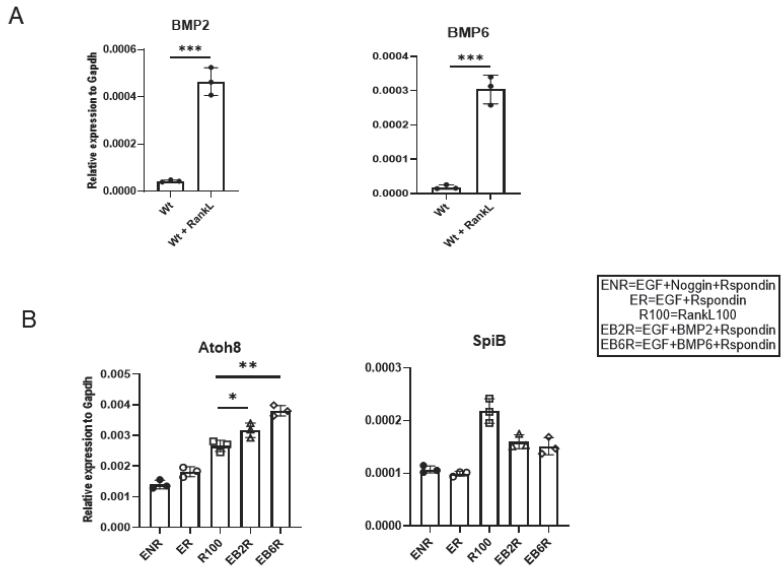

Figure S3. Atoh8 signaling is mediated by RankL/BMP2-BMP6 signaling. (**A**) Organoids from small intestinal crypts of wildtype mice were cultured with or without RANKL for 3 d. (**A**) qPCR analysis of M cell–associated genes expressed in the organoid cultures. Values are presented as the mean ± SD; *** $p < 0.005$; unpaired two-tailed Student's *t* test, $n = 3$. Data are representative of two independent experiments. (**B**) Organoids isolated Wt mice were grown for 3 days in EGF, Noggin, Rspondin media, EGF, Rspondin media, 100ug of Rankl media, Egf, BMP2 Rspondin media and Egf, BMP6, Rspondin media. Values are presented as the mean ± SD; *, $p < 0.05$, **, $p < 0.001$; unpaired two-tailed Student's *t* test, $n = 3$. Data are representative of two independent experiments.

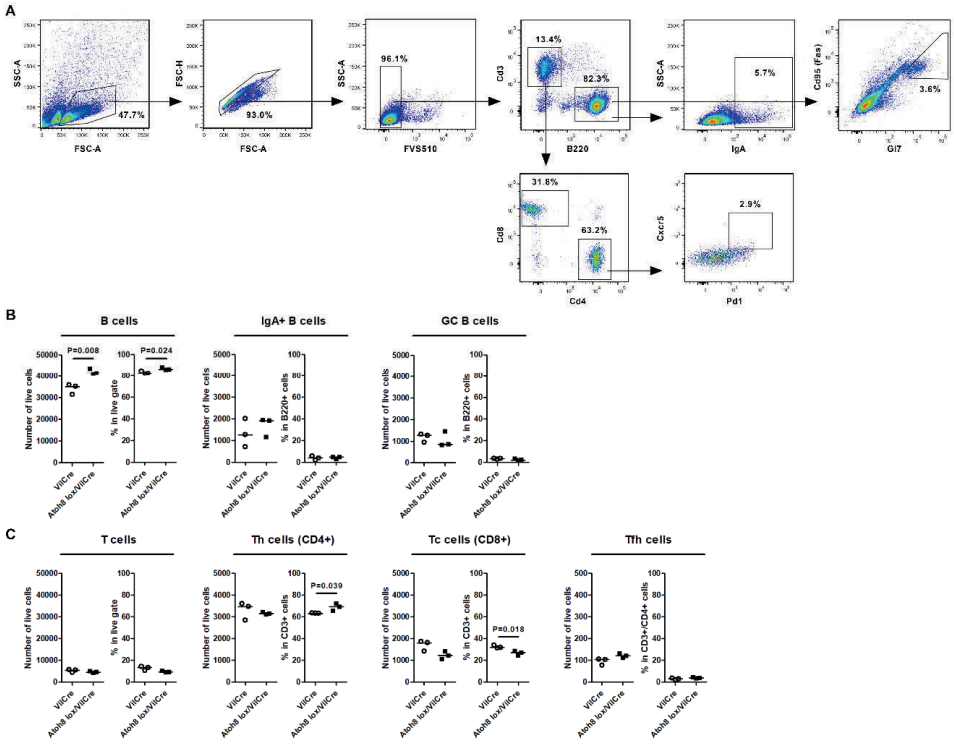

Figure S4. The loss of Atoh8 did not change the composition of lymphoid cells in 10-week-old mice. (**A**) Gating scheme for analysis of B and T cell populations in *VilCre* and Atoh8 *lox/VilCre* ileal PPs. **B** (CD3ε−B220+) cells were analyzed for IgA+ B and CD95+GL7+ GC B cells. T (CD3ε+B220−) cells were analyzed for total CD4+ Th, CD8α+ cytotoxic T (Tc), and CD4+CD8α−CXCR5+PD-1+ Tfh cells. (**B** and **C**) Flow cytometry analysis of indicated immune cells in ileal PPs. (**C**) Number of total B cells, GC B cells, and IgA+ B cells. (**D**) Number of total T cells, Tc cells, Th cells, and Tfh cells; n.s., not significant; Student's *t* test, $n = 3$ per group.

Table S1. List of primers.

Oligonucleotide	Sequence (5' to 3')
Gapdh_fwd	TGTGTCCGTCGTGGATCTGA
Gapdh_rev	CCTGCTTCACCACCTTCTTGA
Spi-B_fwd	GGAGTCTTCTACGACCTGGACAG
Spi-B_rev	GCAGGATCGAAGGCTTCATAGG
Sox8_fwd	GGACCAGTACCCGCATCTCC
Sox8_rev	TTCTTGTGCTGCACACGGAGC
GP2_fwd	GTGTACAAGTTACAGGGTACCCC
GP2_rev	GACAAGTAATCTCACAATTCTTGG

CCL9_fwd CCL9_rev	GCCCAGATCACACATGCAAC AGGACAGGCAGCAATCTGAA
MarcksL1_fwd MarcksL1_rev	CCCGTGAACGGAACAGATGA CCCACCCTCCTTCCGATTTC
Esrrg_fwd Esrrg_rev	GTGTCTCAAAGTGGGCATGC GCTGTTCTCAGCATCTATTCTGC
Aif1_fwd Aif1_rev	GGATTTGCAGGGAGGAAAA TGGGATCATCGAGGAATTG
CCL20_fwd CCL20_rev	TGTACGAGAGGCAACAGTCG TCTGCTCTTCCTTGCTTTGG
TNFAIP2_fwd TNFAIP2_rev	GTGCAGAACCTCTACCCCAATG TGGAGAATGTCGATGGCCA
18s rRNA_fwd 18s rRNA_rev	GTAACCCGTTGAACCCCATT CCATCCAATCGGTAGTAGCG
Atoh8_fwd Atoh8_rev	CGGGGGAAAGTTCCTACTCGTC CGGAAGAATCCGGGTGGTTATT
OPG_fwd OPG_rev	ACCCAGAAACTGGTCATCAGC CTGCAATACACACACTCATCACT

PUBLICATION III

Maf is a regulator of differentiation for gut immune epithelial cell Microfold cell (M cell)

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Maf is a regulator of differentiation for gut immune epithelial cell Microfold cell (M cell)

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Abstract: Microfold cells (M cells) are a specialized subset of epithelial intestinal cells responsible for immunosurveillance of the gastrointestinal tract. M cells are located in the Peyer's patches and are crucial for monitoring and the transcytosis of antigens, microorganisms, and pathogens via their mature receptor GP2. A mature M cell with Gp2 receptor aids in the uptake of antigens, which are passed through the single layer of epithelium and presented to underlying antigen-presenting cells and processed further downstream with B cells, T cells, and dendritic cells. Recent studies revealed several transcription factors and ligands responsible for the development and differentiation of mature M cells however, an exhaustive list of factors remains to be elucidated. Our recent work on the epigenetic regulation of M cell development found 12 critical transcription factors that were controlled by the polycomb recessive complex 2. Musculoaponeurotic fibrosarcoma transcription factor (Maf) was identified as a gene regulated by the polycomb repressive complex (PRC2) during the development of M cells. In this paper, we explore Maf's critical role in M cell differentiation and maturation. Maf falls under the purview of RANKL signaling, is localized in the Peyer's patches of the intestine, and is expressed by M cells. Given that, complete knockout of the Maf gene leads to a lethal phenotype, organoids isolated from Maf knockout mice and treated with RANKL exhibited impaired M cell development and a significant decrease in Gp2 expression. These findings reveal that Maf is an important regulator for M cell development and differentiation.

Keywords: Organoids; *in vitro* modeling; Gut immunity; M cells; Peyer's patch; Maf

1. Introduction

The mucosal lining of the gastrointestinal tract is a constant battlefront where intestinal tissues are up against microbes, viruses, antigens, and other harmful pathogens. One of the defense mechanisms it employs is the immune-inductive sites that are found in the gut-associated lymphoid tissue also known as Peyer's patches (PP). Peyer's patches compose of three distinct regions; a germinal center (GC), an interfollicular region abundant in T cells and the sub-epithelial dome (SED) which houses lymphoid cells such as B cells, T cells, and other antigen-presenting cells [1–3]. Since the PP's lack a lymphatic system where antigens could potentially be transported, the PP's directly sample mucosal antigens through specialized immune epithelial cells, these cells are known as Microfold Cell (M cell) [2,4]. M cells are phagocytic epithelial cells that enable the uptake and transcytosis of luminal antigens into the gut-associated lymphoid tissue (GALT), they are responsible for the rapid transport of bacterial antigens to antigen-presenting immature dendritic cells [5–7]. Transcytosis is achieved through their surface receptor Glycoprotein 2 (GP2) which binds to antigens and aids in uptake, a mature M cell is characterized by the presence of a functioning Gp2 [8,9]. M cells form a part of the adaptive immune system as they provide residence to B cells, T cells, and dendritic cells. They are uniquely different in morphology compared to neighboring epithelial cells as they are characterized by short and irregular microvilli and a lack of mucus layer to aid in transcytosis. they have pocket-shaped invagination under which B cells, T cells, macrophages, and dendritic cells are present [10–13].

M cell differentiation is dependent on the stimulation of Receptor activator of nuclear factor κB ligand (RANKL) which is a member of the tumor necrosis factor (TNF) family cytokine [14,15]. Cycling intestinal stem cells in the crypts that express Lgr5 and Rank receptors undergo differentiation to M cells after exposure to RANKL which is secreted by stromal cells under the follicle-associated epithelium (FAE) known as M cell inducer cells (MCi) [16]. Recent studies demonstrated that Spi-B and Sox8 are transcription factors necessary for M cell development and differentiation. Spi-B null mice lacked transcytosis capacity due to the lack of M cells and demonstrated an impaired mucosal response to *S. Typhimurium* [15,17]. Sox8 null mice gave rise to an immature phenotype of M cells without Gp2 receptor thereby indicating a loss in immune response in the PP's of mice orally infected with *S. Typhimurium*[18]. However, Sox8 expression is active in Spi-B null mice and Spi-B is actively transcribed in Sox8 KO mouse yet both sets of mice show a lack of Gp2 receptor, impaired mucosal response and a severe lack of transcytosis capacity [18]. This implies that there are additional transcription factors involved in M cell development and they remain to be characterized.

Recently, we published our work looking at the epigenetic regulation of M cell differentiation and found that the polycomb repressive complex 2 (PRC2) regulates 12 transcription factors critical for M cell development. PRC2-regulated Estrogen related receptor gamma (Esrrg) was necessary for Sox8 expression and Gp2 expression for mature M cells [19] . Atonal BHLH Transcription Factor 8 (Atoh8), another transcription factor regulated by PRC2, negatively regulated the M cell population in the Peyer's patches; Atoh8 null mice showed an increase in M cell population and a higher transcytosis capacity [20]. Maf was another gene identified to be regulated by the PRC2 complex. Maf has been shown to be critical for the development and differentiation of various signaling mechanisms with tissues such as the cornea and lens development, mechanoreceptors involved in touch sensations, differentiation of chondrocytes [21–24]. Maf has been identified as an immune regulator and a transcription factor for T helper 2 cells (Th2), additionally, it has also been found to play a key role in the differentiation and development of innate immune cell types, B lymphocytes, and T- cell subsets in Peyer's patches [25–30]. Our findings here identify Maf to be an additional regulator of M cell differentiation that is dependent on Rank-RANKL signaling. Maf expression was localized to the Peyer's patch and necessary for the expression of early markers of M cell differentiation and expression of mature M cell marker Gp2. These observations indicate that Maf is a novel player essential for the differentiation and development of M cells in the follicle-associated epithelium.

2. Results

2.1. Maf is regulated by the PRC2 and localized in the Peyer's patch

Our recent data from the chromatin immunoprecipitation assays with sequencing (ChIP- seq) and global run-on sequencing (GRO-seq) analysis of M cells (RANKL treated cells) and crypt/ISC (WENRC treated cells) revealed how the epigenome regulates the differentiation and development M cells in the gut[19]. Furthermore, PRC2-regulated *Maf* turned up as one of the genes highly expressed in the analysis (log2 fold change -2.66 RANKL vs WENRC (crypt/ISCs) (Fig.1A). Leveraging the Maf LacZ mouse model where the Maf gene is disrupted by positioning LacZ gene in the Maf locus, we traced the gene expression of Maf in the GALT of Maf heterozygous mice through β-galactosidase activity[31]. The staining revealed Maf expression to be localized in the Peyer's patch (Fig. 1B). Organoids treated with RANKL underwent immunofluorescence staining of GP2 and Maf. The confocal images revealed their expression to be localized together (Fig. 1C).

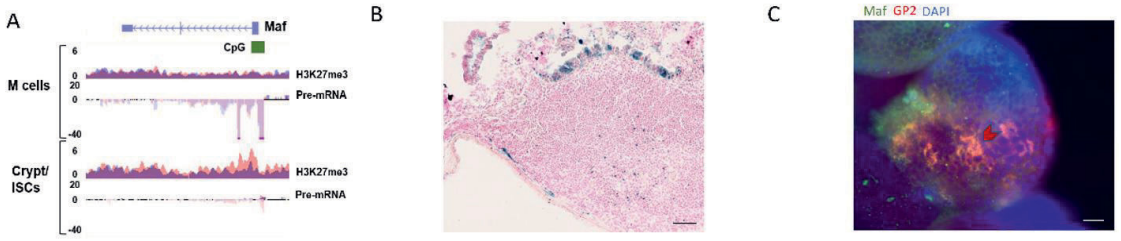

Figure 1. Maf is PRC2 regulated and localized in the FAE**.** A) H3K27me3 occupancy at CpG islands spanning the promoter and first exon of the Maf gene in organoids treated with RANKL (M cells) and treated with Wnt3a and Chir99021 (Crypt/ISCs). Below, pre-mRNA expression of Maf in organoids treated as above (y-axis: normalized tag count, ENR500 = R-spondin 500ng/ml, R100 = RANKL 100ng/ml). Original data published in George et al. 2021 CMGH 10.1016/j.jcmgh.2021.05.014 B) Sections of Peyer's Patch from Maf heterozygous mice were processed and stained with β-galactosidase staining assay to trace Maf expression in the FAE. Bar, 100 μm C) immunofluorescence of GP2 and Maf in RANKL treated organoids, Bar, 100 μm.

2.2. Maf expression is induced by RANKL stimulation and is under the Rank-RANKL signaling axis

To investigate if Maf is expressed by RANKL stimulation, intestinal organoids derived from wildtype mice were cultured with and without RANKL treatment for 3 days. qPCR analysis of isolated RNA shows significant upregulation of *Maf* as well as *Gp2* in organoids treated with RANKL. (Fig.2A) β-galactosidase experiments to trace Maf expression in Maf heterozygous organoids grown in RANKL conditions revealed Maf expression in cells leading to the lumen of the organoids (Fig.2B). Rank-KO mouse intestinal organoids were generated using the Lenti V2 CRISPR/Cas9 system, RANKL treatment of RANK KO did not activate *Maf* expression suggesting that Maf falls under the control of Rank-RANKL signaling (Fig. 2C).

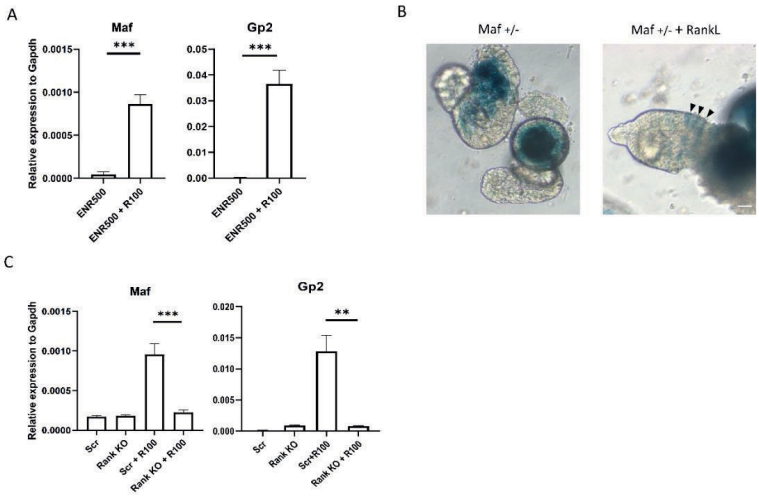

Figure 2. Maf expression in M cells is dependent on Rank- RANKL signaling A) Organoids generated from wild-type mice were stimulated with 100ng of RANKL for 4 d. B) Organoids isolated from Maf heterozygous mice were processed into paraffin blocks and stained with β-galactosidase staining assay to trace Maf expression in the organoids. Arrowheads indicate Maf expressing M cells. Bar, 100 μm C) Rank KO organoids and Scrambled organoids generated by lentiCRISPR v2 were incubated with RANKL for 4 days, *Maf* and *GP2* expression was analyzed by RT-qPCR. In (A&C) unpaired two-tailed Student's t-test was performed for three independent experiments, ***, $P < 0.005$; **, $P < 0.01$.

2.3. Maf deficiency impairs M cell differentiation and other M cell-associated factors.

Given that Maf is prominently expressed in Peyer's patches, we looked to see if the abolition of Maf had any effect on the development of M cells. Complete knockout of the Maf gene produce a lethal phenotype and pups were born stillborn or only survived for up to 4 hours. However, organoids were isolated from both Maf WT and Maf KO pups as soon as the mothers went into labor and cultured in media conditioned with Egf, Noggin, and R-spondin, Wnt, and Chir99021. After propagation, the organoids were grown in the presence and absence of RANKL for 3 days. Rt-qPCR analysis of the isolated RNA reveals a significant decrease in *Gp2* expression; *SpiB*, *Sox8*, and *Esrrg*, transcription factors that are critical for functionally mature M cells showed significantly reduced expression as well (Fig.3A). Early developmental markers of M cell differentiation such as *MarcksL1* and *Tnfaip2* showed diminished expression in the Maf knockout organoids compared to its wildtype counterparts. Immunofluorescence analysis of Gp2 in Maf WT and Maf KO depicted a lack of Gp2 expression. Our observations reveal that Maf is required for the expression of early markers as well as maturation genes associated with M cell differentiation.

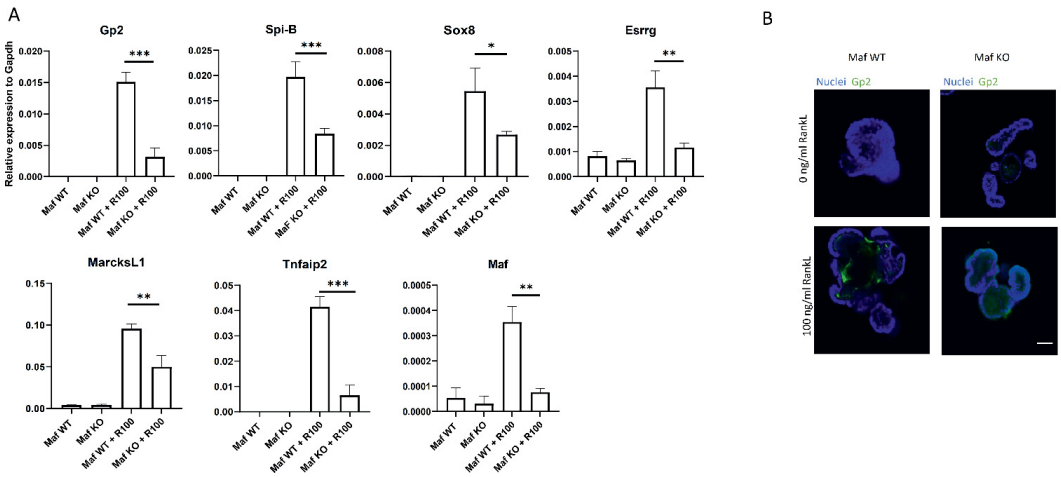

Figure 3. Impaired M cell maturation in Maf KO results from epithelium intrinsic defect. A) qPCR analysis of M cell-associated genes from RNA of organoids derived from Maf WT and Maf KO mice cultured with and without RANKL for 3 d. Values are presented as the mean ± SD; ***, $P < 0.005$; **, $P < 0.01$; *, $P < 0.05$; unpaired two-tailed Student's t-test, n = 3. Data are representative of two independent experiments. (B) Immunostaining images for GP2 (green) on organoids. Bar, 100 μm.

3. Discussion

M cells are specialized intestinal epithelial cells that are a part of the adaptive immunity and on the frontlines of the gut epithelium initiating a mucosal immune response against pathogenic bacteria. These epithelial cells partake in gut immunity by transcytosis of pathogens and commensal bacteria via the receptor Gp2. Recent research into M cells has revealed critical transcription factors such as Spi-B, Sox8, Esrrg, and Atoh8 and various other ligands like membrane-bound RANKL from lymphoid cells and S100A4 from Dock8 cells essential for M cell development. [14,17–19,32–34] However, complete elucidation of the differentiation and development of M cells remains to be understood. Recently we explored the epigenetic regulation of M cells and the analysis revealed several PRC2-regulated transcription factors controlling M cell development and differentiation (6 silenced and 6 upregulated). Maf was found upregulated in the list of genes identified[19]. From this study, we describe that M cells in the FAE express Maf and are under the overview of RANKL signaling and critical for the maturation of functional M cells.

Maf (musculoaponeurotic fibrosarcoma) gene encodes for the transcription factor Maf (c-Maf). The Maf transcription family comprises of 7 members divided into 2 subclasses based on their size; large Maf proteins include MAFA/L-MAF, MAFB, MAF/c-Maf, and NLR (neural retina leucine zipper), and the small Maf proteins, MAFK, MAFG, and MAFF. The small Maf proteins are also distinct by the lack of amino-terminal transactivation domain. The Maf family is highly conserved and has a unique leucine zipper structure (bZIP). In the pancreas, c-Maf regulates glucagon hormone production and in the liver, it is critical for erythropoiesis[35–37]. Maf also plays a role as an immune regulator and a transcription factor necessary for T helper 2 cells (Th2), additionally, it has also been found to play a key role in the differentiation and development of innate immune cell types, B lymphocytes, and T- cell subsets [25–28]. In line with Maf being a major contributor to cellular, signaling, and physiological process, Maf knockout mice with C57BL/6 background are lethal embryonically or perinatally [21,22,36]. They are often born stillborn or only live up to 4 hours. Maf KO with BALB/c background lives to adulthood.

Our work here characterizes the role of Maf in M cell differentiation and development. X-gal assay of Maf-LacZ heterozygous mice show Maf expression in the Peyer's patch of the follicle-associated epithelium and organoids isolated from the Maf heterozygous mice showed that Maf was expression was inducible upon RANKL stimulation. Maf tracing via X-gal assay showed Maf expressing cells away from the crypt and closer towards the lumen in organoids treated with RANKL. As expected, pups with complete knockdown of Maf were stillborn or only lived 2-4 hours after birth but

intestinal crypts isolated and cultured in RANKL were able to provide insights into Maf's critical role in M cells development. Maf deficit organoids showed a significant decrease in the maturation of M cells as Gp2 expression was significantly reduced indicating impaired and immature M cells. Lack of Gp2 expression in M cells have been shown to result in attenuation of antigen sampling and transcytosis and increased rates of infection to *S. typhimurium*. Since this conclusion of impaired M cell development was reached using epithelial organoids, it can also be assumed that loss of Gp2 in Maf KO organoids is an epithelium-intrinsic defect. Key transcription factors required for M cell maturation such as *Spi-B, Sox8*, and *Esrrg* showed absent or reduced activation without Maf. Early developmental markers of M cells such as *MarcksL1* and *Tnfaip2* were also affected and showed reduced activation. In line with key transcription factors and early developmental factors showing absent or reduced expression, it is reasonable to conclude that Maf is a critical factor for M cell differentiation. In ocular lens differentiation and osteogenic differentiation Maf expression is regulated by BMP signaling [38,39]. Atoh8, a transcription factor critical for maintaining the density of the M cell population was also found to be under the regulation of BMP signaling [32]. It is possible that RANKL induced BMP signaling could be responsible for regulating the expression of Maf in M cells. However, further experiments with Maf KO in mice with BALB/c background are required to understand the physiological significance of Maf KO in-vivo.

In conclusion, PRC2 regulated Maf has major implications in M cell maturation and mucosal immunity. The transcription factor is necessary for M cell development and lack of Maf could lead to attenuation of transcytosis of antigens and commensal bacteria. Reduced transcytosis has been shown to lead to an impaired immune response to pilli-expressing pathogenic bacteria such as *E.coli* and *S. typhimurium*[40].

4. Materials and Methods

4.1. Mice

All animal experiments were approved by the Finnish National Animal Experiment Board (Permit: ESAVI/5824/2018). Maf lacZ mice were caged in standard light-dark conditions at the pathogen-free animal facility of the faculty of Medicine and Health Technology. Food, water ad libitum was followed in a regularly timed schedule. B6.129-Maftm1Gsb/J heterozygous mice were purchased from Jackson laboratories (Cat number: 004158 | Maf^{tm1Gsb}). To generate Maf wildtype line, heterozygous line, and homozygous line, B6.129-Maftm1Gsb/J heterozygous were mated with, B6.129-Maftm1Gsb/J heterozygous, F1 generation was backcrossed with heterozygous mice. Littermates with Maf wildtype were used as control. Maf genotypes were confirmed by qPCR.

4.2.β-. galactosidase staining of Peyer's patches and organoids

Ileal PP's were isolated from the ileal section of the small intestine and transferred to a 10cm dish with 30ml of cold PBS. Excess fat was cut from the tissues and embedded into paraffin blocks. Organoids grown in RANKL was washed with PBS and made into paraffin blocks. The blocks were cut at 10um sections and mounted on a slide. X-gal (5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside, Goldbio) was dissolved in dimethylformamide at 50 mg/ml. Paraffin blocks were fixed with 4% PFA for 10 minutes. The slides were washed 3 times with 3 changes of PBS for 5 minutes wash and the final rinse in distilled water. After drying the slides were incubated in X-gal working solution at 37 C for 24 hours in a chamber with adequate moisture content. The sections were washed in PBS solution 2 times for 5 minutes each. After rinsing with distilled water, the sections were counter-stained with nuclear fast red for 3-5 minutes followed by further rinsing and washing in distilled water for 2 minutes. The sections were finally dehydrated for 3 minutes each in 70%, 95%, and twice with 100% ethanol and thrice with xylene. The sections were mounted Permount and covered with a coverslip and examined by digital slide scanner Hamamatsu Nanozoomer.

4.3. Mouse Intestinal Organoid culture

Mouse intestinal crypts were isolated and cultured in an *in vitro* setting as previously described [15,41] Crypts were isolated as soon as the stillborn pups were delivered. Isolated duodenums were washed in PBS and longitudinally cut. Villi were gently scraped with a glass slide. Following washes with PBS, the tissue was cut into 2mm pieces and pipetted up and down with a 10 ml pipette. After repeated changes of the PBS till the suspension was clear, the tissues were suspended in 10mM EDTA in PBS for 20 minutes rocking at room temperature. The crypts were separated from the rest of the tissues using a 70-μm cell strainer (Fisher Scientific). The crypts were counted and cultured on a 24 well plate by embedding them in 30ul of cold Matrigel (Corning). Organoids were cultured in an optimal medium consisting of advanced DMEM/F12 (Thermo Fisher Scientific) that contained HEPES (10mM, Sigma-Aldrich), Glutamax (2mM, Thermo Fisher Scientific), Penicillin-streptomycin (100U/ml, Sigma-Aldrich), B-27 supplement minus Vitamin A (Thermo Fisher

Scientific), N-2 supplement (Thermo Fisher Scientific), N-acetylcysteine (1 mM; Sigma-Aldrich), recombinant murine EGF (50 ng/ml; Thermo Fisher Scientific), recombinant murine Noggin (100 ng/mL; PeproTech), recombinant mouse R-spondin1 (1 μg/mL; R&D Systems). Media were changed every 2 days. For M cell differentiation, recombinant mouse RANKL (100ng/ml, Peprotech) was added to the media and incubated for 4 days.

4.4. CRISPR–Cas9 gene knockout of intestinal organoids

Guide RNAs for gene encoding RANK were designed using CRISPR design tool (http://crispr.mit.edu)[42]. These were cloned into the vector lentiCRISPR v2 (Addgene, 52961). The cloned product was transfected into HEK 293FT cells (ThermoFisher R7007). The supernatant was collected after 48 h and the Lenti-X concentrator (Clontech) was added to the suspension. The 293FT cell line was tested for mycoplasma. Intestinal organoids were cultured in ENCY (EGF, Noggin, Chir-99021, and Y-27632) prior to transduction. After the organoids were dissociated into single cells using TrypLE Express (Thermo Fisher Scientific) and supplemented with 1,000 U/ml DnaseI at 32 °C for 5 mins, the cells were washed once with Advanced DMEM and resuspended in a transduction medium (ENR media supplemented with 1mM nicotinamide, Y-27632, Chir99021, 8 μg/ml polybrene (Sigma-Aldrich)) and mixed with the previously collected concentrated virus. The mixture was centrifuged at 600 x g 32 °C for 1 hr followed by 3 hr incubation at 37°C, after which they were collected and plated on 60% Matrigel with enriched transduction medium without polybrene. On day 2 and day 4, RANK transduced organoids were selected with 2 μg/ml of puromycin (Sigma-Aldrich). Surviving clones were expanded in cultured with ENR medium. RANK KO organoids were confirmed by western blot to validate the expression of deleted gene.

4.5. Immunofluorescence of Organoids

Maf WT and Maf KO Intestinal crypt organoids were analyzed by whole-mount immunostaining. The organoids were cultured for 4 hours in an 8-well chamber plate in the presence and absence of RANKL 100ng/ml following fixation with 4% PFA for 15 minutes, followed by permeabilization with 0.1% Triton X-100 for another 15 minutes. The organoids were stained with Gp2 (MBL, D278-3) antibodies overnight at +4 degrees Celsius. This was followed by 2-hour incubation of anti-Rabbit secondary for Gp2 (and Anti-Rat for the secondary antibody. Gp2 expressing cells were analyzed by Nikon A1R+ Laser Scanning Confocal Microscope after mounting with ProLong Diamond with Dapi mounting solution (Molecular Probes P36962).

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Abbreviations

M cells= Microfold cells**; PP=** Peyer's Patch; **FAE=** Follicle associated epithelium; **VE=** villous epithelium; **RANKL=** Receptor activator of nuclear factor kappa B ligand; **Rank=** Receptor activator of nuclear factor kappa B; **PRC2=** polycomb repressive complex 2, **Musculoaponeurotic fibrosarcoma**= Maf

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