FINDING A NICHE FOR KAISO IN THE INTESTINAL EPITHELIUM

MOLECULAR & BIOLOGICAL CHARACTERIZATION OF THE POZ-ZF TRANSCRIPTION FACTOR KAISO IN INTESTINAL HOMEOSTASIS

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ABSTRACT

We recently reported that intestinal-specific overexpression of the POZ-ZF transcription factor Kaiso produced two prominent phenotypes in 1-year old mice: Kaiso transgenic (*Kaiso*^{Tg}) mice presented with chronic intestinal inflammation, and an increase in secretory cell types – a trait typical of Notch signalling inhibition. Despite these findings however, the factor(s) responsible for Kaiso-mediated inflammation and secretory cell increases had not been elucidated. The primary goal of this thesis was to begin filling in this knowledge gap, by shedding mechanistic insight on Kaiso's role in governing these two prominent phenotypes.

First, we elucidated Kaiso's role in the Notch signalling pathway and found that Kaiso inhibited the expression of the Notch1 receptor, and its ligand Dll-1, but promoted the expression of the Jagged-1 ligand. We postulated that the Kaiso-mediated reduction in Dll-1 might be responsible for the increase in secretory cell types, whereas Kaiso-mediated regulation of Jagged-1, which is dispensable for cell fate decisions, may be implicated in colon cancer progression. Importantly, we also found that Kaiso's effects on Notch pathway inhibition occurred prior to the onset of chronic intestinal inflammation.

Our analyses of the chronic inflammatory phenotype in *Kaiso*^{Tg} mice demonstrated that Kaiso overexpression drives pathogenic neutrophil-specific recruitment (as evidenced by increases in neutrophil-specific enzymatic activity, the formation of crypt abscesses, and augmented expression levels of the neutrophils-specific chemokine, MIP2); an increase in the pore-forming Claudin-2; reduction of the cell adhesion protein E-cadherin; and abnormal intestinal epithelial repair mechanisms. Together, these findings imply that the pathogenesis of Kaiso-mediated intestinal inflammation is a multi-factorial process.

A secondary goal of this thesis was to initiate studies to elucidate how the Kaiso binding partner, Znf131, might play a role Kaiso-mediated transcriptional regulation. We found that Znf131 indirectly associated with several Kaiso target genes, including *Cyclin D1* (*CCND1*). Importantly, Znf131 activated a minimal *CCND1* promoter previously shown to be inhibited by Kaiso. Moreover, Kaiso overexpression attenuated Znf131-mediated transcriptional activation and Znf131 expression in intestinal cells. Together, these findings hint that Znf131 and Kaiso may exert opposing biological functions, which may have implications in Kaiso-mediated intestinal homeostasis and disease.

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Pheeeew!!! On that note, let's get to the juicy stuff!

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No such thing as a dream too big. If I can do it, you can, too!

It always seems impossible, until it's done. - Nelson Mandela

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LIST OF ABBREVIATIONS

Abbreviation	Full Term
5'-aza-dC	5'-aza-**d**eoxy**c**ytidine
ADAM	**A** **d**isintegrin **a**nd **m**etalloprotease
AJ	**A**dherens **j**unction
Amp	**Amp**icillin
ANOVA	**An**alysis **o**f **va**riance
APAF1	**A**poptotic **p**rotease **a**ctivating **f**actor 1
APC	**A**denomatous **P**olyposis **C**oli
Atoh1	**Ato**nal **h**omologue 1
Axin	**Ax**is **in**hibition protein
Bmi1	**B** cell-specific **M**oloney murine leukemia virus **i**ntegration site 1
BrdU	**br**omo**d**eoxy**u**ridine
BTB/POZ	**B**road Complex, **T**ramtrak, **B**ric à Brac/**Po**xvirus and **z**inc **f**inger
C/EBPα	**C**CAAT-**e**nhancer-**b**inding **p**rotein alpha
CD	**C**rohn's **d**isease
CDKN1A	**C**yclin-**d**ependent **k**inase i**n**hibitor 1A
CGI	**C**p**G** **I**sland
ChIP	**Ch**romatin **I**mmuno**p**recipitation
CK1	**C**asein **K**inase 1
CRC	**C**olo**r**ectal **c**ancer
CRE	**c**yclic AMP (cAMP) **R**esponse **E**lement
CSL	**C**BF1/**S**uppressor of Hairless/**L**ag2
DAPI	4, 6- **D**i**a**midino-2-**p**henyl**i**ndole
DBZ	**D**i**b**en**z**azepine
DLL	**D**elta-**l**ike **l**igand
DMEM	**D**ulbecco's Modified **E**agle's **M**edium
DSL	**D**elta/**S**errate/**L**ag2
DSS	**D**extran **S**odium **S**ulfate
EGF	**E**pidermal **G**rowth **F**actor
EMSA	**E**lectrophoretic **m**obility **s**hift **a**ssay
EPLIN	**E**pithelial **p**rotein **l**ost **i**n **n**eoplasm
ERα	**E**strogen **R**eceptor alpha
Fz	**F**ri**z**zled
GFP	**g**reen fluorescent protein
GSK3	**G**lycogen **S**ynthase **K**inase 3
H&E	**H**ematoxylin & **e**osin
HDAC	**H**istone **d**e**ac**etylase
HES	**H**airy **e**nhancer of **s**plit

Hh	**H**edge**h**og
HMBS	**H**ydroxy**m**ethyl**b**ilane **s**ynthase
HTAB	**h**exadecyl**t**rimethyl**a**mmonium **b**romide
IBD	**I**nflammatory **B**owel **D**isease
IEC	**I**ntestinal **e**pithelial **c**ell
IF	**I**mmuno**f**luorescence
IHC	**I**mmuno**h**isto**c**hemistry
Ihh	**I**ndian **h**edge**h**og
IL	**I**nterleukin
IPTG	**i**so**p**ropyl β-D-**t**hio**g**alactoside
IRES	**i**nternal **r**ibosomal **e**ntry **s**ite
ISC	**I**ntestinal **s**tem **c**ell
KaisoTg	Kaiso **t**rans**g**enic
KBS	**K**aiso **B**inding **S**ite
LB	**L**uria **b**roth
Lgr5/6	**L**eucine-rich repeat-containing **G**-protein-coupled **r**eceptor **5/6**
LRP 5/6	**L**ow-density lipoprotein **r**eceptor-related **p**rotein **5/6**
Math1	**M**ouse **at**onal **h**omologue 1
meCpG	**me**thyl CpG
MMP7	**M**atrix **M**etallo**p**roteinase 7
mo.	**mo**nth
MPO	**M**yelo**p**er**o**xidase
MTG16	**M**yeloid **T**ranslocation **G**ene
Myc	**My**elo**c**ytomatosis oncogene
N1-ICD	**N**otch1 **i**ntra**c**ellular **d**omain
NCoR	**N**uclear **co**-**r**epressor
NFκB	**N**uclear **f**actor kappa B
NICD	**N**otch **i**ntra**c**ellular **d**omain
NonTg	Non-**t**rans**g**enic
Oligo	**Oligo**nucleotide
p120^{ctn}	p120 **c**a**te**n**i**n
PAS	**P**eriodic **a**cid-**S**chiff
PCR	**P**olymerase **C**hain **R**eaction
Pofut1	**p**rotein ***O*-fu**cosyl**t**ransferase 1
POZ-ZF	**Po**xvirus and **z**inc finger-**z**inc **f**inger
PPARγ	**P**eroxisome **P**roliferator-**A**ctivated **R**eceptor gamma
PPRE	**P**eroxisome **P**roliferator **R**esponse **E**lement
qRT-PCR	**q**uantitative **r**eal **t**ime-PCR
rapsyn	**r**eceptor **a**ssociated **p**rotein of the **syn**apse
RBP-J	**R**ecombination signal **b**inding **p**rotein for immunoglobulin kappa **J**

RXR	**R**etinoid **X** **R**eceptor
SAMP1/YitFc	**S**enescence **a**ccelerated **m**ice senescence-**p**rone, YitFc substrain
TCF/LEF	**T** **c**ell **f**actor/**l**ymphoid **e**nhancer **f**actor
TFGBR1/2	**T**ransforming **g**rowth **f**actor-**b**eta **r**eceptor 1/2
TJ	**T**ight **j**unction
TLE	**T**ransducin-**l**ike **e**nhancer of split
TNBS	2, 4, 6-**t**ri**n**itro**b**enzene **s**ulfonic acid
TNFα	**T**umour **n**ecrosis **f**actor alpha
TSS	**T**ran**s**cription **s**tart **s**ite
TZD	**t**hia**z**oli**d**inediones
UC	**U**lcerative **c**olitis
VKA/E	**v**illin-**K**aiso line **A**/ line **E**
WNT	**W**ingless-type MMTV i**nt**egration site
ZBE	**Z**nf131 **B**inding **E**lement
ZEB1	**Z**inc finger **E**-box **b**inding homeobox 1
ZF	**Z**inc **f**inger
Znf131	**Zi**n**c** **f**inger protein 131
ZO	**Z**onula **o**ccludens

CHAPTER 1 INTRODUCTION

OVERVIEW

From mouth to anus, the gastrointestinal (GI) tract is a hollow continuous tube, responsible for the digestion of food, elimination of waste and absorption of nutrients – the latter of which occurs over the folded epithelia of the small and large intestines. The intestines undergo rapid turnover, and several highly conserved signalling pathways are indispensible for ensuring homeostasis. Among them is the canonical Notch signalling pathway, which is critical for mediating proliferative and cell differentiation cues. Differentiated intestinal epithelial cells (IECs) consist of absorptive cells, or enterocytes, and three main types of secretory cells – namely the antimicrobial Paneth and goblet cells, and hormone-secreting enteroendocrine cells (Barker 2014, de Santa Barbara *et al.,* 2003, Gassler *et al.,* 2006, Noah *et al.,* 2011).

In addition to digestion, the intestinal epithelium itself also forms a physical barrier, which plays a fundamental role in preventing luminal microbes from entering the body (Gersemann *et al.,* 2011, Landy *et al.,* 2016, Michielan & D'Incà 2015, Peterson & Artis 2014, Su *et al.,* 2009). Deregulated homeostasis can compromise the integrity of the epithelial barrier, and may thus contribute to disorders such as inflammatory bowel disease (IBD) – a chronic and debilitating disease characterized by persistent inflammation along the GI tract. The precise etiology for IBD is currently unknown. However, the prevailing hypothesis is that IBD pathogenesis is triggered by multiple contributing factors, including dysbiosis of the intestinal microbiome, and mutation of

susceptible loci, including *CDH1*, which encodes E-cadherin (Barrett *et al.*, 2009, Cho & Brant 2011, McGovern *et al.*, 2010, Muise *et al.*, 2009). Unfortunately, there is no cure for IBD, and thus a better understanding of the key players that drive intestinal inflammation warrant detailed investigation.

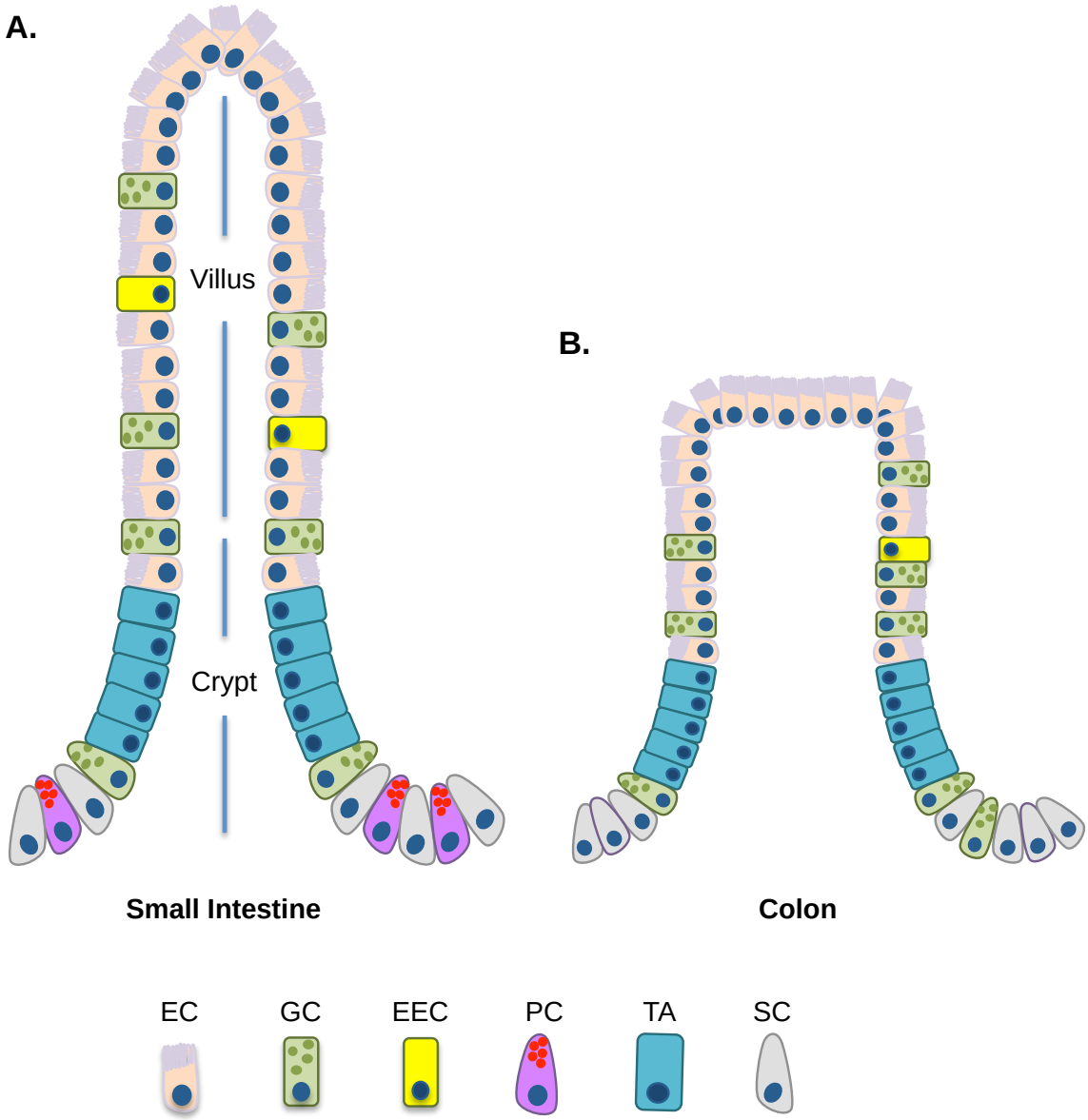
Recent studies have uncovered a novel role for the transcription factor Kaiso in intestinal homeostasis. Notably, constitutive intestinal-specific Kaiso overexpression in 1-year old mice promotes both spontaneous inflammation and increased numbers of secretory cell types (Chaudhary *et al.*, 2013). However, the molecular mechanisms that underlie Kaiso-mediated intestinal homeostasis have not yet been explored. Herein, this work seeks to shed light on a mechanistic role for Kaiso in intestinal homeostasis and disease.

MAINTAINING THE EQUILIBRIUM – HOMEOSTASIS OF THE MAMMALIAN GUT

1.1 The Intestinal Epithelium

The gut is divided into the small and large intestine, and is well known for its functions in digestion, nutrient and water absorption, and waste excretion. In the small intestine, fingerlike projections called villi serve to maximize the surface area for nutrient uptake (Barker 2014, Gassler *et al.*, 2006). Neighboring villi are separated by deep invaginations known as crypts of Lieberkühn, which house stem and progenitor cell populations (discussed in detail in **Section 1.2.3.1**) (Barker 2014, Barker *et al.*, 2008a, Barker *et al.*, 2012, Marshman *et al.*, 2002). The colon, which is continuous with the small intestine, forms the longest portion of the large intestine. Unlike the small intestine however, the

FIGURE 1.1 Schematic representations of the small intestine, colon, and the cells that constitute their epithelium. (A) The small intestine is formed of finger-like projections called villi. Neighboring villi are separated by crypts of Lieberkühn, which contain stem (SC) and progenitor (transit amplifying – TA) cell populations. **(B)** The colon lacks villi, but instead has invaginations/crypts that are separated by flattened surfaces. EC – enterocyte; GC – goblet cell; EEC – enteroendocrine cell; PC – Paneth cell. Original artwork.


A.
Villus
Crypt
B.
Small Intestine
Colon
EC
GC
EEC
PC
TA
SC

the colon lacks villi, but rather has a folded, flat surface epithelium separated by crypts (**Figure 1.1**).

The intestinal epithelium consists of 4 major cell types – absorptive enterocytes and 3 main types of secretory cells: goblet, Paneth, and enteroendocrine cells (Cheng & Leblond 1974) (**Figure 1.1**). Enterocytes (known as colonocytes in the colon) constitute ~85-90% of the epithelium and are the predominant cell type in the intestine. These specialized cells are characterized by their columnar shape and apical brush border of microvilli, (Cheng & Leblond 1974, Umar 2010), which serve to further maximize the surface area for nutrient and water absorption (Kiela & Ghishan 2016).

Both goblet and Paneth cells are critical participants in the innate immunity of the intestine. Mucins secreted by goblet cells contribute to the mucous layer that separates commensal luminal bacteria from the underlying intestinal mucosa, hence providing the first line of defense in intestinal innate immunity (Johansson *et al.,* 2011, Pelaseyed *et al.,* 2014). Paneth cells (which are absent in the colon) reside in the small intestinal crypts where they secrete an arsenal of bactericidal peptides (e.g. lysozyme) into the crypt lumen. As such, Paneth cells control microbial density of the small intestine, and, given their proximity to the stem cell niche, also protect this zone against microbial infection (Ayabe *et al.,* 2000, Ouellette *et al.,* 1994, Ouellette *et al.,* 1992).

Enteroendocrine cells (which make up less than 1% of the intestinal epithelium) secrete various hormones (e.g. serotonin and chromogranins) in response to luminal products, and thus mediate communication between the enteric nervous and endocrine systems (Gribble & Reimann 2016). While they are well known for their endocrine roles,

enteroendocrine cells have also been implicated in immunity. For example, serotonin has been shown to act as a chemoattractant for various immune cell types, and increased serotonin has been detected in both humans and rodents with IBD (Manocha & Khan 2012).

In addition to the four main cell types, a fifth differentiated cell, known as the tuft cell, also forms part of the intestinal mucosa (Gerbe *et al.,* 2012). Even more rare than enteroendocrine cells, the precise role of tuft cells currently remains largely elusive. However, a recent study has implicated tuft cells in intestinal mucosal immunity against parasitic infection (Gerbe *et al.,* 2016).

With the exception of Paneth cells, which reside at the base of the crypt interspersed among intestinal stem cells, terminally differentiated epithelial cells undergo collective sheet migration towards the villus tip. There, they eventually undergo anoikis and are sloughed off into the lumen (Barker 2014, Scoville *et al.,* 2008, Umar 2010). As such, rapid renewal of the epithelium is required to ensure homeostasis, and several highly conserved signalling pathways are essential for maintaining this equilibrium. Among them, the Wnt and Notch signalling pathways are critical for proliferation of the stem and progenitor cell compartments, and for dictating intestinal cell fate decisions (Crosnier *et al.,* 2006, de Santa Barbara *et al.,* 2003, Koch *et al.,* 2013, Nakamura *et al.,* 2007).

1.2 The Canonical Wnt and Notch signalling pathways coordinate to ensure intestinal homeostasis

1.2.1 The Canonical Wnt Signalling Pathway

Wingless-type MMTV i**nt**egration site (Wnt) proteins are a highly conserved family of secreted cysteine-rich ligands that participate in various cellular processes, such as cell proliferation and differentiation (Crosnier *et al.,* 2006, Krausova & Korinek 2014, Logan & Nusse 2004). The main downstream effector of the canonical Wnt pathway is the Armadillo protein, β-catenin, which exists in two pools - a membrane-associated pool (**Section 1.3.2**) and a free cytosolic pool (Nusse & Clevers 2017).

In unstimulated cells, cytosolic β-catenin is maintained at low levels by the constitutive action of the multiprotein Destruction Complex, comprised of the scaffolding proteins, **ax**is **in**hibition protein (Axin) and **a**denomatous **p**olyposis **c**oli (APC); and kinases, **g**lycogen **s**ynthase **k**inase 3 (GSK3) and **c**asein **k**inase 1 (CK1) (Nusse & Clevers 2017) (**Figure 1.2 A**). Phosphorylation of β-catenin by Axin-CK1 complexes primes it for further serine/threonine phosphorylation by GSK3 (Amit *et al.,* 2002). This signals β-catenin for polyubiquitination by β-Trcp (β-**t**ransducin **r**epeat-**c**ontaining **p**rotein), thus targeting it for proteasomal degradation and preventing its nuclear translocation (Liu *et al.,* 1999). In the absence of nuclear β–catenin, **T c**ell **f**actor/**l**ymphoid **e**nhancer **f**actor (TCF/LEF) proteins remain associated with the transcriptional repressor, Groucho/TLE (**T**ransducin-**l**ike **e**nhancer of split), which together repress Wnt target gene expression (Cavallo *et al.,* 1998).

Activation of the Wnt/β-catenin pathway is initiated upon binding of Wnt ligand to its heterodimeric receptor complex, comprised of **F**ri**z**zled (Fz) and **l**ow-density

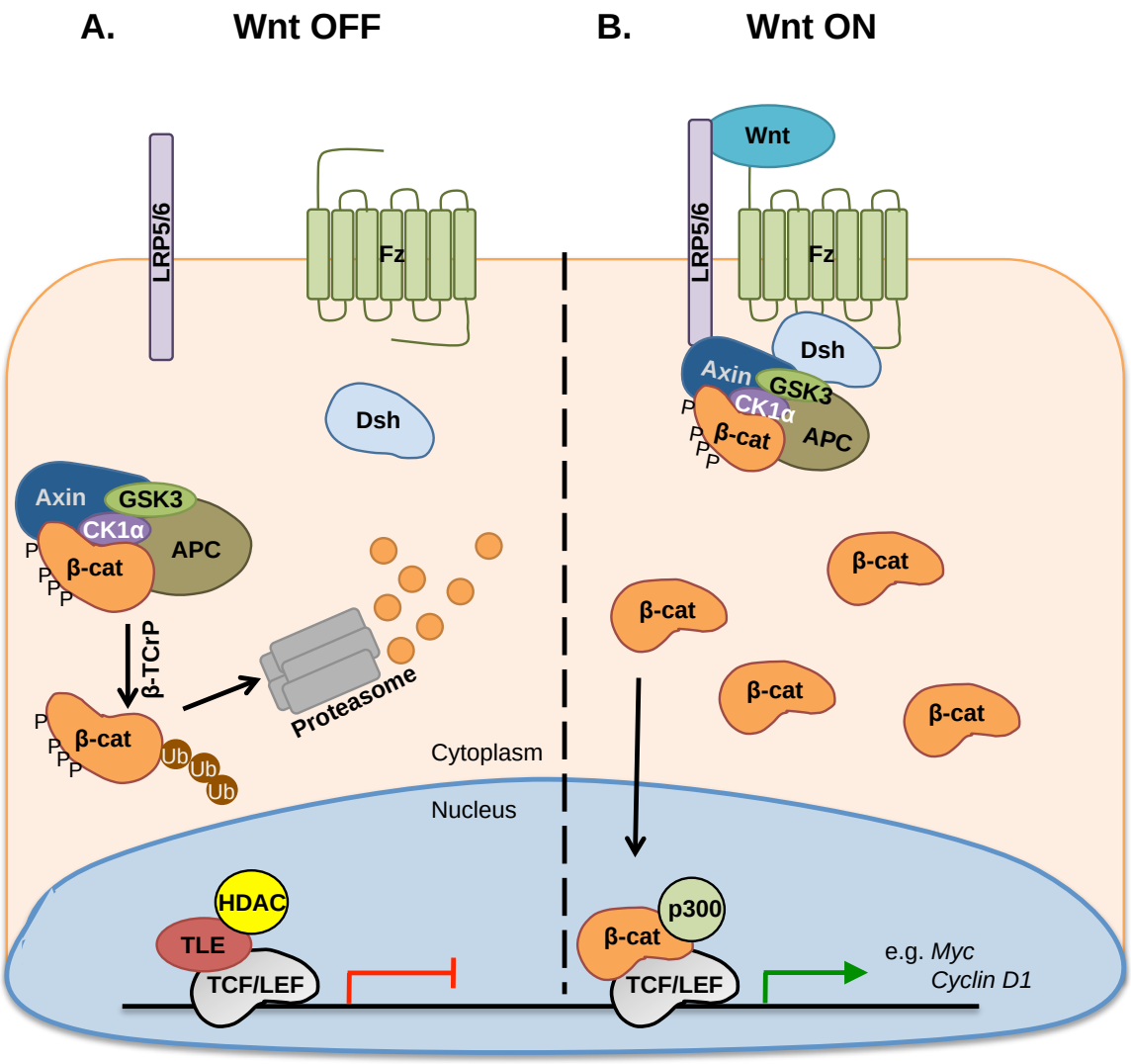
lipoprotein **r**eceptor-related **p**rotein (LRP) 5/6 (Nusse & Clevers 2017) (**Figure 1.2 B**). This leads to the membrane recruitment of **D**i**sh**evelled (Dsh), which promotes formation of signalosomes and leads to CK1- and GSK3-mediated phosphorylation of LRP6. Phospho-LRP6 directs Axin to the plasma membrane, permitting cytosolic accumulation and subsequent nuclear translocation of β-catenin (Cliffe *et al.,* 2003, Gao & Chen 2010). In the nucleus, β-catenin displaces TLE and binds the TCF/LEF transcription factors to activate expression of canonical Wnt target genes (Billin *et al.,* 2000, Daniels & Weis 2005).

Wnt/β-catenin activity is highest at the crypt base and gradually decreases as cells travel toward the crypt-villus junction. In the crypts, Wnt signalling engages with a number of other pathways, including the Notch signalling pathway, to achieve intestinal homeostasis. Together, the canonical Wnt and Notch signalling pathways coordinate physiological processes, including cell proliferation and differentiation (**Section 1.2.3**) (Crosnier *et al.,* 2006, Horvay & Abud 2013, Nakamura *et al.,* 2007, Sancho *et al.,* 2015).

1.2.2 The Canonical Notch Signalling Pathway

The Notch receptor was discovered 100 years ago by Thomas Hunt Morgan, shortly after John Dexter first described the *Notch* mutant phenotype in *Drosophila melanogaster* (Ntziachristos *et al.,* 2014). In mammals, there are four Notch paralogues (Notch 1-4), each encoded by a different gene (Kopan & Ilagan 2009). Notch receptors are single-pass type I transmembrane proteins. The **e**pidermal **g**rowth **f**actor (EGF) repeat-containing extracellular domain mediates binding with one of the five Notch ligands belonging to the **D**elta/**S**errate/**L**ag-2 (DSL) family (**D**e**l**ta-**l**ike [Dll] 1, -3, and -4; and Jagged-1, and -2).

FIGURE 1.2 The canonical Wnt signalling pathway. (A) In the absence of ligand binding, cytosolic β-cat (β-catenin) is maintained at low levels by the action of the multiprotein Destruction Complex, comprised of Axin, CK1, GSK3 and APC. CK1- and GSK3-mediated phosphorylation of β-catenin targets it for β-TCrP-mediated polyubiquitination and proteasomal degradation. Thus, TLE:TCF/LEF:HDAC complexes inhibit target gene expression. **(B)** Pathway activation is stimulated upon Wnt binding to the LRP5/6:Fz heterodimeric receptor, leading to the recruitment and binding of Dsh to Fz. Through its interaction with Axin, Dsh promotes the sequestration of the Destruction Complex at the plasma membrane. This permits cytosolic β-catenin accumulation and nuclear translocation, where it then interacts with TCF/LEF and co-activator proteins to activate target gene expression. Original artwork.

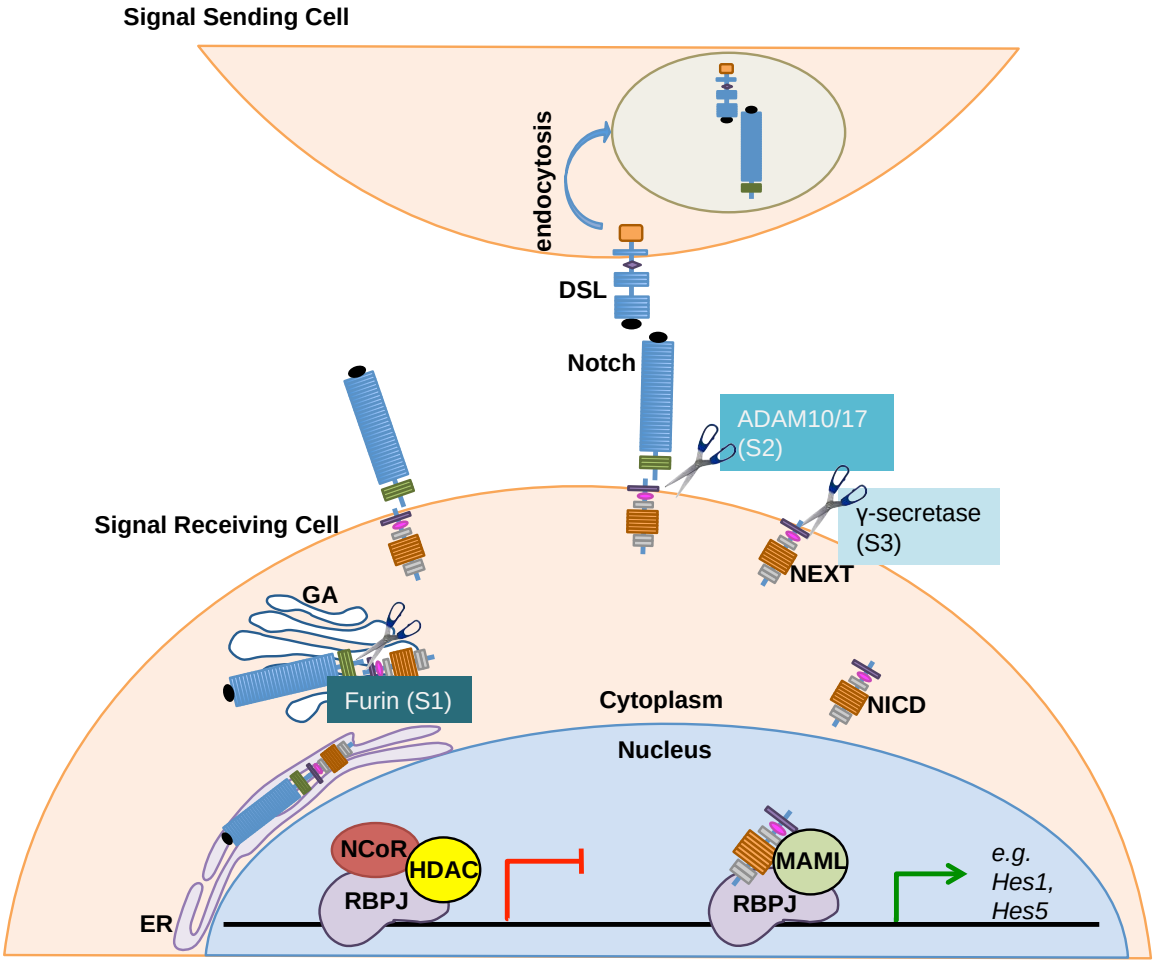

A.
Wnt OFF
B.
Wnt ON
LRP5/6
Fz
Dsh
Axin
GSK3
CK1α
APC
β-cat
P
β-TCrP
Proteasome
Ub
Cytoplasm
Nucleus
HDAC
TLE
TCF/LEF
Wnt
p300
e.g. Myc
Cyclin D1

DSL ligands are also single-pass transmembrane proteins, and thus Notch pathway activation is initiated upon ligand/receptor interactions between adjacent cells (Kopan & Ilagan 2009).

Notch undergoes three proteolytic cleavages in order to activate the signalling pathway. Initially translated as a single protein, Notch first undergoes ligand-independent Furin-mediated cleavage in the Golgi apparatus (**S**ite **1** [S1] cleavage), and is subsequently presented at the cell membrane as a heterodimeric single-pass type I transmembrane receptor (Blaumueller *et al.,* 1997, Logeat *et al.,* 1998) (**Figure 1.3**). Ligand binding exposes the S2 site on Notch for **A d**isintegrin **a**nd **m**etalloprotease (ADAM)-mediated cleavage (Brou *et al.,* 2000), which triggers the release and subsequent trans-endocytosis of the **N**otch **E**xtra**c**ellular **D**omain (NECD) into the neighboring cell (Nichols *et al.,* 2007). Consequently, S2-cleavage results in the formation of the **N**otch **E**xtra**c**ellular **T**runcation (NEXT), which remains tethered to the plasma membrane. NEXT then undergoes S3-cleavage by the γ-secretase protein complex, resulting in the cytoplasmic release of the **N**otch **I**ntra**c**ellular **D**omain (NICD), the active downstream effector of the Notch signalling pathway (Kidd *et al.,* 1998, Struhl & Greenwald 1999).

In the absence of ligand binding, the Notch pathway is silenced by RBP-J (**R**ecombination signal **b**inding **p**rotein for immunoglobulin kappa **J** region, also called CSL – **C**BF1/**S**uppressor of Hairless/**L**ag2), which interacts with co-repressors to inhibit Notch target gene expression (Hsieh *et al.,* 1999, Kao *et al.,* 1998). However, cytoplasmic release of NICD upon Notch pathway activation permits its nuclear

FIGURE 1.3 The canonical Notch signalling pathway. Notch is translated as a single protein in the endoplasmic reticulum (ER). Once in the Golgi apparatus (GA), Notch is cleaved at site 1 (S1) by Furin, and is subsequently presented at the plasma membrane as a heterodimer. The Notch signalling pathway is activated upon binding of Notch to the transmembrane Delta/Serrate/Lag2 (DSL) ligand on the neighboring signal-sending cell. Ligand binding triggers trans-endocytosis of the Notch extracellular domain into the signal-sending cell, and thus exposes S2 for ADAM10/17-mediated cleavage. S2 cleavage results in the formation of the Notch Extracellular Truncation (NEXT), which exposes S3 for cleavage by the γ-secretase protein complex. This frees the Notch Intracellular Domain (NICD), which then translocates into the nucleus of the signal-receiving cell. NICD subsequently interacts with Rbp-J and the coactivator Mastermind-like (MAML) to activate target gene expression. Original artwork.


Signal Sending Cell
endocytosis
DSL
Notch
ADAM10/17
(S2)
γ-secretase
(S3)
NEXT
Signal Receiving Cell
GA
Furin (S1)
Cytoplasm
NICD
Nucleus
NCoR
HDAC
RBPJ
MAML
RBPJ
e.g.
Hes1,
Hes5
ER

translocation, where it displaces transcriptional co-repressors and interacts with RBP-J. NICD:RBP-J complexes recruit co-activators such as **M**aster**m**ind **l**ike (MAML) to drive the expression of Notch target genes – most notably, members of the Hes and Hey family of transcription factors (Fischer & Gessler 2007, Jarriault *et al.*, 1995, Kageyama *et al.*, 2007, Kao *et al.*, 1998) (**Figure 1.3**).

1.2.3 Wnt and Notch strike the balance between proliferation and differentiation in the intestinal epithelium

Intestinal epithelial cells turnover every 3-5 days, making mammalian intestine the most rapidly renewing epithelium in the body. Intricate coordination between the canonical Wnt and Notch signalling pathways is central to this homeostasis. Equilibrium between proliferation and differentiation is key for intestinal regeneration, and ablation of either pathway grossly disturbs the capacity of the epithelium to repair itself. Herein, the roles of Wnt and Notch signalling as they pertain to intestinal proliferation and differentiation will be briefly summarized.

1.2.3.1 Intestinal proliferation in a Wnt & Notch niche.

Counterbalanced by anoikis at the villus tip, renewal of the intestinal epithelium is propelled by proliferation of stem cells (Horvay & Abud 2013). It is currently held that there are two distinct types of intestinal stem cells (ISCs): **B** cell-specific **M**oloney murine leukemia virus **i**ntegration site **1** ($Bmi1^{+ve}$) stem cells, located 4 cell positions from the crypt base (also called +4 stem cells), and **L**eucine-rich repeat-containing **G**-protein-coupled **r**eceptor **5** ($Lgr5^{+ve}$) crypt base columnar stem cells, which reside intermingled among the Paneth cells (Barker 2014, Horvay & Abud 2013, Li & Clevers

2010). Lgr5^{+ve} cells are actively cycling multipotent stem cells that give rise to all cell types of the intestinal epithelium (Barker *et al.,* 2008b, Barker *et al.,* 2007), whereas +4/Bmi1^{+ve} stem cells are quiescent, slow cycling, multipotent cells that undergo proliferation and replace Lgr5^{+ve} stem cells upon crypt injury (Yan *et al.,* 2012).

Both the canonical Wnt and Notch signalling pathways tightly control ISC proliferation. In fact, *LGR5* itself is a canonical Wnt target gene whose protein product, upon binding to R-spondin1, serves to potentiate the Wnt signal (de Lau *et al.,* 2011). Indeed, inhibition of Wnt signalling in the intestine results in a loss of Lgr5-positivity in the crypts (van Es *et al.,* 2012, Yan *et al.,* 2012). Coupled with the observation that Lgr5^{+ve} cells also stain positively for proliferation markers, Ki67 and **br**omo**d**eoxy**u**ridine (BrdU) (Barker *et al.,* 2007), it is not surprising that targeted loss of Wnt signalling in the intestines results in attenuated cell proliferation and crypt loss (de Lau *et al.,* 2011, Ireland *et al.,* 2004, Kuhnert *et al.,* 2004, Pinto *et al.,* 2003, van Es *et al.,* 2012). Conversely in murine models of constitutive Wnt signalling, mice exhibit hyperproliferation and adenoma formation (Peignon *et al.,* 2011, Sansom *et al.,* 2004); and the adenomas themselves display enhanced, albeit patchy, expression of Lgr5 (Baker *et al.,* 2015, Becker *et al.,* 2008, Fan *et al.,* 2010).

While Wnt plays a fundamental role in the proliferation of the intestinal epithelium, several studies have highlighted that intimate crosstalk with the Notch signalling pathway is crucial in modulating this process. Perhaps the first clue to suggest crosstalk between the two pathways was the observation that, like the Wnt gradient, Notch activity is highest in the proliferative crypt compartment (Jensen *et al.,* 2000).

Mice with intestinal-specific Notch pathway over-activation show increased cell proliferation (Fre *et al.,* 2005). Conversely, genetic and chemical ablation of Notch signalling causes reduced Ki67 positivity in *Rbp-J*$^{-/-}$ and **d**i**b**en**z**azepine (DBZ)-treated mice, respectively, suggesting reduced cell proliferation (van Es *et al.,* 2005, VanDussen *et al.,* 2012). This is supported by the observation that combined loss of both Notch1 and Notch2 induced cell cycle arrest, de-repression of cell cycle inhibitors, p27 and p57, and loss of progenitor cells (Riccio *et al.,* 2008). Notably, in Lgr5-GFP (**g**reen **f**luorescent **p**rotein) reporter mice, Notch inhibition caused a reduction in GFP-positivity in the crypts (VanDussen *et al.,* 2012), which might be due to the recent observation that Notch signalling is also required for the interconversion between +4/Bmi1^{+ve} and Lgr5^{+ve} stem cells (Srinivasan *et al.,* 2016).

While the above studies highlight a critical role for Notch signalling in intestinal proliferation, the propagative effects of Notch require active Wnt signalling, as Notch hyper-activation in a *Tcf4*$^{-/-}$ background fails to prompt proliferation (Fre *et al.,* 2009). This finding suggests that Wnt signalling produces a signal required for Notch-mediated proliferation. Indeed, several studies have shown that the Notch ligand, Jagged-1 is a direct Wnt/β-catenin target gene (Estrach *et al.,* 2006, Katoh 2006, Pannequin *et al.,* 2009, Rodilla *et al.,* 2009), a finding supported by the observation that Jagged-1 is elevated in Wnt-induced murine models of **c**olo**r**ectal **c**ancer (CRC) (Guilmeau *et al.,* 2010, Rodilla *et al.,* 2009).

Together, the highlighted studies underscore the importance and complex interplay of both Wnt and Notch signalling in ISC proliferation, which ultimately has

implications for intestinal homeostasis, repair, and disease. To summarize, loss of either Wnt or Notch signalling attenuates cell proliferation. The observation that Notch cannot induce proliferation in the absence of Wnt signalling (Fre *et al.,* 2009) suggests that Wnt is the master regulator of proliferation in the intestinal epithelium. However, Notch signalling seems to provide permissive cues for cell division, as loss of Notch signalling also interferes with this process (Riccio *et al.,* 2008, van Es *et al.,* 2005, VanDussen *et al.,* 2012). The intricate crosstalk between Wnt and Notch signalling may thus serve as a safety check to ensure intestinal homeostasis.

As cells journey from the crypt to the villus, they stop dividing and differentiate into one of the four primary cell types. Similar to proliferation, the Wnt and Notch signalling pathways are also vital in the differentiation process. Whereas Wnt is the key driver of proliferation in the intestine, Notch is the master regulator of cell differentiation, as discussed below.

1.2.3.2 *Notch – the master regulator of binary cell fate decisions in the intestine*.

Lateral inhibition describes the biological process in which one cell type adopts a particular cell fate, while simultaneously precluding its neighboring from adopting the same fate (Cabrera 1990). Importantly, the Notch signalling pathway is pivotal in the process of lateral inhibition, and is essential in mediating binary cell fate decisions of several tissue types, including the intestine (Cabrera 1990, de Santa Barbara *et al.,* 2003).

The first report implicating Notch signalling in mammalian intestinal homeostasis was a study by Jensen *et al.* (2000), which revealed that Hes-1, the downstream effector of Notch signalling, localized in the proliferative compartment of wildtype mouse

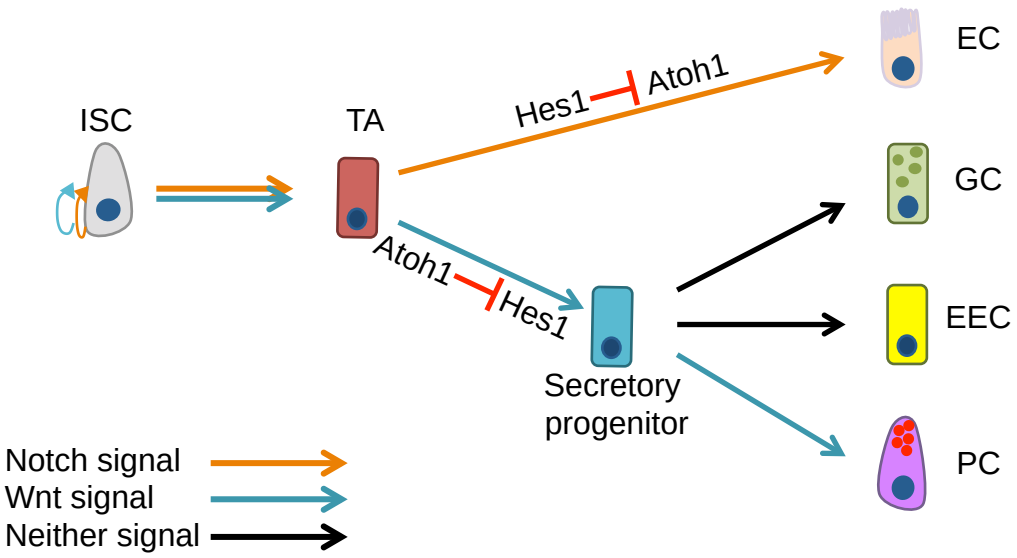
embryos (Jensen *et al.*, 2000). Complete loss of *Hes-1* results in embryonic lethality, with some homozygous animals surviving until only shortly after birth (Ishibashi *et al.*, 1995). However, among the various phenotypes observed in *Hes-1*$^{-/-}$ embryos, was a reduction in enterocytes, an increase in goblet cells, and enhanced expression of the enteroendocrine cell marker, chromogranin A, as early as embryonic day 17 (Jensen *et al.*, 2000). This finding has been corroborated by numerous subsequent studies in which genetic and pharmacological inactivation of Notch signalling produces an increase in the number of secretory cells. Indeed, combined inactivation of both Notch1 and Notch2 (Riccio *et al.*, 2008, Tian *et al.*, 2015, VanDussen *et al.*, 2012); loss of Dll-1 (Pellegrinet *et al.*, 2011) and RBP-J (van Es *et al.*, 2005); combined loss of Hes-1, - 3, and -5 (Ueo *et al.*, 2012); or treatment with γ-secretase inhibitors (van Es *et al.*, 2005, VanDussen *et al.*, 2012) have all been shown to drive secretory cell hyperplasia, which in this context refers to the increase in the number of secretory cells (VanDussen *et al.*, 2012). Conversely, Notch gain-of-function in the intestine, as demonstrated by intestinal-specific overexpression of Notch1, disrupts secretory cell differentiation, and results in a loss of secretory cell types (Fre *et al.*, 2005).

In progenitor cells with active Notch signalling, Hes-1 suppresses the expression of the transcription factor, **ato**nal **h**omologue 1 (Atoh1; **Figure 1.4**) (Fre *et al.*, 2005, van Es *et al.*, 2005, Yang *et al.*, 2001). These Hes-1 positive progenitors are fated to become absorptive enterocytes. Progenitor cells that lack Notch signalling accumulate Atoh1, and are thus fated towards the secretory lineage, reviewed in (Demitrack & Samuelson 2016). In fact, in addition to secretory cell hyperplasia, Notch signalling inhibition in mice is

also accompanied by an increase in **m**ouse **Ato**h1 (Math1) (Fre *et al.*, 2005, van Es *et al.*, 2005). It is thus not surprising that Math1 loss-of-function phenocopies Notch gain-of-function – i.e. *Math1* null mice present with a reduction of secretory cell types in the intestinal epithelium (Fre *et al.*, 2005, Yang *et al.*, 2001). The finding that Math1 is required for secretory cell differentiation was strengthened by observations that *Math1* null mice are refractory to Notch signalling inhibition, as pharmacological inhibition of Notch was unable to rescue the loss of secretory cells caused by *Math1* deletion (van Es *et al.*, 2010).

While Notch dictates the absorptive versus secretory fate, differentiation of secretory cells requires the presence of active Wnt signalling, which has been demonstrated in studies employing different genetic mouse models of Wnt pathway inhibition. For instance, transgenic mice with intestinal-specific overexpression of Wnt antagonist Dickkopf (Pinto *et al.*, 2003), and those with targeted deletion of β-catenin (Ireland *et al.*, 2004) present with a reduced number of secretory cells. Intriguingly however, constitutive activation of the Wnt pathway does not produce an increase in secretory cell types. Intestinal-specific disruption of *Apc*, which drives uninhibited Wnt/β-catenin activity, results in a concomitant reduction in goblet cells and Math1 expression, and increased Hes-1 expression (Peignon *et al.*, 2011, van Es *et al.*, 2005). While regulated by Hes-1, Math1 is also regulated by GSK3, which, in the context of

FIGURE 1.4 The canonical Notch and Wnt signalling pathways coordinate cell fate in the intestine. Both Notch and Wnt activity are required for the maintenance of long-lived, slow proliferating, intestinal stem cells (ISCs), which give rise to shorter-lived and rapidly proliferating transit amplifying (TA). TA cells with high Notch and low Wnt signalling produce enterocyte (EC), while those with low Notch and high Wnt signalling levels produce secretory cell progenitors. Paneth cell (PC) differentiation requires continued Wnt pathway activity, while differentiation of goblet (GC) and enteroendocrine (EEC) cells do not. Original artwork.


ISC
TA
Hes1
Atoh1
Atoh1
Hes1
Secretory
progenitor
EC
GC
EEC
PC
Notch signal
Wnt signal
Neither signal

Wnt pathway activation, is available to phosphorylate Math1 and target it for degradation (Peignon *et al.,* 2011). Thus, in progenitor cells lacking Notch activity, Math1 is stabilized as cells move away from the Wnt source at base of crypt, and are hence programmed to differentiate into one of the three secretory cells (Crosnier *et al.,* 2006).

These studies highlight the delicate and complex interplay between the canonical Wnt and Notch pathways in tightly modulating cell fate decisions in the intestine. To summarize, Notch signalling induces the expression of Hes-1, which in turn inhibits Math1 in cells fated to become enterocytes. Conversely, progenitors that lack Notch signalling accumulate Math1 as they move away from the Wnt signal at the crypt base, and are consequently fated toward the secretory cell lineage. These studies also suggest that, with the exception of Paneth cells which require Wnt signalling for terminal differentiation, Wnt confers competence upon those programmed for secretory fate once they escape the proliferative cues of the crypt (Crosnier *et al.,* 2006) (**Figure 1.4**).

1.3 Let's Stick Together - Apical Junctional Complexes in the Intestinal Epithelium

Epithelial cells, including IECs, are a polarized cell type characterized by differences in their apical, lateral, and basal plasma membranes. At their apical surface, IECs of the small intestine are characterized by their microvilli brush border (Granger & Baker 1950). Similar to other epithelial cell types, the IEC basolateral membrane consists of intricate multi-protein complexes (**Figure 1.5 A**), which dictate paracellular permeability (i.e. tight junctions, Landy *et al.,* 2016), cell-cell adhesion (i.e. adhesion and desmosomal junctions, Bondow *et al.,* 2012, Mehta *et al.,* 2015, Spindler *et al.,* 2015, Ungewiß *et al.,*

2017), and intercellular communication (i.e. gap junctions, Al-Ghadban *et al.,* 2016). While all are essential for intestinal homeostasis, tight junctions and adherens junctions are particularly crucial for preventing the translocation of microbes into the underlying *lamina propria*, and hence serve as a physical barrier against pathogenic infection (Berkes *et al.*, 2003). This section will focus particularly on the main constituents of both tight and adherens junctional complexes, as well as their contributions to intestinal homeostasis.

1.3.1 Tight Junctions

Tight **j**unctions (TJ) are highly organized multiprotein adhesion complexes located at the apical-most region of the lateral membrane (**Figure 1.5 B**; Schneeberger & Lynch 2004). Comprised of >50 proteins, the TJ is formed of several intimate membrane appositions (known as "kissing" points) between neighboring cells, resulting in an organized belt-like network of TJ strands that effectively obliterates the intercellular space (Anderson & Van Itallie 2009, Ulluwishewa *et al.,* 2011). As such, TJs are the gatekeepers of paracellular permeability.

The complexity and organization of the TJ varies along the intestinal tract and crypt-villus axis. In fact, the depth and number of TJ strands increases from the jejunum to ileum; and both were found to increase as cells migrate from crypt to villus tip (Madara *et al.*, 1980). While the occurrence of multiple TJ strands has been proposed to provide redundancy to the barrier (Anderson & Van Itallie 2009), the selective exclusion property of the TJ has been largely attributed to the membrane profile of a subset of proteins, known as Claudins.

The Claudin protein family is comprised of 26 members (Günzel & Yu 2013). These ~20-27 kDa, Ca^{2+}-independent transmembrane proteins are primarily responsible for imparting the selective exclusion properties of the TJ (Furuse *et al.*, 1998a, Furuse *et al.*, 1998b, Kubota *et al.*, 1999) and are stratified into two functional subgroups: barrier- and pore-forming Claudins (Anderson & Van Itallie 2009). In the murine gut, transcripts for 16 Claudins are expressed at varying levels, which differ in space (i.e. along the longitudinal and crypt-villus axes) and time (i.e. during development) (Fujita *et al.*, 2006, Holmes *et al.*, 2006). Of these, Claudins 1 and 5 are involved in barrier formation (Günzel & Yu 2013), while Claudin-2 mediates permeability (Conrad *et al.*, 2016, Krug *et al.*, 2012).

Occludin is another component of the TJ complex but its precise role remains controversial since it not required for TJ formation *per se* (Schulzke *et al.*, 2005). However the Occludin carboxy-terminal tail is a site of extensive phosphorylation (Cummins 2012), and its phosphorylation status dictates paracellular permeability, and Claudin-1 and -2 stability (Raleigh *et al.*, 2011).

Claudins and Occludin are tetraspan membrane proteins, with both the amino- and carboxy-termini residing in the cytoplasm (Furuse *et al.*, 1993, Furuse *et al.*, 1998b). Their PDZ-containing carboxy-termini interact with scaffolding proteins, such as **z**onula **o**ccludens-1 (ZO-1), which anchor them to the actin cytoskeleton (**Figure 1.5, B**; Fanning *et al.*, 1998, Itoh *et al.*, 1999). Indeed, stable ZO-1 depletion in MDCK cells leads to apical actin reorganization and increased solute flux (Van Itallie *et al.*, 2009). In addition to TJ proteins, ZO-1 has also been found to associate with α- and p120-catenin, integral

components of the adherens junction (Maiers *et al.,* 2013, Rajasekaran *et al.*, 1996, Van Itallie *et al.,* 2013).

1.3.2 Adherens Junctions

While the TJ is pivotal for establishing the epithelial barrier, the **a**dherens **j**unction (AJ) governs cell-cell adhesion. AJs are typically localized basal to TJs and are comprised of cadherin-catenin complexes, with the transmembrane **e**pithelial (E)-cadherin as the central cell-cell adhesion molecule. E-cadherin is a member of the type I classical cadherin family, and as such, contains 5 **e**xtracellular **c**adherin (EC) repeats which mediate cell-cell adhesion in a Ca^{2+}-dependent manner (Halbleib & Nelson 2006, Pokutta *et al.,* 1994). E-cadherin homodimers on neighboring cells engage in homotypic interactions, thus imparting adhesive and mechanical strength to the epithelium (Halbleib & Nelson 2006). The turnover and membrane stability of E-cadherin, and therefore the strength of the AJ, is governed by members of a family of cytosolic proteins, known as catenins (**Figure 1.5 C**).

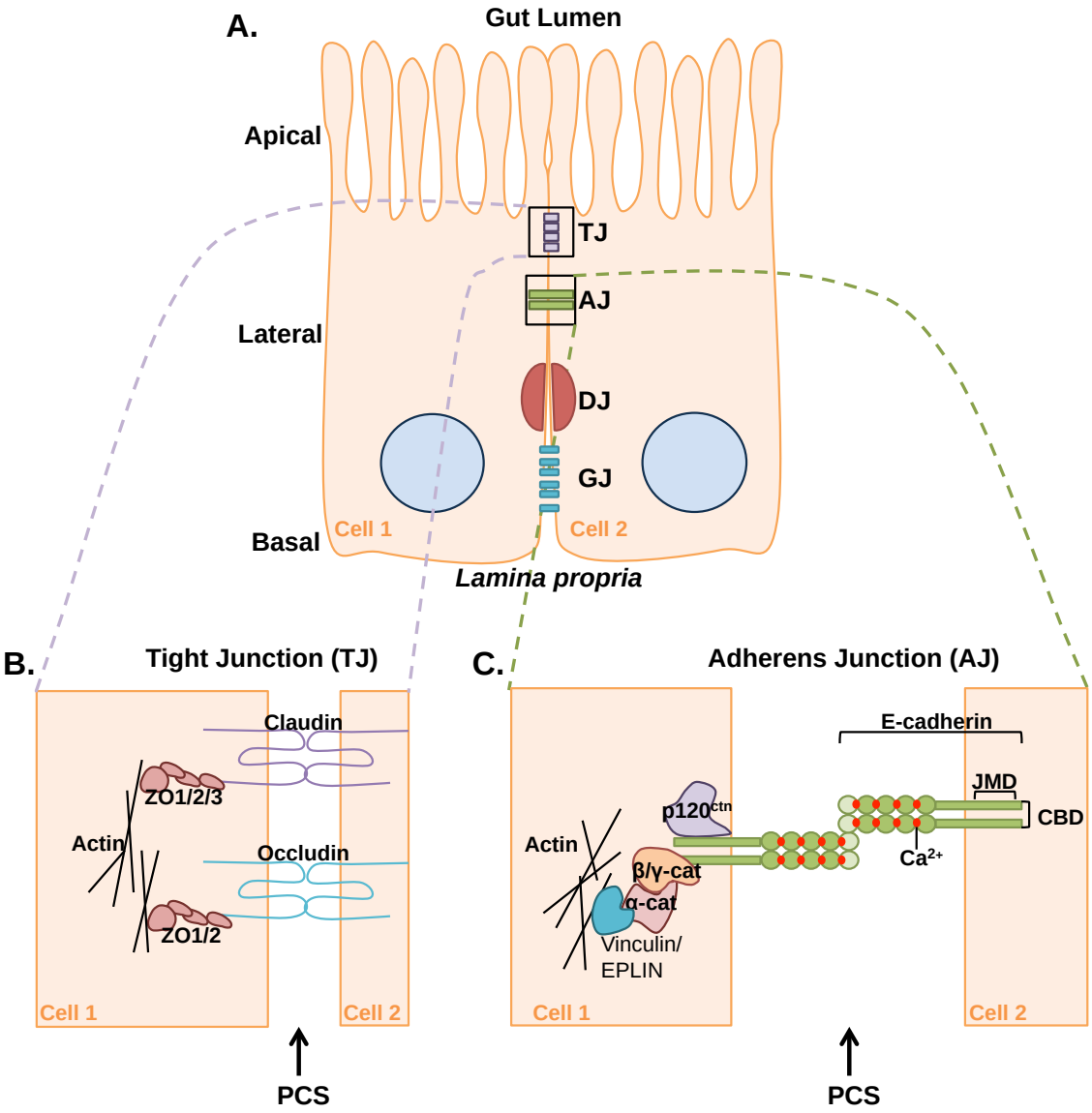
Catenins are a family of proteins that impart stability and turnover of AJ-associated cell adhesion proteins, including E-cadherin. Most catenins belong to the Armadillo family of proteins, which, similar to their *Drosophila* homologue, are characterized by a variable number of central Armadillo repeats (Tewari *et al.,* 2010), and are classified into three main subfamilies: (1) the plakophillin subfamily, which includes plakophilin 1-3; (2) the β-catenin subfamily, which includes β-catenin and plakoglobin; and (3) the p120-**ca**t**en**in ($p120^{ctn}$) subfamily, which includes $p120^{ctn}$, ARVCF, δ-catenin, and p0017, reviewed in (Miller *et al.,* 2013). In contrast to plakophillins, which associate

with desmosomes, the p120^{ctn} and β-catenin subfamilies are cadherin-associated catenins (with the exception of plakoglobin, which can also associate with desmosomes). Notably, p120^{ctn} and β-catenin directly bind to E-cadherin via their respective Arm domains, reviewed in (Miller *et al.,* 2013).

β-catenin exists in two pools – a free cytosolic pool that is involved in canonical Wnt signalling (see **Section 1.2.1**), and a membrane-bound pool, which is associated with the AJ (Kim *et al.*, 2013). Membrane-associated β-catenin stabilizes E-cadherin via two mechanisms: first, β-catenin confers secondary structure to the E-cadherin cytoplasmic domain, thereby stabilizing E-cadherin before it is targeted to the plasma membrane. This is mediated by co-translational formation of β-catenin:E-cadherin complexes in the Golgi apparatus (Curtis *et al.,* 2008). Indeed, in the absence of β-catenin, E-cadherin is targeted for proteolytic degradation prior to integrating into the plasma membrane (Choi *et al.,* 2006, Huber *et al.,* 2001). Secondly, β-catenin functions to stabilize membrane-localized E-cadherin by indirectly anchoring it to the actin cytoskeleton through its interaction with α-catenin (Ozawa *et al.,* 1990) (**Figure 1.5 C**).

α-catenin is a cadherin-associated catenin, but unlike β-catenin or p120^{ctn}, it lacks an Arm domain and is thus not a member of the Armadillo protein family, reviewed in (Miller *et al.,* 2013). In contrast to the previously held hypothesis that α-catenin directly links the AJ to the actin cytoskeleton, isolated E-cadherin/β-/α-catenin ternary complexes do not associate with actin filaments (Drees *et al.*, 2005, Yamada *et al.*, 2005). Instead it has been proposed that α-catenin associates with other actin-associated proteins such as EPLIN (**e**pithelial **p**rotein **l**ost **i**n **n**eoplasm) or vinculin (Ratheesh & Yap 2012).

FIGURE 1.5 Depiction of cell-cell junctional complexes. (A) Schematic diagram highlighting adhesion and communication between two neighboring IECs, mediated by tight junctions (TJ), adherens junctions (AJ), desmosomal junctions (DJ) and gap junctions (GJ), respectively. (**B)** TJ's are the apical-most adhesion complex along the lateral membrane. Claudins are integral for controlling charge- and size-selective permeability between the paracellular space (PCS). TJs are stabilized at the plasma membrane via the scaffolding protein zonula occludens (ZO), which anchors the TJ to the actin cytoskeleton. While occludin is found at the TJ, it's precise role in intestinal barrier integrity remains to be elucidated. **(C)** AJs are found immediately basal to TJs, and they function to control intercellular adhesion via Ca^{2+}-dependent cadherins. E-cadherin is key for IEC adhesion, and is stabilized at the membrane by the simultaneous binding of β/γ catenin (cat) to the catenin binding domain (CBD) and to α-cat, which indirectly associates with the actin cytoskeleton. $p120^{ctn}$ associates with the juxtamembrane domain (JMD), blocking E-cadherin's endocytic signal, and thus controls E-cadherin turnover. Original artwork.


A.
Gut Lumen
Apical
TJ
AJ
Lateral
DJ
GJ
Basal
Cell 1
Cell 2
Lamina propria
B.
Tight Junction (TJ)
Claudin
ZO1/2/3
Actin
Occludin
ZO1/2
Cell 1
Cell 2
PCS
C.
Adherens Junction (AJ)
E-cadherin
JMD
CBD
p120ctn
Actin
β/γ-cat
α-cat
Vinculin/
EPLIN
Ca2+
Cell 1
Cell 2
PCS

Nevertheless, α-catenin is a fundamental component of the AJ, as its loss compromises AJ formation and integrity in various tissues and organisms (Scott & Yap 2006).

The Reynolds group was the first to establish that $p120^{ctn}$ associated with E-cadherin protein complexes, together with α- and β-catenin (Daniel & Reynolds 1995, Reynolds *et al.,* 1994). However unlike α- and β-catenin, which serve to provide mechanical strength to the AJ, $p120^{ctn}$ associates with E-cadherin's juxtamembrane domain and blocks an endocytic signal, thereby regulating E-cadherin stability and turnover at the plasma membrane (Nanes *et al.,* 2012, Yap *et al.,* 1998). Indeed, ectopic $p120^{ctn}$ expression in the $p120^{ctn}$-deficient SW48 colon cancer cell line led to enhanced E-cadherin expression and membrane localization, and induced a cobblestone (epithelial) cell morphology (Ireton *et al.,* 2002). These findings were supported by a subsequent study, which revealed that loss of $p120^{ctn}$ resulted in lysosomal degradation of E-cadherin, and reduced expression of α- and β-catenin (Davis *et al.,* 2003).

1.3.3 Keeping gaps closed – physiological functions of apical junctional complexes

Once migrating IECs reach the villus tip, they are shed into the intestinal lumen (Barker 2014). Apical junctional complexes are essential for ensuring barrier integrity, and while the process of cell shedding causes transient gaps in the epithelium, barrier function is maintained (Guan *et al.,* 2011, Watson *et al.,* 2005). This is accomplished, in part, by the redistribution of apically localized ZO-1 along the basolateral membrane of the extruding cell (Guan *et al.,* 2011). As a cell is shed from the epithelial monolayer, neighboring cells protrude their cytoplasm and establish ZO-1-expressing cell-cell contacts to maintain barrier integrity (Bullen *et al.,* 2006, Madara 1990). Indeed, using the membrane-

impermeable dye Lucifer Yellow, Guan and colleagues showed that the dye failed to permeate across the epithelium even during cell extrusion (Guan *et al.*, 2011).

Cell shedding is caused by anoikis, which is mediated by loss of the AJ protein, E-cadherin (Fouquet *et al.*, 2004, Lugo-Martínez *et al.*, 2009). This was first definitively demonstrated by the Thenet group in 2004, where, using *ex vivo* analyses of living tissues, they showed that E-cadherin loss preceded anoikis, which was diminished upon E-cadherin engagement (Fouquet *et al.*, 2004). Indeed, chimeric transgenic mice expressing dominant negative N-cadherin (NCADΔ) (which leads to reduced endogenous E-cadherin levels) exhibit cell detachment from the underlying *lamina propria* and increased cell extrusion (Hermiston & Gordon 1995).

In addition to the normal shedding of enterocytes, loss of apical junctional complexes also affects the integrity of the intestinal barrier by perturbing cell-cell adhesion. This was first demonstrated by Hermiston and Gordon using their chimeric NCADΔ mice, which presented with a defective epithelial barrier (Hermiston & Gordon 1995). Intestinal-specific E-cadherin knockout (*VilCre:Cdh1*$^{loxP/loxP}$) causes perinatal death likely due to impaired barrier function. Indeed, by E18.5, *VilCre:Cdh1*$^{loxP/loxP}$ mice have already developed gaps in the intestinal barrier and a deterioration of the epithelium (Bondow *et al.*, 2012). In agreement with findings from *VilCre:Cdh1*$^{loxP/loxP}$ embryos, inducible E-cadherin inactivation in adult mice also caused disintegration of the intestinal epithelium (Schneider *et al.*, 2010). The consequences of defective cell adhesion will be emphasized in greater detail below, in **Section 1.5.1**. However, these findings highlight

the importance of apical junctional complexes as they pertain to the normal physiology of the intestinal barrier.

1.4 Crosstalk between Notch signalling and tight junctions regulate intestinal homeostasis.

While apical junctional proteins are essential for barrier integrity, accumulating evidence demonstrate crosstalk between Notch signalling and barrier functions in order to maintain homeostasis. For instance, the Radkte group used keratinocyte-specific Notch pathway knockout mouse models to demonstrate that loss of Notch signalling produced a phenotype reminiscent of atopic dermatitis, or eczema – a skin condition known to present with defective TJ function (De Benedetto *et al.*, 2011, Dumortier *et al.*, 2010). Crosstalk between cell adhesion and Notch signalling is not unique to the epidermis, and similar interactions have also been demonstrated in the intestinal epithelium.

In a recent study, Pope and colleagues created an intestinal-specific Claudin-1 overexpressing mouse (Cl-1Tg) to further elucidate the role of Claudin-1 in intestinal homeostasis (Pope *et al.*, 2014). The epithelium of Cl-1Tg mice exhibited reduced goblet cell numbers, increased NICD and Hes1 expression, and enhanced expression of the enterocyte marker, carbonic anhydrase (Pope *et al.*, 2014). These findings were corroborated using the SW480 colon cancer cell line, in which Claudin-1 was ectopically expressed. Similar to Cl-1Tg mice, Claudin-1 overexpression in SW480 cells resulted in increased NICD formation, which was accompanied by a concomitant increase in the Notch target gene Hes-1, and decrease in the Hes-1 target gene, Atoh1 (Pope *et al.*, 2014). These findings implicate a role for Claudin-1 in cell differentiation of the

intestinal epithelium and thus highlight communication between TJs and the Notch signalling pathway.

While TJs have been linked to Notch-mediated differentiation in the intestine, communication between the TJ and Notch signalling is bi-directional, as Notch has also been implicated in epithelial barrier function in intestinal cells. For instance, shRNA-mediated Notch1 knockdown in Caco-2 colon cancer cell monolayers resulted in enhanced protein expression of pore-forming Claudin-2, and reduced expression of Occludin and barrier-forming Claudin-5 (Dahan *et al.*, 2011). This was accompanied by lowered transepithelial resistance, hinting that loss of Notch signalling promotes a leaky barrier in cultured cells (Dahan *et al.*, 2011). These observations were upheld by a follow-up *in vivo* study by Mathern and colleagues, where they targeted Notch-1 for RNAi-mediated silencing by intra-rectal injection of Notch-1 siRNA into wildtype mice (Mathern *et al.*, 2014). Expression levels of the Notch effector Hes1, its downstream target gene Atoh-1, and the number of goblet cell were unchanged in Notch-1 siRNA injected mice, possibly due to functional redundancy conferred by Notch-2 on cell differentiation (Riccio *et al.*, 2008). Nonetheless, loss of Notch-1 alone was sufficient to significantly inhibit Claudin-5 expression, and elicit increased flux of FITC-dextran in the targeted epithelium (Mathern *et al.*, 2014)

Together, these findings highlight the complex exchange between Notch signalling and barrier functions in the intestinal epithelium. Defective Notch signalling, barrier function, or both, disturbs the equilibrium of the intestine, and such perturbations

may have implications in intestinal pathologies, such as inflammatory bowel disease, which is discussed in further detail below.

TIPPING THE BALANCE – FROM HOMEOSTASIS TO DISEASE

1.5 Inflammatory Bowel Disease

Inflammatory **b**owel **d**isease (IBD) is an umbrella term used to describe both **u**lcerative **c**olitis (UC) and **C**rohn's **d**isease (CD). While UC and CD differ in their pathology, both debilitating disorders are characterized by chronic, relapsing inflammation along the gastrointestinal tract, marked by intermittent periods of active and quiescent disease (Abraham & Cho 2009, Boirivant & Cossu 2012, Fakhoury *et al.*, 2014). Chronic inflammation observed in UC patients most often affects the rectum, but may also impact parts of, or the entire, colon in a continuous pattern (Xavier & Podolsky 2007). In contrast, patients with CD may exhibit inflammation anywhere along the GI tract, though most often along the terminal ileum of the small intestine. Unlike UC, CD patients present with discontinuous inflammation, which is interrupted by regions of non-inflamed tissue (Xavier & Podolsky 2007).

While the etiology of IBD remains unclear, it is agreed that development of IBD is multifactorial and that perturbations in the environment (e.g. microbiota, diet), immune responses, and genetics may all play a role (Cho & Brant 2011, Groschwitz & Hogan 2009, Manichanh *et al.*, 2012). While altered immune responses underscore the pathogenesis of both CD and UC, this section will place particular emphasis on the roles of defective IEC homeostasis in IBD development, as they pertain to perturbed barrier and Notch pathway functions.

1.5.1 When Things Fall Apart – Pathologies of faulty cell adhesion in the intestinal epithelium

It has long been known that IBD patients exhibit enhanced intestinal permeability (Hollander 1999, Secondulfo *et al.*, 2001, Vivinus-Nébot *et al.*, 2014). Given that the intestine plays an essential role in excluding luminal antigens from entering the body, it is not surprising that a faulty epithelial barrier is a hallmark of this disease (Berkes *et al.*, 2003, Gersemann *et al.*, 2011, Peterson & Artis 2014). Indeed, inflamed intestinal tissues in both human and rodent models of IBD exhibit misexpression of several adhesion-associated proteins, a finding supported by genome-wide association studies of both CD and UC, reviewed in (Mehta *et al.*, 2015). Among the various susceptibility loci for IBD, is the *CDH1* locus, which encodes E-cadherin (Barrett *et al.*, 2009, McGovern *et al.*, 2010, Muise *et al.*, 2009).

Several rodent models of IBD have highlighted the importance of E-cadherin in intestinal inflammation. For instance, in addition to a loss of cell-cell adhesion, NCADΔ mice also presented with defective barrier function and greater infiltration of gram-negative bacteria. Since NCADΔ mice exhibit reduced E-cadherin, it was postulated that loss of E-cadherin in these mice renders the epithelium susceptible to infection and perhaps inflammation (Hermiston & Gordon 1995). In agreement with this study, it was recently found that E-cadherin-deficient (*VilCre:Cdh1*$^{loxP/loxP}$) mice treated with **d**extran **s**odium **s**ulfate (DSS – a chemical inducer of acute UC), exhibited greater neutrophil infiltration into the *lamina propria* than control DSS-treated mice (Grill *et al.*, 2015). Moreover, DSS-treated *VilCre:Cdh1*$^{loxP/loxP}$ presented with worse clinical symptoms (e.g. bloody diarrhea) and more extensive tissue damage (Grill *et al.*, 2015).

Given p120^{ctn}'s role in stabilizing E-cadherin at the AJ, it is not surprising that loss of p120^{ctn} has also been associated with intestinal inflammation (Perez-Moreno *et al.*, 2006, Perez-Moreno *et al.*, 2008, Smalley-Freed *et al.*, 2010). As expected, intestinal-specific loss of p120^{ctn} resulted in a concomitant reduction in E-cadherin, and loss of cell-cell adhesion (Smalley-Freed *et al.*, 2010). Moreover, these mice also exhibited histological features of IBD, including crypt hyperplasia, mucosal thickening and increased neutrophil infiltration (Smalley-Freed *et al.*, 2010). Similar findings were also observed in tamoxifen-induced p120^{ctn} mosaic knockout mice, however these mice also developed colonic adenomas as they aged (Smalley-Freed *et al.*, 2011).

The above studies highlight a role of faulty AJs in intestinal inflammation; however, several reports have demonstrated that defective TJs are also implicated in IBD. For example, a number of studies have reported increased expression of pore-forming Claudin-2 in IBD tissues, which is postulated to contribute to increased leakiness of inflamed intestines (Prasad *et al.*, 2005, Weber *et al.*, 2008b, Zeissig *et al.*, 2007). Indeed, alleviation of inflammation in DSS-treated mice resulted in suppressed Claudin-2 expression, and reduced intestinal permeability (Xiao *et al.*, 2016).

In contrast to Claudin-2, the role of barrier-forming Claudin-1 in IBD is much more complex and depends on whether the inflammation is acute or chronic. Chemical induction of acute colitis in rodents (e.g. animals treated with 2, 4, 6-**t**ri**n**itro**b**enzene **s**ulfonic acid, TNBS, Loeuillard *et al.*, 2014 or DSS, Kang *et al.*, 2015, Mennigen *et al.*, 2009) results in decreased Claudin-1 expression compared to control animals. Moreover, mice recovering from DSS-induced colitis featured an accompanying increase in

Claudin-1 expression (Kang *et al.,* 2015, Mennigen *et al.,* 2009). Similar findings were observed in the **I**nter**l**eukin 10 (IL-10) KO mouse model of colitis at 8-weeks of age, where Claudin-1 expression was reduced relative to wildtype mice (Chen *et al.,* 2010, Shi *et al.,* 2014). While IL-10 KO mice are not a model of acute colitis, it would be interesting to investigate whether expression of Claudin-1 changes as these mice age. Nevertheless, these findings would suggest that loss of the barrier-forming Claudin-1 contributes to impaired epithelial barrier integrity, thereby rendering mice susceptible to inflammation.

Intriguingly however, several studies report enhanced expression of Claudin-1 in many cases of IBD, (Poritz *et al.,* 2011, Weber *et al.,* 2008b), and UC-associated CRC (Kinugasa *et al.,* 2010, Mees *et al.,* 2009). It has been proposed that while Claudin-1 is protective against acute inflammation, increased Claudin-1 observed in chronic inflammation plays a more pathogenic role (Lu *et al.,* 2013). Indeed, Singh and colleagues observed that Claudin-1 inhibits E-cadherin in colon cancer cells by promoting the expression of its transcriptional repressor, **z**inc finger **E**-box **b**inding homeobox 1 (ZEB1) (Singh *et al.,* 2011). While the significance of this interaction has not yet been examined in the context of IBD, constitutive Claudin-1 overexpression in Cl-1Tg mice results in increased susceptibility to DSS-induced colitis (Pope *et al.,* 2014). Furthermore, and as mentioned previously (**Section 1.4**), Cl-1Tg mice also feature a loss of goblet cells due to Notch-pathway activation, which is hypothesized to contribute to IBD (Gersemann *et al.,* 2009, Gersemann *et al.,* 2011, Pope *et al.,* 2014).

1.5.2 Notch Signalling and Inflammation – Is there a link?

Emerging evidence implicates Notch signalling in inflammation, although its precise role in this process remains controversial (Fazio & Ricciardiello 2016). Perhaps the first clue to suggest a potential role for Notch signalling in intestinal inflammation was the observation that UC is characterized by a striking reduction in goblet cells (Gersemann *et al.*, 2009, Xavier & Podolsky 2007). This phenotype contributes to the thinning of the protective mucous layer, and thus contributes to the intestinal damage observed in UC patients (Kim & Ho 2010). While one group also found a modest, but significant, goblet cell reduction in CD (Gersemann *et al.*, 2009), this form of IBD is often reported to exhibit unaltered goblet cell numbers, reviewed in (Kim & Ho 2010). Nonetheless, active Notch signalling, as measured by the expression levels of NICD and Hes1, has been reported in both forms of IBD (Dahan *et al.*, 2011, Gersemann *et al.*, 2009, Okamoto *et al.*, 2009), and a role for Notch signalling in inflammation has been supported by animal models of IBD, which is described in more detail below.

Several studies employing the DSS-model of UC report a significant reduction in goblet cells in treated mice, which is also accompanied by an increase in Hes1 expression (Kaur *et al.*, 2015, Okamoto *et al.*, 2009). However, it appears that Notch signalling levels must be strictly regulated, because while small-molecule inhibition of Notch signalling increased the number of goblet cells, it grossly exacerbated DSS-induced ulcer formation and tissue injury (Okamoto *et al.*, 2009). In agreement with this finding, Mathern and colleagues recently reported that siRNA-mediated knockdown of Notch1 in the colonic epithelium worsened DSS-mediated tissue damage (Mathern *et al.*, 2014).

A role for Notch signalling in inflammation is also supported by genetic mouse models. For example, ablation of Notch signalling in mice with targeted deletion of RBP-J (*RBP-J*$^{\Delta IEC}$) triggers spontaneous intestinal inflammation that is accompanied by increased neutrophil recruitment. Notably, in contrast to DSS-treated mice, *RBP-J*$^{\Delta IEC}$ mice also featured an increased number of goblet cells and other secretory cell types (Obata *et al.,* 2012).

Since Notch is key for proliferation of the stem cell compartment, it is hypothesized that impaired regeneration of the intestinal epithelium due to loss of Notch pathway function renders it susceptible to microbial translocation and injury (Obata *et al.,* 2012, Okamoto *et al.,* 2009). Indeed, although barrier proteins such as ZO-1 and E-cadherin were unperturbed, transmission electron photomicrographs of *RBP-J*$^{\Delta IEC}$ mice demonstrated a defective intestinal epithelial barrier and increased serum levels of 4 kDa **f**luorescein **i**so**t**hio**c**yanate (FITC)–dextran (Obata *et al.,* 2012). Importantly, *RBP-J*$^{\Delta IEC}$ mice also exhibited reduced proliferation and severely delayed repair of the intestinal epithelium (Obata *et al.,* 2012).

There is mounting evidence demonstrating that a defective barrier and altered Notch signalling are implicated in intestinal inflammation. However, whether they induce, or are the consequences of, inflammation is still being actively investigated (Michielan & D'Incà 2015, Shawki & McCole 2017). Recently, we found that overexpression of the transcription factor Kaiso is implicated in both chronic inflammation and Notch signalling regulation in mice, (Chaudhary *et al.,* 2013, **and Chapters 3 & 4)**. The following sections provide a detailed overview on Kaiso, its

modes of transcriptional regulation, and its roles in vertebrate development, with emphasis on intestinal homeostasis.

GENE REGULATORY ROLES OF THE POZ-ZF TRANSCRIPTION FACTOR, KAISO

1.6 Kaiso is a dual-specificity POZ-ZF transcription factor

Kaiso was first identified in a yeast-two hybrid screen as a novel $p120^{ctn}$ binding partner, and has been characterized as member of the BTB/POZ (**B**road complex, **t**ramtrak, **b**ric à brac/**Po**x virus and **z**inc finger) **z**inc **f**inger (hereafter, POZ-ZF) family of transcription factors (Daniel & Reynolds 1999). At its amino-terminus, Kaiso possesses the hallmark amino-terminal BTB/POZ domain that mediates protein-protein interactions, and its carboxy-terminal DNA-binding domain, comprised of three C_2H_2 Krüpple-type ZFs (Daniel & Reynolds 1999) (**Figure 1.6**).

However unlike most POZ-ZF proteins, Kaiso exhibits dual-specificity DNA binding and was the founding member of a unique subfamily of Kaiso-like POZ-ZF proteins that include ZBTB4 and ZBTB38 (Daniel *et al.*, 2002, Filion *et al.*, 2006, Sasai *et al.*, 2010). Kaiso and ZBTB4 both recognize and bind sequence-specific and **me**thyl-CpG (meCpG) dinucleotide DNA motifs (Daniel *et al.*, 2002, Prokhortchouk *et al.*, 2001a, Prokhortchouk *et al.*, 2001b). While initially found to associate with the **K**aiso **B**inding **S**ite (KBS), TC**CTGCNA** (core sequence bolded and underlined) in a sequence-specific manner (Daniel *et al.*, 2002), Kaiso was recently shown to also bind the palindromic sequence, TCTCGCGAGA (Raghav *et al.*, 2012). Notably, studies have

FIGURE 1.6 Schematic representation of Kaiso. At its N-terminus, Kaiso possesses a POZ domain, which mediates homo- and heterodimerization with various proteins such as N-CoR, mSin3a and Znf131. Kaiso possesses 3 C_2H_2-type zinc fingers that mediate DNA binding. $p120^{ctn}$ binds to a region on Kaiso flanking its zinc fingers, thereby inhibiting Kaiso-mediated transcriptional activities. Two acidic regions are present within Kaiso, and they are thought to mediate transcriptional activation of genes. A nuclear localization signal (NLS) allows nuclear translocation of Kaiso, and 8 putative serine/threonine phosphorylation sites (asterisks) are present, the relevance of which are currently unknown. Original artwork.


Protein-Protein
interaction domain
NLS
DNA Binding Domain
N
POZ
* * * * * * * *
ZF
ZF
ZF
C
AR
AR
p120ctn binding sites

demonstrated increased binding of Kaiso to both sequences upon methylation of the sequence (Raghav *et al.,* 2012) and surrounding CpG dinucleotides (Donaldson *et al.,* 2012).

1.6.1 Kaiso inhibits transcription via methyl-CpG and sequence-specific mechanisms

DNA methylation is an epigenetic mechanism whereby methylation of cytosine residues in the context of CpG dinucleotides promotes gene silencing in one of two ways – either via precluding a transcription factor from recognizing its cognate binding site, or via the formation of transcriptionally inaccessible heterochromatin (Buck-Koehntop & Defossez 2013). In the latter case meCpGs serve as a signal for the recruitment and binding of meCpG binding proteins – a class of proteins that is further stratified into three subfamilies based on their methyl-binding motifs: **m**ethyl **b**inding **d**omain (MBD); **S**ET and **R**ING finger-**a**ssociated (SRA) domain; and ZF domain (Buck-Koehntop & Defossez 2013). As previously alluded to, Kaiso is a member of the ZF family and uses its three ZFs to associate with meCpGs.

meCpG binding has been shown to prompt Kaiso's recruitment of co-repressor protein complexes, which subsequently recruit **h**istone **de****ac**etylases (HDACs). For instance, Kaiso directly associates with **N**uclear receptor **co-r**epressor-1 (N-CoR) and guides N-CoR to the **me****t**astasis **a**ssociated protein 2 (MTA2) promoter in a methylation-dependent manner, thus resulting in HDAC-dependent transcriptional repression (Yoon *et al.,* 2003).

While the above study highlights an example of Kaiso-mediated meCpG-dependent gene silencing, several studies also support the hypothesis that Kaiso-mediated

repression can occur by sequence-specific mechanisms alone, or in combination with meCpG dinucleotides. For example, our group and others reported that Kaiso negatively regulates a number of Wnt/β-catenin target genes via the KBS. This was first demonstrated by studies in *Xenopus laevis,* where Kaiso was reported to associate with and repress the canonical Wnt targets *siamois* and *Wnt11* via the KBS (Kim *et al.,* 2004, Park *et al.,* 2005). In fact, treatment with the demethylating agent, **5**'**aza**-**d**eox**c**ytidine (5'-aza-dC) did not abrogate Kaiso binding to the KBS in the *siamois* promoter in *Xenopus* embryos (Park *et al.,* 2005), supporting the hypothesis that Kaiso is capable of KBS-specific transcriptional repression. This was corroborated by our group when we showed that Kaiso directly associated with and repressed the Wnt target gene, ***m****atrix* ***m****etallo****p****roteinase 7 (MMP7),* in a sequence-specific manner (Spring *et al.,* 2005). Importantly, mutation of the core KBS sequence strongly attenuated Kaiso-mediated repression of a minimal *MMP7* reporter construct (Spring *et al.,* 2005). This finding was substantiated by a study by Barrett and colleagues, where they showed that Kaiso-mediated inhibition of MMP7 depended on its association with the co-repressor, **M**yeloid **T**ranslocation **G**ene 16 (MTG16) at the KBS (Barrett *et al.,* 2012).

In addition to methylation- and sequence-specific transcriptional regulation, we also showed that Kaiso is capable of regulating gene expression using a combination of both mechanisms, as observed with the *CCND1* (which encodes Cyclin D1) promoter. Specifically, Kaiso bound the -1067 *CCND1* KBS in a sequence-specific manner, and was unperturbed by treatment with 5'-aza-dC. In contrast, Kaiso's association with the +69 *CCND1* KBS was enhanced upon methylation, and abolished upon treatment with

5'-aza-dC (Donaldson *et al.*, 2012). Interestingly, mutation of the nucleotides surrounding the CpG in the +69 KBS weakened Kaiso's interaction with this site (Donaldson *et al.*, 2012), which suggests that the KBS functions to also strengthen and increase the specificity of Kaiso/meCpG interactions.

While the vast majority of findings to date support a role for Kaiso in transcriptional silencing (Buck-Koehntop & Defossez 2013, Fournier *et al.*, 2012, Kelly & Daniel 2006), mounting evidence suggests that Kaiso can also function as a transcriptional activator (Blattler *et al.*, 2013, Koh *et al.*, 2015, Koh *et al.*, 2014, Rodova *et al.*, 2004).

1.6.2 Kaiso-mediated transcriptional activation

Although most POZ-ZF transcription factors have been characterized as transcriptional repressors, a growing number of POZ-ZF factors, including **M**yc **i**nteracting **z**inc finger-1 (Miz-1), Znf131 and Kaiso, have been reported to exhibit both transcriptional repression and activation capabilities depending on their respective protein-interaction partner. For instance, when heterodimerized with Myc (Herold *et al.*, 2002) or the Kaiso-like protein, ZBTB4 (Weber *et al.*, 2008a), Miz-1 is capable of transcriptional repression via the recruitment of co-repressor proteins such as HDAC-1. However upon binding of the co-activator and histone acetyltransferase, p300, Miz-1 functions as a transcriptional activator (Adhikary & Eilers 2005).

A 2004 study by Rodova and colleagues was the first to report a transcriptional activator role for Kaiso. Specifically, they demonstrated that Kaiso activated the neuromuscular gene, *rapsyn* (**r**eceptor **a**ssociated **p**rotein of the **syn**apse) by directly

associating with the core KBS in the *rapsyn* promoter (Rodova *et al.*, 2004). We also recently reported that Kaiso can activate gene expression by binding to the KBS using methylation-dependent and sequence-specific binding mechanisms, as exemplified with the *TGFBR1* and *TGFBR2* promoters, respectively, in **t**riple **n**egative **b**reast **c**ancer (TNBC) cells (Bassey-Archibong *et al.*, 2016). In addition to transcriptional activation via the KBS (Bassey-Archibong *et al.*, 2016, Rodova *et al.*, 2004), Blattler and colleagues showed that Kaiso associates with unmethylated regions of actively transcribed genes via the palindromic sequence, TCTCGCGAGA (Blattler *et al.*, 2013).

Unlike transcriptional repression, the mechanisms governing POZ-ZF-mediated transactivation are less understood. However, Kaiso possesses two acidic regions (Daniel & Reynolds 1999) that are hypothesized to participate in transactivation, reviewed in (Weake & Workman 2010), though this has not been confirmed empirically. Nonetheless, recent studies have shown that Kaiso activates **c**yclin-**d**ependent **k**inase i**n**hibitor (*CDKN1A/p21*; Koh *et al.*, 2014) and **a**poptotic **p**rotease-**a**ctivating **f**actor 1 (*APAF1*; Koh *et al.*, 2015) in a p53-dependent manner. In both studies, Koh and colleagues demonstrated that Kaiso forms a complex with p53 and p300, where it promotes p300-mediated acetylation of p53. This protein complex binds p53 response elements, leading to subsequent transactivation of *CDKN1A* and *APAF1* (Koh *et al.*, 2015, Koh *et al.*, 2014).

Although Kaiso's association with p300 provides some mechanistic insight into Kaiso-mediated transactivation, Kaiso's interactions with its protein binding partners,

p120^{ctn} and Znf131 add an additional layer of complexity that must be considered when characterizing its transcriptional activity.

1.7 Kaiso's binding partners affect its gene regulatory functions.

1.7.1 The POZ-ZF transcription factors Kaiso and Znf131 act antagonistically

In an effort to identify additional Kaiso binding partners and gain insight into Kaiso's biological roles, the POZ-ZF transcription factor, **Z**i**n**c **f**inger protein 131 (Znf131) was isolated in a yeast-two hybrid screen using Kaiso's POZ domain as bait (Donaldson *et al.,* 2010). Ectopic expression of Znf131 activated an artificial promoter-reporter construct containing 4 tandem copies of the **Z**nf131 **B**inding **E**lement (ZBE). Interestingly however, Znf131-mediated transactivation was attenuated upon co-expression with Kaiso (Donaldson *et al.,* 2010), and unpublished findings from our lab show that Znf131 can also inhibit Kaiso-mediated transcriptional repression (N.S. Donaldson, Ph.D. Thesis).

Although Znf131 remains a largely uncharacterized protein, a recent study highlighted that in contrast to Kaiso, Znf131 inhibited the expression CDKN1A/p21 in mature T cells (Iguchi *et al.,* 2015). The precise mechanism of Znf131-mediated repression of p21 was not clarified in this study and whether Znf131 directly antagonizes Kaiso to regulate CDKN1A remains to be elucidated. However, reporter assays of a minimal *CDKN1A* luciferase construct hint that the effect is transcriptional, as Znf131 overexpression inhibited *CDKN1A* promoter activity (Iguchi *et al.,* 2015).

Additionally, as described in Chapter 5 (Robinson *et al.,* 2017a), we demonstrated that Znf131 associates with a subset of Kaiso target genes via the KBS in their cognate promoters. One such gene was *CCND1,* and we found that Znf131

activates the minimal *CCND1* reporter construct, which we previously showed was repressed by Kaiso (Donaldson *et al.*, 2012). Interestingly, Znf131 did not directly associate with the +69 *CCND1* KBS, suggesting that Znf131 may indirectly associate with Kaiso target genes via a protein interaction with Kaiso. Moreover, we found that Kaiso inhibits Znf131 expression, possibly in a negative feedback loop. Together, these data hint that the Znf131 and Kaiso may possess opposing or antagonistic transcriptional and physiological roles.

1.7.2 p120^{ctn} attenuates Kaiso-mediated transcriptional repression

In contrast to Znf131 and other proteins that bind to Kaiso's BTB/POZ domain, p120^{ctn} interacts with regions flanking Kaiso's DNA-binding domain, suggesting it directly interferes with Kaiso's transcriptional activities (Daniel & Reynolds 1999). This was first hinted in a *Xenopus* study, which showed an interaction between *x*Kaiso and *x*p120^{ctn} (Kim *et al.*, 2002). By exploiting the GAL4-UAS system, Kim and colleagues showed that expression of *x*KaisoGAL4 inhibited a UAS reporter construct, whereas expression of *x*p120^{ctn}GAL4 resulted in transcriptional activation (Kim *et al.*, 2002). This observation has been supported by numerous subsequent studies. Using an artificial promoter-reporter construct with 4 tandem copies of the KBS, our group demonstrated that Kaiso-mediated repression of the 4X KBS was attenuated upon co-expression of p120^{ctn} (Kelly *et al.*, 2004b). This effect was lost upon co-expression of a p120-NLS-mutant that fails to translocate to the nucleus (Kelly *et al.*, 2004b). Moreover, ectopic p120^{ctn} expression attenuated Kaiso's association with the *WNT11* promoter in HeLa cells

(Kim *et al.*, 2004), and relieved *x*Kaiso-mediated repression of *Wnt11* and *siamois* transcripts in *Xenopus* embryos (Kim *et al.*, 2004, Park *et al.*, 2005).

One mechanism by which $p120^{ctn}$ alleviates Kaiso's transcriptional activity was first illustrated by the McCrea group when they demonstrated a $p120^{ctn}$-induced shift in Kaiso's subcellular localization (Park *et al.*, 2006), despite the fact that Kaiso lacks a nuclear export signal (Kelly *et al.*, 2004b). In this study, *x*Kaiso exhibited nuclear localization when transfected into HeLa cells, but was targeted to the cytoplasm upon expression of *x*p120 (Park *et al.*, 2006). However, it has been subsequently shown that nuclear $p120^{ctn}$ is still capable of attenuating Kaiso's activity without shuttling it to the cytoplasm (van de Ven *et al.*, 2015). Thus $p120^{ctn}$ inhibits Kaiso's transcriptional activities by directly blocking its interaction with DNA and/or shuttling it out of the nucleus.

KAISO – AN EMERGING MEDIATOR OF VERTEBRATE HOMEOSTASIS

1.8 Physiological roles for Kaiso in non-intestinal development.

Many of the first intimations regarding Kaiso's role in vertebrate development were gleaned from studies in *Xenopus*. *x*Kaiso transcripts were observed at all stages of embryonic development, and both Kaiso-depletion and -overexpression in *Xenopus* resulted in defective blastopore closure, suggesting it plays a crucial role in gastrulation (Kim *et al.*, 2004, Ruzov *et al.*, 2004).

Intriguingly, many of the genes initially identified as Kaiso targets were also Wnt/β-catenin target genes and this raised the possibility that Kaiso was a negative regulator of canonical Wnt signalling. This hypothesis was confirmed in *Xenopus*, when Park and

colleagues demonstrated that *x*Kaiso overexpression transcriptionally inhibited several canonical Wnt target genes including *Cyclin D1, siamois, c-myc* and *Wnt11* but more importantly *x*Kaiso overexpression rescued the duplicate-axis phenotype induced by constitutive Wnt/β-catenin signalling (Park *et al.,* 2005). Kaiso's negative effects on Wnt signalling were further supported by experiments employing the dominant negative Kaiso mutant, D33N/R47Q, which harbours a double point-mutation in the BTB/POZ domain. Kaiso$^{D33N/R47Q}$-injected embryos failed to repress *siamois* expression or rescue Wnt/β-catenin-induced axis duplication (Park *et al.,* 2005).

Several studies have hinted at a role for Kaiso in central nervous system development. This was first suggested in *Xenopus*, which exhibit pronounced *xKaiso* expression in cells destined for neural fate (Kim *et al.,* 2002). This finding was corroborated by a subsequent study by Della Ragione and colleagues, who observed uniform expression of Kaiso in the human brain (Della Ragione *et al.,* 2006). A more precise role for Kaiso in neural tissue development has been elucidated in a recent study by Zhao and colleagues, who found that Kaiso is essential for oligodendrocyte maturation (Zhao *et al.,* 2016). Specifically, Kaiso was shown to interact with the Wnt/β-catenin co-factor TCF4 at early stages of oligodendrocyte precursor cell differentiation. Formation of Kaiso/TCF4 complexes precluded β-catenin from interacting with TCF4. Consequently, canonical Wnt/β-catenin signalling was inhibited, thereby permitting progression of the oligodendrocyte maturation process (Zhao *et al.,* 2016).

As described earlier (see **Section 1.2**), canonical Wnt signalling is a highly conserved pathway that is vital for homeostasis of various tissues, including the

intestines. Hence the finding that Kaiso engages with and attenuates canonical Wnt signalling suggests that Kaiso might also be integral for the development and homeostasis of the intestines, which is discussed in detail below.

1.9 Kaiso plays an essential role in murine intestinal homeostasis

X-linked, Kaiso maps to chromosome Xq24 in both mice and humans (Della Ragione *et al.*, 2006, Martin Caballero *et al.*, 2009, Prokhortchouk *et al.*, 2006), and its role in mammalian development has been highlighted using transgenic and knockout mouse models. A recent study examining the distribution of Kaiso in murine tissues showed wide expression in the central nervous system, male reproductive organs, and mammary glands, among others (Shumskaya *et al.*, 2015, and our unpublished data). In agreement with previous reports (Chaudhary *et al.*, 2013, Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006), Kaiso was also found expressed along the gastrointestinal tract (Shumskaya *et al.*, 2015), supporting a role for Kaiso in intestinal homeostasis. While Kaiso has been implicated in the homeostasis of a variety of tissues (e.g. breast, Bassey-Archibong *et al.*, 2016, Jones *et al.*, 2014a, Vermeulen *et al.*, 2012; pancreas, Jones *et al.*, 2016; and prostate, Jones *et al.*, 2012), this section will provide detailed insight into Kaiso's role in mammalian development as it pertains specifically to intestinal homeostasis and disease.

1.9.1 Kaiso promotes colon cancer progression

Since Wnt signalling is integral to homeostasis of the intestinal epithelium (**Section 1.2.3**), and Kaiso had been implicated as a negative regulator of canonical Wnt signalling in *Xenopus* (**Section 1.8**), our group and others examined the role of Kaiso in the murine

intestinal epithelium (Chaudhary *et al.*, 2013, Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006). Prokhortchouk and colleagues found that *Kaiso*$^{-/y}$ mice were viable and did not exhibit any gross developmental defects – possibly due to functional redundancy conferred by the related proteins, ZBTB4 and ZBTB38 (Prokhortchouk *et al.*, 2006). However, when mated with the *Apc*$^{Min/+}$ mouse model of Wnt/β-catenin-induced colon cancer, the resulting *Kaiso*$^{-/y}$;*Apc*$^{Min/+}$ progeny lived significantly longer than their *Apc*$^{Min/+}$ siblings, and presented with fewer polyps that were smaller in size.

As a follow-up to this study, our group generated villin-Kaiso transgenic (*Kaiso*^{Tg}) mice, which drive intestinal-specific overexpression of Kaiso (Chaudhary *et al.*, 2013). In agreement with Prokhortchouk's group, but counterintuitive based on the *Xenopus* findings, we found that when *Kaiso*^{Tg} were mated with *Apc*$^{Min/+}$ mice, the *Kaiso*^{Tg};*Apc*$^{Min/+}$ progeny exhibited significantly decreased survival. Moreover, we found that *Kaiso*^{Tg};*Apc*$^{Min/+}$ mice developed more polyps that were smaller in size than *Apc*$^{Min/+}$ siblings (Pierre *et al.*, 2015b). Additionally, expression of Wnt target genes – *Axin2, CD44*, *EphB2* and *MMP7* – were elevated in *Kaiso*^{Tg};*Apc*$^{Min/+}$ mice relative to their *Apc*$^{Min/+}$ counterparts, but not in *Kaiso*^{Tg} versus NonTg (Pierre *et al.*, 2015b). Together these studies highlight that, in contrast to findings in *Xenopus* where Kaiso antagonizes canonical Wnt signalling, Kaiso potentiates canonical Wnt signalling in mammalian intestinal tissues in the context of the *Apc*$^{Min/+}$ background.

1.9.2 Kaiso^{Tg} mice exhibit chronic intestinal inflammation and Notch-depletion phenotypes

The enhanced Wnt signalling observed in *Kaiso^{Tg};Apc$^{Min/+}$* mice was accompanied by increased intestinal inflammation (Pierre *et al.,* 2015b), the latter of which was first demonstrated by Chaudhary *et al.* during their characterization of *Kaiso^{Tg}* mice (Chaudhary *et al.,* 2013). In this study, 1-year old *Kaiso^{Tg}* mice exhibited phenotypes reminiscent of chronic intestinal inflammation; namely fused and blunted villi, increased infiltration of leukocytes into the lamina propria, and increased **m**yeolo**p**er**o**xidase activity (MPO) – a proxy for neutrophil activation (Klebanoff *et al.,* 2013).

Interestingly, Kaiso's binding partner p120^{ctn} exhibited prominent nuclear localization in 1-year old *Kaiso^{Tg}* mice, unlike their NonTg counterparts, which exhibited basolateral localization of p120^{ctn} (Chaudhary *et al.,* 2013). This raised the possibility that Kaiso overexpression may induce a barrier defect by recruiting p120^{ctn} away from the AJ, thus predisposing mice to spontaneous chronic inflammation similar to p120^{ctn} KO mice (Smalley-Freed *et al.,* 2010).

In addition to inflammation, 1-year *Kaiso^{Tg}* mice also exhibited a significant increase in the number of secretory cell types, a phenotype reminiscent of loss of Notch signalling in the intestine (**Section 1.2.3.2**). Given the roles of Notch signalling in epithelial renewal and inflammation, it was also possible that Kaiso overexpression resulted in defective repair of the intestinal epithelium, a possibility that we explore in greater detail **in Chapters 3 and 4, respectively. More precisely, in Chapter 3 (Robinson *et al.,* 2017b) we report that Kaiso is a negative modulator of the Notch signalling pathway in intestinal cells; and in Chapter 4 we report that Kaiso**

overexpression results in neutrophil-specific inflammation and defective intestinal renewal mechanisms.

RATIONALE & RESEARCH GOALS

Most of the evidence to-date implicating Kaiso in vertebrate development was garnered from studies in *Xenopus*, which demonstrated a role for Kaiso as a negative regulator of Wnt-mediated development (Iioka *et al.,* 2009, Park *et al.,* 2006, Park *et al.,* 2005). Intriguingly however, in mice, Kaiso potentiates Wnt-mediated tumourigenesis, as demonstrated in studies using the $Apc^{Min/+}$ model of CRC (Pierre *et al.*, 2015b, Prokhortchouk *et al.,* 2006). As intestinal-specific Kaiso overexpression in mice promoted the development of spontaneous chronic inflammation (Chaudhary *et al.,* 2013, Pierre *et al.,* 2015b), and induced an increase in secretory cell types – a phenotype reminiscent of Notch pathway inhibition (Chaudhary *et al.,* 2013), this led us to **hypothesize** that, in addition to regulating the Wnt pathway, ***Kaiso is also capable of regulating the Notch signalling pathway in intestinal tissues***.

It is well established that a defective epithelial barrier is characteristic of IBD, and while the role of Notch signalling in intestinal inflammation is an area of active investigation, its precise contribution to IBD remains controversial. The observation that 1-year old $Kaiso^{Tg}$ mice exhibit phenotypes consistent with Notch-depletion and chronic inflammation places Kaiso at the nexus of both Notch and inflammation-related pathways. However, the molecular mechanism(s) governing Kaiso-mediated homeostasis in mammalian intestines had not yet been explored. This work sought to begin filling this gap by addressing the following **research aims:**

1. **Define Kaiso's role in the Notch signalling pathway in intestinal cells, and determine whether Kaiso-induced secretory dysplasia is linked to chronic inflammation.**

2. **Elucidate the mechanism(s) of Kaiso-induced intestinal inflammation, by examining whether *Kaiso*^{Tg} mice are pre-disposed to epithelial barrier defects prior to inflammation onset.**

3. **Elucidate the role of the Kaiso-binding partner Znf131 in Kaiso-mediated processes in intestinal cells.**

CHAPTER 2 – MATERIALS AND METHODS

2.1 Mouse Husbandry

All mouse-handling protocols were performed in accordance with the McMaster Animal Research Ethics Board (AREB) under the Animal Utilization Protocol (AUP) #14-08-29. All mice were bred in the disease-free Barrier Facility, and subsequently transferred into a Specific Pathogen Free facility upon weaning. Mice were fed a standard chow diet and maintained on a 12-hour light/dark cycle. *Kaiso*^{Tg} mice were identified by PCR analysis of DNA isolated from ear snips using the following primers: forward SV40-Kaiso 5'-ATC ATC AAA GCC GGG TGG GCA-3' and reverse SV40-Kaiso: 5'-TTT TCT ACT CTC CAT TTC ATT CAA GTC CTC-3'. Animals were sacrificed by CO_2 asphyxiation and cervical dislocation.

2.2 Myeloperoxidase (MPO) Assay

Ileum from age-matched mice was flash frozen in liquid nitrogen. Approximately 50 mg of frozen tissue was sonicated in a solution of 50 mg/mL 0.5% HTAB (**h**exadecylt**r**i-methyl**a**mmonium **b**romide), 50 mM phosphate buffer pH 6.0 at 30 Hz for 4 min. Homogenized samples were cleared at 12,000 RPM, 15 min. at 4 °C. Seven microlitres of each sample was read in triplicate every 30 seconds, in 200 µL o-dianisidine dihydrochlorine buffer (16.8 mg/mL o-dianisidine dihydrochloide, 0.24% H_2O_2, 5mM phosphate buffer, pH 6.0) at absorbance 450 nm. MPO activity was measured in **u**nits (U), where 1 U is the amount MPO required to degrade 1 µmol H_2O_2/minute at 25 °C, giving an absorbance of $1.13X10^{-2}$ nm/min. MPO activity was calculated as the change in

absorbance [ΔA(t2-t1)/Δmin] ÷ ($1.13X10^{-2}$ nm/min), and the amount of activity per tissue was calculated using the formula, MPO U ÷ 0.35 mg of tissue.

2.3 *In vivo* Migration Assay

Age-matched NonTg and *Kaiso*^{Tg} mice were given 50 mg/kg BrdU by intraperitoneal injection. Mice were randomized into three time-point groups, and were euthanized after 2, 24 or 48 hours (hrs) post-injection. Tissues were then processed for immunohistochemistry, as described in **Section 2.4** below. The distance migrated of BrdU label-retaining cells was measured from the base of the crypt to the furthest migrated cell, using Aperio Image Scope software by three blind observers. To control for possible differences in villus height, the average distance migrated was normalized to the average villus height. Comparisons between NonTg and *Kaiso*^{Tg} mice were performed using Student's t-test, and $p<0.05$ was considered statistically significant.

2.4 Tissue Staining

2.4.1 Intestinal Tissue Harvest

Immediately following euthanasia, intestinal tissues were removed and flushed with ice-cold **p**hosphate **b**uffered **s**aline (PBS; pH 7.4). The small intestine was divided into 3 equal sections and all tissue sections were rolled into "Swiss rolls". Tissues were then fixed in 10% phosphate-buffered formalin for 24-48 hrs, and then dehydrated at **r**oom **t**emperature (RT) in 70% ethanol.

Intestinal tissue blocks of 1-month old *Kaiso*$^{-/y}$ mice were generously provided by Dr. Egor Prokhortchouk (Prokhortchouk *et al.,* 2006). For histological comparison of *Kaiso*$^{-/y}$ to NonTg mice, intestines were flushed with ice cold PBS and fixed for 16 hrs at

4 °C in Carnoy's fixative (60% EtOH, 30% Chloroform, 10% glacial acetic acid). Tissues were then dehydrated at RT in 70% ethanol.

All tissues were paraffin-embedded, sliced into 5 µm sections and affixed onto glass slides by the McMaster Core Histology Research Facility.

2.4.2 Immunohistochemistry

Slides were de-waxed in 2X 5-**min**utes (min) xylenes washes and hydrated in a graded ethanol series followed by 3X 1-min washes in distilled water. Tissues were then subject to either heat-mediated or enzymatic antigen retrieval, as summarized in **Table 2.1**. Tissues were allowed to cool to RT and then permeabilized with TBS-T (TBS, 0.05% Tween-20). For staining with BrdU, permeabilization was accomplished with PBST (PBS, 0.1%Triton X-100) for 10 min, and all subsequent wash steps were performed with PBS or PBST, in place of TBS or TBS-T, respectively. Endogenous peroxidase was quenched with 3% H_2O_2, TBS and tissues were subsequently blocked for 1 hr at RT with blocking solution (1% **b**ovine **s**erum **a**lbumin [BSA], and/or 5% normal serum) supplemented with avidin (Vector Laboratories). Primary antibodies were diluted in biotin-supplemented blocking solution (Vector Laboratories) and incubated in a humidified chamber at 4^oC overnight (**Table 2.2**). Tissues were washed 3X 5-min with TBS-T and 3X 3-min with TBS followed by incubation with a biotin-conjugated goat-anti-mouse or donkey-anti-rabbit secondary antibody for 2 hrs at RT. Tissues were washed as previously described and incubated with Vectastain Elite ABC reagent (Vector Laboratories) for 30 min at RT, washed in TBS and incubated with 3, 3-**d**i**a**mino**b**enzine (DAB) substrate (Vector Laboratories) until adequate tissue staining (3-15 min). Tissues

were counterstained with Harris haematoxylin (Sigma Aldrich), differentiated in acid ethanol (70% EtOH, 0.3% HCl), blued in Scott's tap water (23.8 mM $NaHCO_3$, 0.83 M $MgSO_4$), and dehydrated in a graded ethanol series and 2X 5 min xylenes changes. Dried slides were mounted using Polymount (Polysciences Inc), and images were taken with the Aperio Scope slide scanner.

Table 2.1 Methods and conditions of antigen retrieval for immunohistochemistry and immunofluorescence

Antibody	Antigen retrieval method	Incubation time and conditions
β-catenin	10 mM $Na_3C_6H_5O_7$, 0.05%Tween, pH 6.0	Microwave 3 min high power, 12 min 40% power
BrdU moBU-1	10 mM $Na_3C_6H_5O_7$, 0.05%Tween, pH 6.0	Microwave 3 min high power, 12 min 40% power
Chromogranin A	10 mM $Na_3C_6H_5O_7$, pH 6.0	Microwave 2 min 45 sec high power, 8 min 40% power
Claudin-2	TE-0.05% Tween, pH 9.0	Microwave 3 min high power, 15 min 40% power
Dll-1	10 mM $Na_3C_6H_5O_7$, 0.05%Tween, pH 6.0	Microwave 2 min high power, 8 min 40% power
E-Cadherin	10 mM $Na_3C_6H_5O_7$, 0.05%Tween, pH 6.0	Microwave 3 min high power, 12 min 40% power
Hes1	TE-0.05% Tween, pH 9.0	Microwave 1.5 min high power, 15 min 40% power
Hes5	TE-0.05% Tween, pH 9.0	Microwave 1.5 min high power, 15 min 40% power
Jagged-1	20 µg/mL proteinase K, in TE pH 9.0	37°C, 20 min in humidified chamber
Ki67	DAKO Target retrieval solution # S2369	Microwave 3 min high power, 20 min 40% power
Lysozyme	10 mM $Na_3C_6H_5O_7$, pH 6.0	Microwave 2 min high power, 8 min 40% power
NICD	TE-0.05% Tween, pH 9.0	Microwave 3 min high power, 15 min 30% power
Znf131 clone R325	10 mM $Na_3C_6H_5O_7$, pH 6.0	Microwave 3 min high power, 10 min 40% power
ZO-1	TE-0.05% Tween, pH 9.0	Microwave 3 min high power, 15 min 40% power

2.4.3 Immunofluorescence (Tissues)

Slides were de-waxed, and tissues were subject to antigen retrieval (**Table 2.1**) and membrane permeabilization as described above. Tissues were then blocked with 10% BSA for 1 hr at RT, and incubated with primary antibodies overnight at 4^{o}C in humidified chamber (**Table 2.2**). Following 3X5 min 0.05% TBS-T washes and a 5-minute TBS wash, tissues were incubated with the appropriate species-specific Alexa fluor antibody (1:500) for 2 hrs at RT in a dark humidified chamber. Tissues were washed as described above, and incubated with TOTO-3 (1:1500, in TBS) for 30 min at RT in a dark humidified chamber. Slides were then tapped several times to remove TOTO-3 solution, dried in the dark, and mounted with Prolong Gold Anti-fade Reagent (Life Technologies). Immunofluorescence images were acquired using the Leica TCS SP5 II confocal microscope.

2.5 Cell Culture

Human colon carcinoma (HCT116, HT29, SW480), and human breast carcinoma (MCF7) cells were purchased from the American Tissue Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagles Medium (DMEM; Lonza) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco). Stable pRS-shKaiso-scrambled and pRS-shKaiso HCT116 and HT29 cell lines were selected with 2 µg/mL puromycin, and stable SW480 pRS-shKaiso-scrambled and pRS-shKaiso cell lines were selected with 4 µg/mL puromycin. For demethylation studies, culture media was supplemented every 17-24 hours for a total of 72 hours with 5'-aza-dC (final concentration 5µM, Sigma Aldrich). All cell lines were maintained at 37°C and 5% CO_2.

2.6 Protein Isolation and Immunoblotting

For *in vivo* analyses, intestines were harvested and flushed with ice-cold saline solution (150 mM NaCl, 2 mM imidazole, 0.02% NaN_3), cut into 1-2 cm pieces and stirred vigorously in ice-cold sucrose solution (12 mM EDTA, 200 mM sucrose, 20 mM KH_2PO_4, 78 mM Na_2HPO_4, 0.02% NaN_3) for 1-2 hrs at 4°C. **I**ntestinal **e**pithelial **c**ells (IECs) were separated from large tissue segments using a metal sink strainer and pelleted at 200x g for ~20 min at 4°C. IECs were washed 3x with sucrose buffer, pelleted at 200x g at 4°C, lysed in sample buffer (2% SDS, 10% glycerol, 62.5 mM Tris, pH 6.8) containing one cOmplete mini protease inhibitor cocktail tablet (Roche), and then denatured by boiling for 5 min.

Cultured cells were washed twice with cold 1XPBS and pelleted upon centrifugation at 2000 RPM at 4°C for 5 min. Cell pellets were lysed with sample buffer containing one cOmplete mini protease inhibitor cocktail tablet (Roche) on ice for 15 min with periodic vortexing, and denatured by boiling for 5 min.

Denatured lysates were centrifuged at 13, 200 RPM, 4°C for 15 min and supernatants were transferred to new pre-chilled tubes and quantified using the DC Protein Assay, according to manufacturer's protocol (BioRad). Equal amounts of protein were further denatured upon addition of reducing buffer (final concentration 5% β-mercaptoethanol, 62.5 mM Tris pH 6.8, 0.04% bromophenol blue). Samples were then separated by SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were blocked with 4% milk, TBS for 1 hr at RT and primary antibodies were incubated overnight at 4°C (**Table 2.2**). Membranes were then washed 5X 5 min with TBS, incubated with the appropriate

HRP-conjugated secondary antibody (1:5000 – 1:10,000), and washed again as described. Protein bands were visualized using Western Lightning enhanced chemiluminescent reagent (Perkin Elmer), and images were acquired using the ChemiDoc MP Imaging System (Bio-Rad).

2.7 Chromatin Immunoprecipitation (ChIP)

Cells were washed twice with cold PBS and fixed for 10 min at RT with 1% formaldehyde diluted in DMEM lacking supplements. Following two cold PBS washes, fixation was stopped by incubation of cells with 125 mM glycine for 5 min at RT. Cells were scraped with 1 mL PBS containing PIC, and pelleted gently at 2000 RPM, for 5 min at 4°C. Next, cell pellets were lysed with Cell Lysis Buffer (5 mM PIPES, pH8.0, 85 mM KCl, 0.5% NP-40), homogenized 10 times with a dounce homogenizer and incubated on ice for 10 min. Lysates were then centrifuged for 5 min at 5000 RPM at 4ºC, and pelleted nuclei were resuspended and lysed with Nuclei Lysis Buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS) on ice for 10 min. Chromatin was sheared by sonication at 90% max power output, 5% microtip for 5 rounds of 15 pulses. Six micrograms of chromatin were pre-cleared for 1 hr with 5 µg rabbit IgG (Abcam), rotating end-over-end at 4°C. Non-specific proteins were precipitated with protein-A sepharose beads that had been blocked with 5 µg salmon sperm DNA (Rockland Inc.). Pre-cleared lysates were immunoprecipitated overnight at 4°C with the following antibodies: Kaiso 6F mouse mAb (4 µg); Histone H3 rabbit mAb (2 µg; Abcam cat# ab1791); Non-specific rabbit IgG

Table 2.2 Antibodies and their respective dilutions used for immunohistochemistry (IHC), immunofluorescence (IF), and western blotting (WB)

Antibody	IHC	IF	WB	Manufacturer (catalogue #)
β-actin	-	-	1:50, 000	**Sigma Aldrich** (A5441- .2ML)
β-catenin (serum)	1:2000	-	-	**In house** (clone #C2206)
β-catenin	-	-	1:40, 000	**BD** (610153)
BrdU moBU-1	1:100	-	-	**Thermo Fisher** (B35128)
Chromogranin A	1:500	-	-	**Abcam** (ab15160)
Claudin-2	1:50	-	-	**Abcam** (ab53032)
Cleaved Notch 1 Val 1744 (NICD)	1:100	-	1:500	**CST**[1] (4147)
Dll-1	1:100	-	1:500	**Abcam** (ab84620)
E-cadherin	1:100	-	-	**BD** (610182)
Hes1	1:100	-	-	**CST** (11988S)
Hes5	1:125	-	-	**Abcam** (ab 65077-100)
Jagged-1	-	1:1000	1:500	**Santa Cruz** (sc-8303)
Kaiso	-	-	1:1000	**In house**
Ki67	1:150	-	-	**Spring Biosciences** (SP6M3060)
Lysozyme	1:50	-	-	**Pierce** (PA1-29680)
$p120^{ctn}$	1:500	-	-	**BD** (610133)
ZO-1	1:400	-	-	**Proteintech** (21773-1-AP)
Znf131	1:300	-	1:300	**In house** (clone R325)

1. CST – Cell Signalling Technologies

(4 µg; Santa Cruz cat# sc-2027). For IPs using a mouse antibody, Rabbit α-mouse IgG was incubated with washed protein A-sepharose beads for 30 min at 4°C (RαM bridge).

Protein-DNA complexes were precipitated with washed protein-A sepharose beads or RαM bridge for 1 hr end-over-end at 4°C. Precipitated immunocomplexes were then washed end-over-end at 4°C as follows: 10 min with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 1 mM EDTA); 10 min with High Salt buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 1 mM EDTA); 5 min with LiCl buffer (50 mM Tris, pH 8.0, 250 mM LiCl, 0.5% deoxycholate, 1% NP-40, 1 mM EDTA); and 2X 10 min with Tris EDTA (TE; pH 8.0). Samples were centrifuged at 4°C for 5 min at 5000 RPM between washes. Protein was denatured and degraded with 0.05% SDS and 0.25 µg/µL proteinase K at 42°C for 4 hrs, and crosslinks were reversed at 65°C overnight. DNA was then subject to two rounds of phenol chloroform extraction followed by ethanol precipitation with cold 100% EtOH at -20°C overnight. DNA was rinsed twice with cold 70% EtOH, air-dried at RT overnight, resuspended with Gibco water (Invitrogen), and then subject to PCR amplification. The primer pairs and their respective annealing temperatures are listed in **Table 2.3**.

2.8 Electrophoretic Mobility Shift Assay (EMSA)

2.8.1 Purification of GST-fusion proteins

Dr. Juliet Daniel and Dr. Nickett Donaldson generated the GST-Kaiso and -Znf131 expression constructs, respectively (Daniel *et al.*, 2002, Donaldson *et al.*, 2010). Twelve millilitres of transformed GST-Kaiso and -Znf131 bacterial stocks were cultured for

approximately 16 hrs in **L**uria **b**roth (LB) containing **amp**icillin (Amp) and shaken at 37 °C, 200 RPM. Bacterial cultures (10 mL each) were grown in 90 mL fresh LB/Amp for 90 min at 30 °C, 200 RPM, and GST-protein expression was induced with 10 µM **i**so**p**ropyl β-D-**t**hio**g**alactoside (IPTG) for 3 hrs at 30 °C, 200 RPM. Bacteria were pelleted at 6000 RPM, 15 min, 4 °C and lysed by sonication (6 pulses, 30 seconds each, at 40% output, 40% duty) in 0.1%NP-40, PBS. Lysates were cleared at 10,000 RPM, 20 min, 4 °C, and the pellet was discarded.

Table 2.3 Primers used for ChIP-PCR and their corresponding annealing temperatures

Target	**Primer sequence (5'– 3')**	**Annealing temperature**
DLL1	Fwd: AAG CTC TGC AGC TCT CTT GG Rev: GGC GAC TTT CGT TTT CCT C	63.5 °C
JAG1 KBS1	Fwd: GAG AAG GAC CCG GAG AGC R: AGT TCC TCC TCG CAC TAC CC	66.0 °C
JAG1 KBS2	Fwd: CCC AAC ATT TCC CTT TCC TC Rev: CCT GAG GGT GTA AGT GAT AGG C	63.5 °C
JAG1 KBS3	Fwd: CCT CAG GCA CTA CCT CCA GA Rev: AGC TCT TGT GGC CTC ACT TC	66.0 °C
+69 CCND1 KBS	Fwd: CAC ACG GAC TAC AGG GGA GTT Rev: CTC GGC TCT CGC TTC TGC TG	53 °C
ID2	Fwd: CAG CCC CGC ACT TAC TGT ACT GTA Rev: CAT TGG CGG AAG GTG GCA CGT	53 °C
MTA2	Fwd: GCT AAG GCG CGC GAG TCT TTG Rev: GAG AAC AAG GCC CAC TGC TCG GC	53 °C
MMP7	Fwd: AAG GAG ACC CAA AGA AGG GA Rev: GAA CAT CAC CAA AAT CCT GTG G	45 °C
WNT11	Fwd: TGT CTG TTT GTT TGA GAC GGA Rev: CGT CTC TAC TAC GAA TAC AA	45 °C
ZNF131	Fwd: TTC CCC CAT GGT TTC ATC TA Rev: TTG CTG GAT AGA CGC ACA AG	63.5 °C

Lysates were rotated end-over-end at 4 °C for 1 hr with Glutathione Sepharose 4B beads (GE Healthcare) equilibrated in 0.1%NP-40, PBS. Proteins were eluted with 10 mM Glutathione, 50 mM Tris pH 8.0 for 10 min at RT and centrifuged at 2,000 RPM for 2 min to pellet beads. Supernatants were collected and stored at -80 °C. A 20 µL aliquot of each sample was denatured upon addition of Laemmli sample buffer (final concentrations – 50 mM Tris pH 6.8, 1% SDS, 5% sucrose, 1.2M β-mercaptoethanol, 0.004% bromophenol blue) and separated by SDS-PAGE with BSA standards of known concentration. Gels were Coomassie stained (50% methanol, 10% acetic acid, 0.2% Coomassie Brilliant Blue) and microwaved for 3-5 min at medium power with fast de-stain solution (30% methanol, 7% acetic acid), until sufficiently de-stained. Gels were then dried onto blotting paper using the Bio-Rad Model 583 Gel Dryer, and GST-protein concentrations were estimated by comparing band intensities against those of the known BSA standards.

2.8.2 Oligonucleotide Radiolabelling and EMSA

End-labelling of double-stranded **oligo**nucleotides (oligos) corresponding to the *CCND1* +69 core KBS **(Table 2.4**) was performed by incubation at 37 °C for 45 min with γ^{32}-P ATP, 10 units T4 **p**oly**n**ucleotide **k**inase (PNK; New England Biolabs) and 1X PNK Buffer (New Englad Biolabs). Labelling reactions were stopped upon addition of 20 mM EDTA. Oligos were purified using the Chromaspin TE-10 column (Clontech) and counted using a scintillation counter.

GST-proteins (200 ng) were incubated with 30,000 cpm radiolabelled DNA probes in binding reaction buffer (25 mM HEPES pH 7.5, 100 mM KCl, 1 mM EDTA,

10 mM $MgCl_2$, 0.1% NP-40, 5% glycerol, 1mM DTT, 150 ng/µL poly-dI-dC, 0.2 µg/µL BSA) for 30 min on ice, followed by 25 min at RT. Reactions were then electrophoresed on a 4% non-denaturing gel, and dried onto blotting paper at 80 °C for 1.5 hrs. DNA Shifts were visualized by exposing gel to XAR film at -80 °C and developed using the SRX-101A Konica Minolta developer.

Table 2.4 WT and mutant *CCND1* +69 KBS sequences used for EMSA in **Chapter 3**.

Oligo name	Oligo Sequence
+ 69 WT KBS	5' CTG TCG GCG CAG TAG CAG CGA GCA GCA GAG 3' 3' GAC AGC CGC GTC ATC GTC GCT CGT CGT CTC 5'
+69 KBS MUT	5' CTG TCG GCG CAG TAA AAT CGA GCA GCA GAG 3' 3' GAC AGC CGC GTC ATT TTA GCT CGT CGT CTC 5'

Underlined in red – WT or mutated KBS sequences

2.9 Plasmid Constructs

Reporter constructs

pGLuc-4XCSL: The pGL2-4XCSL-luciferase construct was purchased from Addgene (Plasmid #41726; deposited by Raphael Kopan). The 4XCSL sites were subcloned into pGLuc-basic using HindIII and EcoRI.

pGLuc(-)1748CD1: The pGL3(-)*1748CD1* construct was created by Dr. Abena Engmann (née Otchere; A. Otchere MSc. Thesis), which was subsequently subcloned into pGLuc-basic by Dr. Nickett Donaldson (Donaldson *et al.*, 2012).

Expression constructs

pcDNA3-hKaiso: The human Kaiso expression construct was created by Dr. Kevin Kelly (Kelly *et al.*, 2004a).

pCAGGS-NICD: The pCAGGS-NICD expression vector, which contains the murine Notch1 intracellular domain, was purchased from Addgene (Plasmid #26891) and deposited by Nicholas Gaiano.

pcDNA3-mZnf131: murine Znf131 was PCR amplified from the pCS2+ MT mZnf131 construct using the following primers: Znf131 N-term (Fwd) : 5' TTA GGA TCC ACC ACC ATG GCA ATG GAG GCT GAA GAG ACG 3' and Znf131 C-term (Rev) with STOP codon: 5' TACG CGC GGC CGC TCA TTC TAA AAC CGG 3'. Full-length mZnf131 was subcloned into the NotI and BamHI sites of the pcDNA3 expression vector.

2.9.1 Molecular Subcloning

Plasmid inserts and backbone vectors were digested with the appropriate restriction enzymes. Both insert and vector were then separated on a low-melt agarose gel, and the appropriate bands were identified and excised using a UV transilluminator. Excised DNA bands were pelleted at 14,000 RPM for 30 seconds and melted at 65°C for 10 min with periodic vortexing. Insert and backbone vector were then combined at a 1:5 – 1:7 ratio, incubated at 65°C and vortexed 5X 2min. Reactions were incubated at 37°C for 3 min and ligated using 10 units/µL T4 DNA ligase supplemented with 1 mM ATP (Life Technologies). Reactions were gently agitated by hand to mix, spun at 14,000 RPM for 30 seconds to pellet the reaction mixture, and incubated overnight at RT. Ligation reactions were stopped the following morning by incubation at 65°C for 10 min.

TCM buffer (10 mM **T**ris, pH 7.5, 10 mM **C**aCl_2, 10 mM **M**gCl_2) was added to ligation reactions to a final concentration of 1 mM, and the DNA mixture was used to

transform competent *E. Coli*. Briefly, bacteria were incubated with the DNA mixture on ice for 30 min, heat shocked at 42°C for 2 min, and placed back on ice for 2 min. Eight hundred microliters SOC media were added to the competent *E. Coli*, and the mixture shaken at 37°C, 200 RPM for 1 hr. Bacterial cells were pelleted at 5000 RPM for 5 min, resuspended in 200µL SOC and plated onto agar containing the appropriate antibiotic. Plated bacteria were incubated at 37°C in the dark overnight for 16-18 hrs, after which colonies were screened by a crude DNA extraction followed by restriction digest with the same enzymes used for subcloning. Positive clones were sequenced by the Mobix Lab, McMaster University.

2.10 Reporter Assays

$4X10^5$ HCT116 and SW480 cells/well **(Chapter 3 & 5 Robinson *et al.*, 2017a, b)**, and $5X10^5$ MCF7 cells/well **(Chapter 5, Robinson *et al.*, 2017a)** were seeded into a 6-well dish. Cells were co-transfected with 0.5µg pRSV/β-galactosidase and the indicated amounts of reporter & expression constructs 12-15 hrs post-seeding, using TurboFect Transfection Reagent according to manufacturer's protocols (Thermo Fisher Scientific). Briefly, DNA was first diluted and mixed well in DMEM without supplements. Upon addition of TurboFect, reactions were vortexed briefly and allowed to incubate at RT for 20 min. Transfection complexes were added drop-wise to the cells, which were agitated gently to ensure even distribution. Cells were then incubated at 37°C, and 5% CO_2 for 24 hrs. Secreted *Gaussia* luciferase was assayed 24 hrs. post-transfection using BioLux *Gaussia* luciferase assay kit (New England Biolabs) and read using an LB Luminometer

(Thermo Fisher). Relative light units were normalized to β-galactosidase, and statistical significance was determined by one-way ANOVA with Tukey's post-hoc test.

2.11 Quantitative Real-Time PCR.

Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel) according to manufacturer's protocols. mRNA was then converted to cDNA using the SensiFAST™ cDNA synthesis kit, according to the manufacturers instructions (Bioline). qRT-PCR analysis was performed using the SensiMix™SYBR® Hi-ROX kit (Bioline) using the primer sequences and annealing temperatures listed in **Table 2.5**. Gene expression changes were normalized to the endogenous control gene and quantified using the standard curve method. Student's T-test was used for statistical calculations and a p-value ≤ 0.05 was considered statistically significant.

Table 2.5 Primer sequences used for qRT-PCR and their annealing temperatures

Target	Primer sequence (5'– 3')	Annealing temperature
hβ-Actin	Fwd: CTC TTC CAG CCT TCC TTC CT Rev: AGC ACT GTG TTG GCG TAC AG	55 °C
mDll-1	Fwd: GCG ACT GAG GTG TAA GAT GGA A Rev: TCT CAG CAG CAT TCA TCG GG	60.5 °C
hDll-1	Fwd: AGA AAG TGT GCA ACC CTG GC Rev: CAC TCT GCA CTT GCA TTC CCC	63.5 °C
mDll-4	Fwd: GCA AAC TGC AGA ACC ACA CA Rev: TGG CTT CTC ACT GTG TAA CCG	52.5 °C
mHes1	Fwd: AAA ATT CCT CCT CCC CGG Rev: TTT GGT TTG TCC GGT GTC G	60.8 °C
mHes5	Fwd: TGC AGG AGG CGG TAC AGT TC Rev: GCT GGA AGT GGT AAA GCA GCT T	60.8 °C
mHMBS	Fwd: GAT GGG CAA CTG TAC CTG ACT G Rev: CTG GGC TCC TCT TGG AAT G	63 °C
hJagged1	Fwd: CCA GGT CTT ACT ACG GAG CAC ATT Rev: CGC AAG CGA TGT AGA TTG AAT ATT	62.0 °C
MIP-2	Fwd: CAC TCT CAA GGG CGG TCA AA Rev: GGT TCT TCC GTT GAG GGA CA	61 °C
mNotch1	Fwd: ACA GTG CAA CCC CCT GTA TG Rev: TCT AGG CCA TCC CAC TCA CA	63 °C
mNotch2	Fwd: ACA GTG TTG GCT CCC TGT TC Rev: ATC GTT TAC CTT GCC AGC CA	63 °C
mTFIID	Fwd: CCA CGG ACA ACT GCG TTG AT Rev: GGC TCA TAG CTA CTG AAC TG	55 °C
Znf131[1]	Fwd: GAT TCT GCT CTA GCA CTG TTG GC Rev: GCT TCT TAC TGA CTC CAC CTT CTT	53 °C

1 These primers were used to amplify both murine and human *Znf131*, and they recognize both the long and short *Znf131* isoforms

CHAPTER 3 KAISO TAKES IT DOWN A NOTCH

Elucidating Kaiso's role in the Notch signalling pathway

PREFACE

This chapter describes the work that resulted in the following manuscript, which was published on June 21 2017 in *Journal* of *Cell Communication and Signaling*. The manuscript has been re-formatted to maintain consistency with other chapters. Significant changes include removal of materials and methods (which is included in **Chapter 2**), list of abbreviations (included on page xvii), author declarations, and reference list, and re-formatting of the in-text citations and order of figures.

Shaiya C. Robinson, Kristina Klobucar, Christina C. Pierre, Amna Ansari, Svetlana Zhenilo, Egor Prokhortchouk and Juliet M. Daniel**.** 2017 **Kaiso differentially regulates components of the Notch signalling pathway in intestinal cells**. *Journal* of *Cell Communication and Signaling* 15 (1): 24.

Summary: In this work we sought to elucidate Kaiso's potential role and mechanism(s) of action in the Notch signalling pathway in intestinal cells. We found that 3-month old *Kaiso*^{Tg} mice exhibit secretory cell dysplasia and reduced expression of Notch target genes prior to the onset of inflammation. Intriguingly, *Kaiso*^{Tg} mice were found to exhibit reduced Notch1 and Dll-1, but increased Jagged-1 expression. Moreover, we found that Kaiso associates with the *DLL1* and *JAG1* promoters, implicating both genes as putative Kaiso targets. Since Dll-1 and Jagged-1 are implicated in cell differentiation and colon

cancer progression, respectively, we hypothesize that Kaiso's role in intestinal homeostasis is two-fold: Kaiso regulates cell fate via Dll-1-mediated Notch signalling, while Kaiso-mediated Jagged-1 expression renders mice susceptible to colon cancer.

***Author contributions**:* Dr. Christina Pierre performed the MPO assay in Fig. 3.1B, and provided the quantification of goblet cells in Fig. 3.1D (which were scored by three blind observers). Ms. Kristina Klobucar assisted with IHC, ChIP of HT29 cells in Fig. 3.6, and played an instrumental role in assisting with the preparation of reagents necessary for this manuscript. Ms. Amna Ansari stained for Paneth cells in Fig. 3.1B. She also was one of three blind observers who quantified the Paneth & enteroendocrine cells in Fig 3.1B and Jagged-1 positive cells in Fig. 3.5A. Dr. Juliet M. Daniel and I provided significant contribution to the conceptualization, critical assessment, writing and revision of the manuscript. I also performed all other experiments, analyzed and interpreted the data and assembled all the figures therein.

3.1 ABSTRACT

Background

In mammalian intestines, Notch signaling plays a critical role in mediating cell fate decisions; it promotes the absorptive (or enterocyte) cell fate, while concomitantly inhibiting the secretory cell fate (i.e. goblet, Paneth and enteroendocrine cells). We recently reported that intestinal-specific Kaiso overexpressing mice (*Kaiso*^{Tg}) exhibited chronic intestinal inflammation and had increased numbers of all three secretory cell types, hinting that Kaiso might regulate Notch signaling in the gut. However, Kaiso's precise role in Notch signaling and whether the *Kaiso*^{Tg} secretory cell fate phenotype was linked to Kaiso-induced inflammation had yet to be elucidated.

Methods

Intestines from 3-month old Non-transgenic and *Kaiso*^{Tg} mice were "Swiss" rolled and analysed for the expression of Notch1, Dll-1, Jagged-1, and secretory cell markers by immunohistochemistry and immunofluorescence. To evaluate inflammation, morphological analyses and myeloperoxidase assays were performed on intestines from 3-month old *Kaiso*^{Tg} and control mice. Notch1, Dll-1 and Jagged-1 expression were also assessed in stable Kaiso-depleted colon cancer cells and isolated intestinal epithelial cells using real time PCR and western blotting. To assess Kaiso binding to the DLL1, JAG1 and NOTCH1 promoter regions, chromatin immunoprecipitation was performed on three colon cancer cell lines.

Results

Here we demonstrate that Kaiso promotes secretory cell hyperplasia independently of

Kaiso-induced inflammation. Moreover, Kaiso regulates several components of the Notch signaling pathway in intestinal cells, namely, Dll-1, Jagged-1 and Notch1. Notably, we found that in *Kaiso*^{Tg} mice intestines, Notch1 and Dll-1 expression are significantly reduced while Jagged-1 expression is increased. Chromatin immunoprecipitation experiments revealed that Kaiso associates with the DLL1 and JAG1 promoter regions in a methylation-dependent manner in colon carcinoma cell lines, suggesting that these Notch ligands are putative Kaiso target genes.

Conclusion

Here, we provide evidence that Kaiso's effects on intestinal secretory cell fates precede the development of intestinal inflammation in *Kaiso*^{Tg} mice. We also demonstrate that Kaiso inhibits the expression of Dll-1, which likely contributes to the secretory cell phenotype observed in our transgenic mice. In contrast, Kaiso promotes Jagged-1 expression, which may have implications in Notch-mediated colon cancer progression.

3.2 BACKGROUND

The mammalian intestine is a rapidly self-renewing epithelium that undergoes continual turnover every 3-5 days (Crosnier *et al.,* 2006, de Santa Barbara *et al.*, 2003). The mucosa of the small intestine is folded into finger-like projections known as villi (de Santa Barbara *et al.,* 2003), which serve to maximize the surface area over which nutrient uptake occurs (Gassler *et al.,* 2006). Most intestinal epithelial cells (IECs) migrate toward the tip of the villus, where they undergo apoptosis and are shed into the intestinal lumen (Barker 2014). The majority of IECs in the intestinal tract consists primarily of absorptive cells known as enterocytes. The remaining cells are categorized as secretory cells and include mucus-secreting goblet cells, hormone-secreting enteroendocrine cells, and anti-microbial Paneth cells (Barker 2014, de Santa Barbara *et al.,* 2003, Gassler *et al.,* 2006, Noah *et al.,* 2011). Unlike most terminally differentiated IECs, Paneth cells migrate downward toward the base of intervillar crypts, where intestinal stem and progenitor cell populations also reside (Gassler *et al.,* 2006, Scoville *et al.,* 2008, Umar 2010). The constant turnover of cells within the intestinal epithelium requires strict regulation of the signaling pathways that govern stem and progenitor cell proliferation and differentiation. While several pathways are critical for intestinal homeostasis, the Notch signaling pathway is indispensible for dictating intestinal cell fate decisions (Hansson *et al.,* 2004, Okamoto *et al.,* 2009, Pellegrinet *et al.,* 2011, Riccio *et al.,* 2008, Ueo *et al.,* 2012, Yang *et al.,* 2001).

Notch receptors are heterodimeric single-pass transmembrane proteins (Fleming 1998, Hansson *et al.,* 2004, Kageyama *et al.,* 2007, Kopan & Ilagan 2009) with four

receptors present in mammals (Notch1-4), each encoded by a separate gene (Kopan & Ilagan 2009). Active Notch signaling is initiated upon binding of the Notch receptor to the transmembrane Delta/Serrate/Lag2 (DSL) family of Notch ligands, including Delta-like ligand (Dll)-1, Dll-4 and Jagged-1(Fleming 1998, Kopan & Ilagan 2009). Activated Notch then undergoes multiple proteolytic cleavages, which culminates in the cytoplasmic release of the Notch intracellular domain (NICD) fragment (Fleming 1998). NICD subsequently translocates to the nucleus and interacts with RBPJ (recombination signal binding protein for immunoglobulin kappa J region; also known as CSL – CBF1, suppressor of hairless, Lag2) to transactivate the expression of Notch target genes, most notably, the Hes (Hairy enhancer of split) family of transcriptional repressors (Hansson *et al.*, 2004, Kageyama *et al.*, 2007, Yang *et al.*, 2001).

In the intestines, Notch signaling dictates binary cell fate decisions – progenitor cells that lack Notch signaling are fated toward the secretory cell lineage (i.e. goblet, Paneth and enteroendocrine cells), while those with active Notch signaling are fated to become enterocytes (Demitrack & Samuelson 2016). Thus, misregulated Notch signaling in the intestines perturb cell fate decisions. For example, loss of Dll-1 and combined loss of Hes1, Hes3 and Hes5 result in increased goblet, Paneth and enteroendocrine cells (Pellegrinet *et al.*, 2011, Stamataki *et al.*, 2011, Ueo *et al.*, 2012). We recently reported that ectopic expression of the poxvirus and zinc finger (POZ)-zinc finger (hereafter, POZ-ZF) transcription factor Kaiso in the intestines of 12-month old mice (*Kaiso*^{Tg}) resulted in chronic intestinal inflammation and a significant increase in secretory cell types compared to non-transgenic (NonTg) mice. This finding, coupled with our observation

that expression of the Notch target gene *Hes1* was reduced in *Kaiso*^{Tg} compared to NonTg mice, implicated Kaiso as a negative regulator of Notch signaling (Chaudhary *et al.*, 2013).

Since Kaiso overexpression in 12-month old mice is reminiscent of loss of Notch pathway activity, we sought to further investigate Kaiso's role in Notch-mediated intestinal homeostasis and cell fate decisions. We found that the Kaiso-induced increase in intestinal secretory cells occurs prior to the onset of chronic intestinal inflammation, suggesting that the secretory cell phenotype does not manifest as a consequence of Kaiso-induced chronic inflammation. Notably, we found that Kaiso inhibits Dll-1 expression in the intestine, and we postulate that this inhibition contributes to the Kaiso-induced increase in secretory cell types. Surprisingly however, we found that Kaiso promotes Jagged-1 expression, which has been previously implicated in colon cancer progression (Dai *et al.*, 2014, Guilmeau *et al.*, 2010, Rodilla *et al.*, 2009, Sugiyama *et al.*, 2016). Collectively, these data highlight novel roles for Kaiso in regulating Notch-mediated intestinal homeostasis.

3.3 RESULTS

3.3.1 Ectopic Kaiso expression decreases Notch signaling and increases secretory cell numbers independent of Kaiso-induced inflammation.

Our finding that Kaiso overexpression in the murine gut (*Kaiso*^{Tg}) results in chronic intestinal inflammation and an increase in secretory cells in 12-**mo**nth (mo.) old mice compared to age-matched NonTg mice (Chaudhary *et al.*, 2013), was paradoxical since chronic inflammation is typically associated with a reduction in goblet cells (Dorofeyev

et al., 2013, Gersemann *et al.*, 2011). Thus, we sought to determine whether the *Kaiso*^{Tg} secretory cell phenotype occurred independently, or as a consequence of the Kaiso-induced chronic inflammation by analyzing subclinical *Kaiso*^{Tg} mice. Thus, we first analysed the small intestine of 3-mo. old mice for signs of chronic inflammation. The small intestines of 3-mo. old *Kaiso*^{Tg} mice do not exhibit signs of widespread chronic intestinal inflammation visible in 12-mo. old *Kaiso*^{Tg} mice – i.e. extensive villus blunting, thickened submucosa, extensive neutrophil infiltration, etc. (Chaudhary *et al.,* 2013) (Fig. 3.1 A). Consistent with the lack of inflammation-related tissue damage, there was no significant difference in myeloperoxidase (MPO) activity (a measure of activated neutrophils and a surrogate marker for inflammation) between 3-mo. old NonTg and *Kaiso*^{Tg} mice ($p= 0.792$; Fig. 3.1 B).

Since we did not observe neutrophil-specific intestinal inflammation in 3-mo. old *Kaiso*^{Tg} mice, we next assessed the secretory cell types in these mice. Small intestines were stained with periodic acid-Schiff stain (PAS), or labeled with antibodies against lysozyme and chromogranin A to identify goblet, Paneth and enteroendocrine cells, respectively. We observed a significant increase in all three types of secretory cells ($p<0.0001$) in 3-mo. old *Kaiso*^{Tg} mice compared to NonTg siblings (Fig. 3.1 C, D and E).

Given that Kaiso overexpression drives an increase in secretory cells, we hypothesized that loss of Kaiso would result in a reduced number of secretory cells. We thus examined age-matched *Kaiso*$^{-/y}$ and NonTg mice for goblet cell numbers using alcian blue. Intriguingly, we did not observe a significant difference in the number of goblet cells (Fig. 3.2).

FIGURE 3.1 Kaiso phenocopies loss of canonical Notch signaling independently of intestinal inflammation. (A) Hematoxylin & eosin (H&E) staining of 3-month (mo.) old *Kaiso*^{Tg} intestines do not show widespread intestinal tissue damage due to chronic inflammation. **(B)** Myeloperoxidase (MPO) activity of 3-mo. old *Kaiso*^{Tg} is unchanged relative to age-matched NonTg mice. **(C-E)** 3-mo. old *Kaiso*^{Tg} mice exhibit an increase goblet (PAS - periodic acid Schiff), Paneth (Lysozyme) and enteroendocrine (EEC; ChrA - chromogranin A, yellow arrowheads) cell numbers relative to age-matched NonTg mice. Statistical significance determined using student's t-test. Error bars are SEM, ***$p<0.0005$. Scale bar, 50 μm.

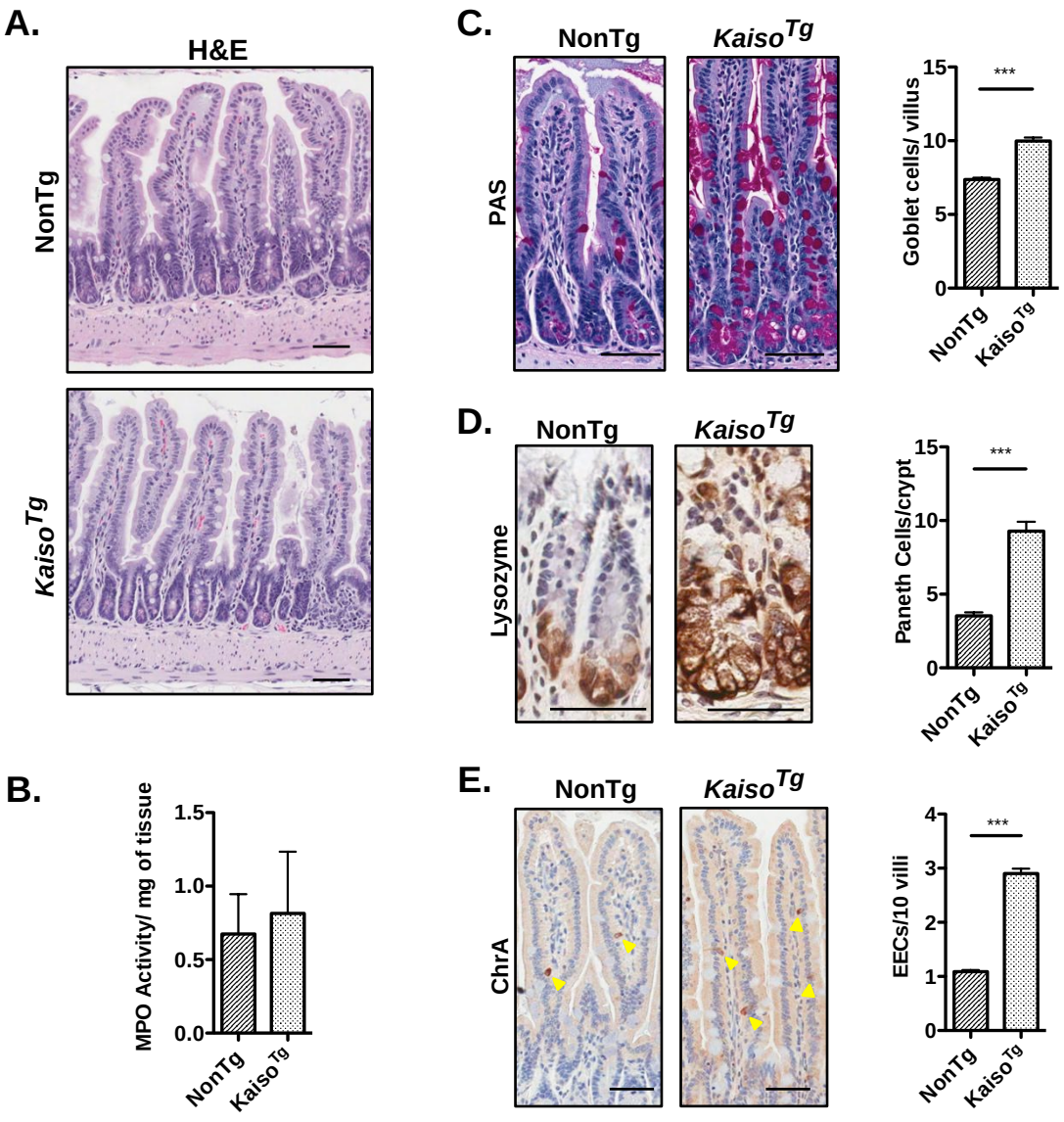
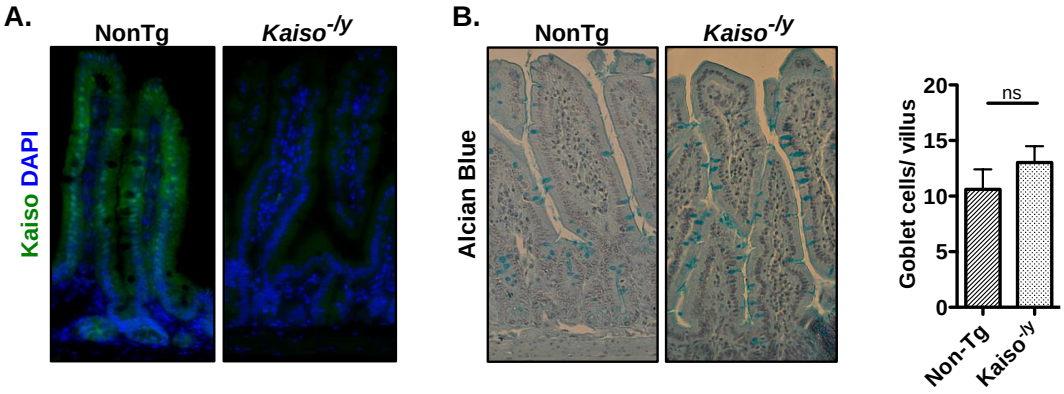

A.
H&E
NonTg
Kaiso^Tg
B.
MPO Activity/ mg of tissue
1.5
1.0
0.5
0.0
NonTg
Kaiso Tg
C.
NonTg
Kaiso^Tg
PAS
Goblet cells/ villus
15
10
5
0
***
NonTg
Kaiso Tg
D.
NonTg
Kaiso^Tg
Lysozyme
Paneth Cells/crypt
15
10
5
0
***
NonTg
Kaiso Tg
E.
NonTg
Kaiso^Tg
ChrA
EECs/10 villi
4
3
2
1
0
***
NonTg
Kaiso Tg

FIGURE 3.2 *Kaiso*$^{-/y}$ mice do not exhibit a goblet cell defect. (A) Immunofluorescence staining of *Kaiso*$^{-/y}$ mice confirm Kaiso-depletion in the intestinal epithelium. Intestines were counterstained with DAPI (4, 6-diamidino-2-phenylindole) to label the nuclei. (**B)** Goblet cells from three 4-week old NonTg and *Kaiso*$^{-/y}$ mice were labeled with alcian blue and quantified. *Kaiso*$^{-/y}$ mice do not exhibit a significant change in goblet cells, as determined by student's t-test.

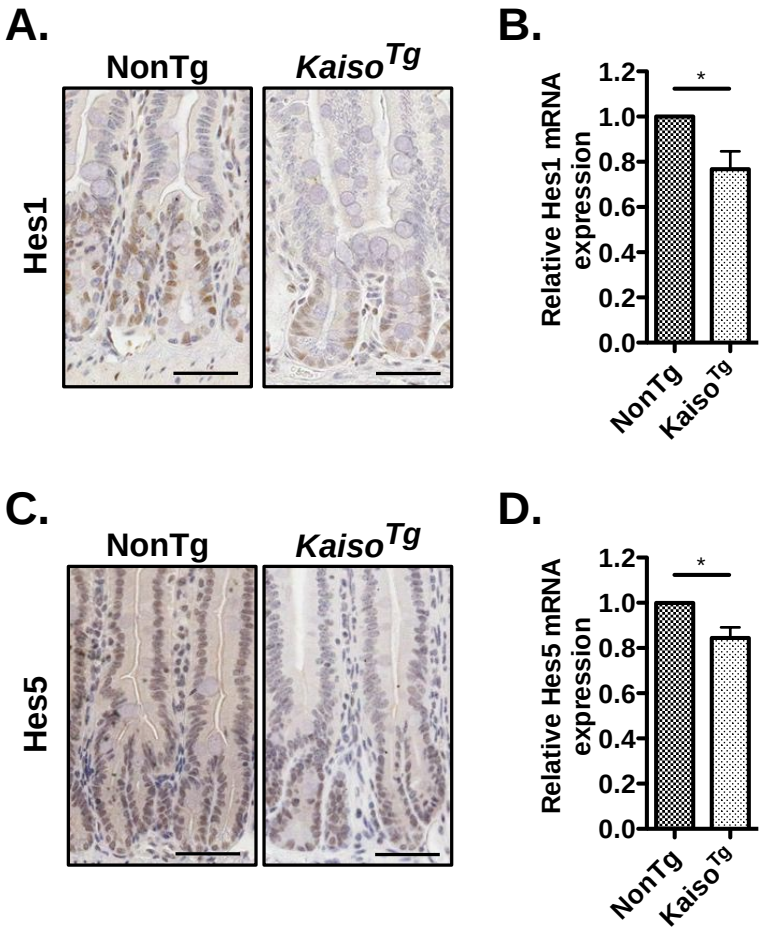

A.
NonTg
Kaiso-/y
Kaiso DAPI
B.
NonTg
Kaiso-/y
Alcian Blue
Goblet cells/ villus
20
15
10
5
0
ns
Non-Tg
Kaiso-/y

Several studies have highlighted the critical role of Notch signaling in mediating intestinal cell fate decisions, by demonstrating that loss of Notch signaling produces an increase in secretory cell types (de Santa Barbara *et al.*, 2003, Fre *et al.*, 2011, Hansson *et al.*, 2004, Kazanjian *et al.*, 2010, Okamoto *et al.*, 2009, Pellegrinet *et al.*, 2011, Riccio *et al.*, 2008, Stamataki *et al.*, 2011, Ueo *et al.*, 2012, Yang *et al.*, 2001). Thus, we examined *Kaiso*^{Tg} intestinal tissues for the expression levels of two downstream Notch pathway effectors, the Hes1 and Hes5 transcription factors, which are known to inhibit secretory cell fates (Ueo *et al.*, 2012). Indeed, we observed reduced Hes1 and Hes5 expression in *Kaiso*^{Tg} compared to NonTg mice as determined by IHC (Fig. 3.3 A, C). Examination of *Hes1* and *Hes5* transcript levels using mRNA isolated from intestinal epithelial cells (IECs) revealed marginal, but significant, reductions in the relative expression levels of both *Hes1* and *Hes5* in *Kaiso*^{Tg} mice compared to NonTg siblings (Fig. 3.3 B, D). Together, these data suggest that Kaiso overexpression inhibits, but does not completely abolish, Notch pathway activation in intestinal cells. More importantly, our data indicate that Kaiso's effects on Notch signaling precede the onset of Kaiso-induced intestinal inflammation.

3.3.2 Kaiso inhibits Notch1 but not Notch2 expression in the small intestine.

Since our data suggest that Kaiso overexpression suppresses Notch signaling in the intestine of 3-mo. old mice, we next sought to determine how Kaiso regulates this pathway. Although all four Notch receptors are expressed in the intestinal mucosa, Notch1 and Notch2 are specifically expressed in IECs and are responsible for governing

FIGURE 3.3 Notch pathway effectors Hes1 and Hes5, are reduced in *Kaiso*^{Tg} mice. Comparison of Hes1 and Hes5 expression in the small intestine of 3-mo. old NonTg and *Kaiso*^{Tg} mice by immunohistochemistry **(A, C)** and qRT-PCR **(B, D)** demonstrate decreased expression of both Notch effectors in *Kaiso*^{Tg} mice relative to NonTg siblings. Statistical significance determined using student's t-test. Error bars are SEM, *$p<0.05$. Scale bar, 50 μm.

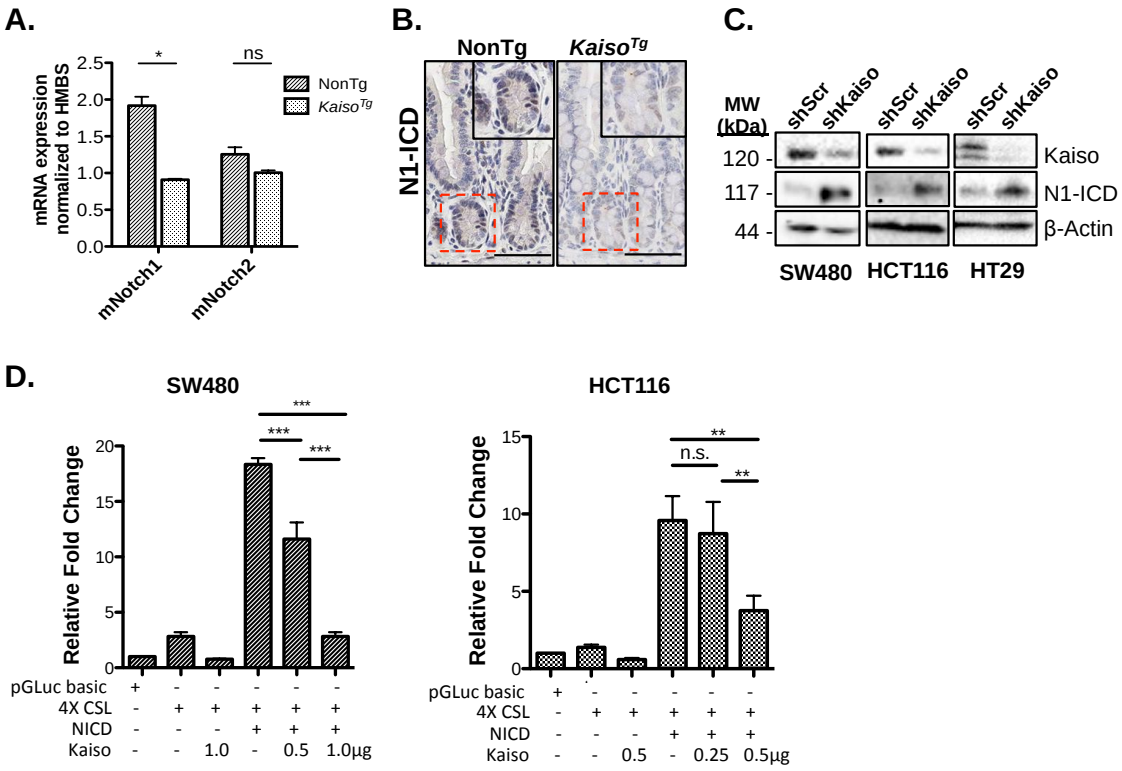

A.
NonTg
Kaiso^Tg
Hes1
B.
Relative Hes1 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
NonTg
Kaiso^Tg
C.
NonTg
Kaiso^Tg
Hes5
D.
Relative Hes5 mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
NonTg
Kaiso^Tg

cell fate decisions in the intestines (Fre *et al.*, 2011, Riccio *et al.*, 2008, Sander & Powell 2004, Schröder & Gossler 2002). IECs from 3-mo. old *Kaiso*^{Tg} and NonTg mice were isolated and assayed for *Notch1* and *Notch2* mRNA levels. We observed a significant reduction in *Notch1* mRNA expression in *Kaiso*^{Tg} compared to NonTg mice ($p=0.014$, Fig. 3A). While *Notch2* mRNA levels were also reduced, the change was not statistically significant ($p=0.072$, Fig. 3.4 A).

Notch pathway activation is characterized by the cytoplasmic release and nuclear translocation of the NICD (Mumm & Kopan 2000). Since we observed a reduction in *Notch1* mRNA expression levels, we next assessed Notch1-ICD (N1-ICD) levels in *Kaiso*^{Tg} mice by examining the tissue distribution of N1-ICD in *Kaiso*^{Tg} mice using IHC. As expected, N1-ICD was restricted to the nuclei of crypt epithelial cells in both NonTg and *Kaiso*^{Tg} mice (Fig. 3.4 B). However, *Kaiso*^{Tg} intestinal tissues exhibited an overall reduction in N1-ICD expression compared to their NonTg counterpart (Fig. 3.4 B). To further validate our observations, we examined N1-ICD expression in three stable Kaiso-depleted colon cancer cell lines, as we postulated that loss of Kaiso would result in increased N1-ICD expression. Indeed, N1-ICD expression was increased in stable Kaiso-depleted (shKaiso) SW480, HCT116 and HT29 cells compared to control cell lines that stably express a scrambled shKaiso sequence (shScr) (Fig. 3.4 C).

To examine Kaiso's effects on N1-ICD function, we performed artificial promoter-reporter luciferase assays using parental HCT116 and SW480 colon cancer cells. HT29 cells were omitted from this analysis since these cells exhibited poorer transfection efficiency compared to HCT116 and SW480 cells. A *Gaussia* luciferase

FIGURE 3.4 Kaiso inhibits Notch1 expression and N1-ICD levels. (A) qRT-PCR analysis revealed decreased mNotch1 and mNotch2 in isolated IECs of *Kaiso*^{Tg} compared to NonTg mice. **(B)** IHC revealed reduced N1-ICD protein levels in *Kaiso*^{Tg} mice. Insets are enlarged images of the area boxed with a dotted line. (**C)** Stable Kaiso-depleted (shKaiso) SW480, HCT116, and HT29 cells exhibit increased levels of N1-ICD as determined by western blot (gamma changes employed). (**D)** Kaiso overexpression attenuates N1-ICD-mediated transactivation of the 4xCSL artificial promoter in SW480 and HCT116 cells. Cells were co-transfected with 0.5 µg pGLuc-4xCSL, 0.25 µg pCAGGS-N1-ICD, and the indicated amounts of pcDNA3-hKaiso. Statistical significance was determined by student's t-test or one-way ANOVA with Bonferroni post-test. Error bars are SEM, ns -not significant, *$p<0.05$, **$p<0.001$, ***$p<0.0001$. Scale bar, 50 µm**.**


A.
mRNA expression normalized to HMBS
2.5
2.0
1.5
1.0
0.5
0.0
*
ns
NonTg
Kaiso^Tg
mNotch1
mNotch2
B.
NonTg
Kaiso^Tg
N1-ICD
C.
MW (kDa)
shScr
shKaiso
shScr
shKaiso
shScr
shKaiso
120 -
117 -
44 -
Kaiso
N1-ICD
β-Actin
SW480
HCT116
HT29
D.
SW480
Relative Fold Change
20
15
10
5
0
***
***
***
pGLuc basic + - - - - -
4X CSL - + + + + +
NICD - - - + + +
Kaiso - - 1.0 - 0.5 1.0µg
HCT116
Relative Fold Change
15
10
5
0
**
n.s.
**
pGLuc basic + - - - - -
4X CSL - + + + + +
NICD - - - + + +
Kaiso - - 0.5 - 0.25 0.5µg

vector containing 4 tandem copies of the *RBP-J/CSL* consensus sequence (pGLuc-4XCSL) was co-transfected with expression vectors encoding N1-ICD (pCAGGS-N1-ICD) and Kaiso (pcDNA3-hKaiso). Co-expression of pCAGGS-N1-ICD and pGLuc-4XCSL resulted in a ~15- and ~18-fold increase in pGLuc-4XCSL activity in HCT116 and SW480 cells, respectively ($p<0.0001$) (Fig. 3.4 D). However, co-expression with pcDNA3-hKaiso attenuated N1-ICD-mediated transactivation of pGLuc-4XCSL in both cell lines, in a dose-dependent manner ($p<0.0001$) (Fig. 3.4 D). Collectively, these data demonstrate that Kaiso inhibits Notch1 signaling in intestinal cells.

3.3.3 Dll-1 expression is reduced in Kaiso^{Tg} mice.

Previous reports have shown that Notch1 and Notch2 act redundantly in the intestine; thus the single loss of either receptor is insufficient to attenuate Notch signaling (Riccio *et al.,* 2008). Hence, we surmised that the secretory cell phenotype observed in *Kaiso^{Tg}* mice (Fig. 3.1) could not be attributed to a reduction in Notch1 expression alone, and that Kaiso may be inducing the secretory cell phenotype via regulation of other components of the Notch pathway. Since Dll-1 and Dll-4 are also implicated in cell fate decisions in the intestine (Pellegrinet *et al.,* 2011), we interrogated the effects of Kaiso overexpression on Dll-1 and Dll-4 expression in our *Kaiso^{Tg}* mice. We first quantified *Dll-1* and *Dll-4* transcript levels by qRT-PCR analysis of harvested IECs from 3-mo. old NonTg and *Kaiso^{Tg}* mice. Interestingly, we observed an ~2-fold reduction in *Dll-1* transcript levels in *Kaiso^{Tg}* mice compared to NonTg siblings ($p=0.04$), while *Dll-4* transcript levels remained relatively unchanged ($p=0.303$) (Fig. 3.5 A). We next assessed the *in vivo* expression levels of Dll-1 and Dll-4 in our *Kaiso^{Tg}* intestines by IHC and found a striking

reduction in Dll-1 positive cells in intestines from *Kaiso*^{Tg} mice compared to NonTg siblings (Fig. 3.5 B). Consistent with qRT-PCR analyses, Dll-4 tissue expression was relatively unchanged (Fig. 3.5 B). In support of our findings, western blot analysis also revealed a marked reduction in Dll-1 protein levels in isolated *Kaiso*^{Tg} IECs relative to NonTg (Fig. 3.5 C).

To further validate our *in vivo* findings, Dll-1 transcript and protein levels were also assessed in stable Kaiso-depleted SW480, HCT116 and HT29 cells. Increased levels of *Dll-1* transcripts were observed in all three Kaiso-depleted cell lines (SW480, HCT116 and HT29) relative to the scrambled controls, but the change in *Dll*-1 mRNA levels was only statistically significant in Kaiso-depleted HT29 cells (p=0.012) (Fig. 3.5 D). Immunoblot analysis of Dll-1 protein expression revealed an ~1.2-, ~1.5- and ~2.6-fold increase in Kaiso-depleted SW480, HCT116 cells, and HT29 cells, respectively (Fig. 3.5 E). Together, these data demonstrate that Kaiso inhibits Dll-1 expression in intestinal cells, and raise the possibility that the observed increase in secretory cell types is due to Kaiso-mediated Dll-1 loss.

3.3.4 Kaiso associates endogenously with the DLL1 but not the NOTCH1 promoter.

Our group and others have shown that Kaiso possesses dual-specificity DNA-binding abilities, and recognizes and binds both the sequence-specific Kaiso binding site (KBS – TCCTGCNA, core sequence underlined) and methylated CpG dinucleotides (Daniel *et al.*, 2002, Donaldson *et al.*, 2012, Filion *et al.*, 2006, Jiang *et al.*, 2012, Prokhortchouk *et al.*, 2001a). Given Kaiso's effects on the expression of Dll-1 and Notch1 in intestinal cells, we next assessed whether either of these Notch pathway components might be putative

Kaiso target genes. Examination of the *DLL1* and *NOTCH1* gene promoters revealed the presence of one or several putative KBS sequences, and numerous CpG dinucleotides. We assayed whether Kaiso associated with these loci in a sequence- or methylation-specific manner. To this end, chromatin was isolated from untreated SW480, HCT116 and HT29 cells, and from cells treated with 5 μM 5'-aza-cytidine for 5 consecutive days. Chromatin immunoprecipitation (ChIP) was performed using the Kaiso-specific 6F monoclonal antibody (Daniel *et al.,* 2001), or with histone H3 and non-specific IgG as positive and negative controls, respectively. The *DLL1* gene promoter spanning -1000 to +400 bp of the transcriptional start site (TSS) contains 2 CpG islands (CGIs; Fig. 3.5 F & Fig. 3.6), and has one full KBS located 256 bp downstream of the TSS which is not only surrounded by 6 CpG dinucleotides, but also itself encompasses a CpG dinucleotide, TCCTGCGA (Fig. 3.5 F). Thus, ChIP-PCR was performed on the region encompassing the KBS and surrounding 6 CpG dinucleotides to ascertain whether Kaiso binding occurred in a sequence-specific or methylation-dependent manner. ChIP analysis revealed that Kaiso associated endogenously with the *DLL1* KBS site in untreated HCT116 and HT29 cells, but not in SW480 cells (Fig. 3.5 G). However, Kaiso binding was abolished upon de-methylation with 5'-aza-cytidine (Fig. 3.5 G, 5'aza-treated), suggesting that Kaiso associates with the *DLL1* promoter in a methylation-dependent manner despite the presence of the KBS.

FIGURE 3.5 Kaiso inhibits Dll-1 expression in intestinal cells. (A, B) Dll-1, but not Dll-4, is reduced in intestinal tissues from *Kaiso*^{Tg} mice compared to NonTg as revealed by qRT-PCR **(A)** and IHC **(B)**. **(C)** Dll-1 protein levels are reduced in intestinal epithelial cells from *Kaiso*^{Tg} relative to NonTg. **(D & E)** Stable Kaiso-depleted (shKaiso) SW480, HCT116 and HT29 colon cells express increased Dll-1 transcripts **(D)** and protein **(E)** compared to controls as determined by qRT-PCR (normalized to β-actin) and western blot analyses, respectively. Western blots were quantified by densitometry (as relative fold change over control). **(F)** Schematic representation of the human *DLL1* promoter spanning -1000 to +400 bp of the transcription start site (+1). Arrowheads denote regions amplified by PCR; vertical lines – CpG dinucleotides; CGI – CpG island. **(G)** Kaiso associates with the *DLL1* KBS in HCT116 and HT29 cells, but not SW480 cells. De-methylation with 5'aza-cytidine (5'aza) abolishes Kaiso binding; Histone H3 (H3) and IgG serve as positive and negative controls, respectively. Asterisk (*) denotes primer dimers. Statistical significance was determined by student's t-test. Error bars are SEM, ns – not significant, **p<0.005. Scale bar, 50 μm.

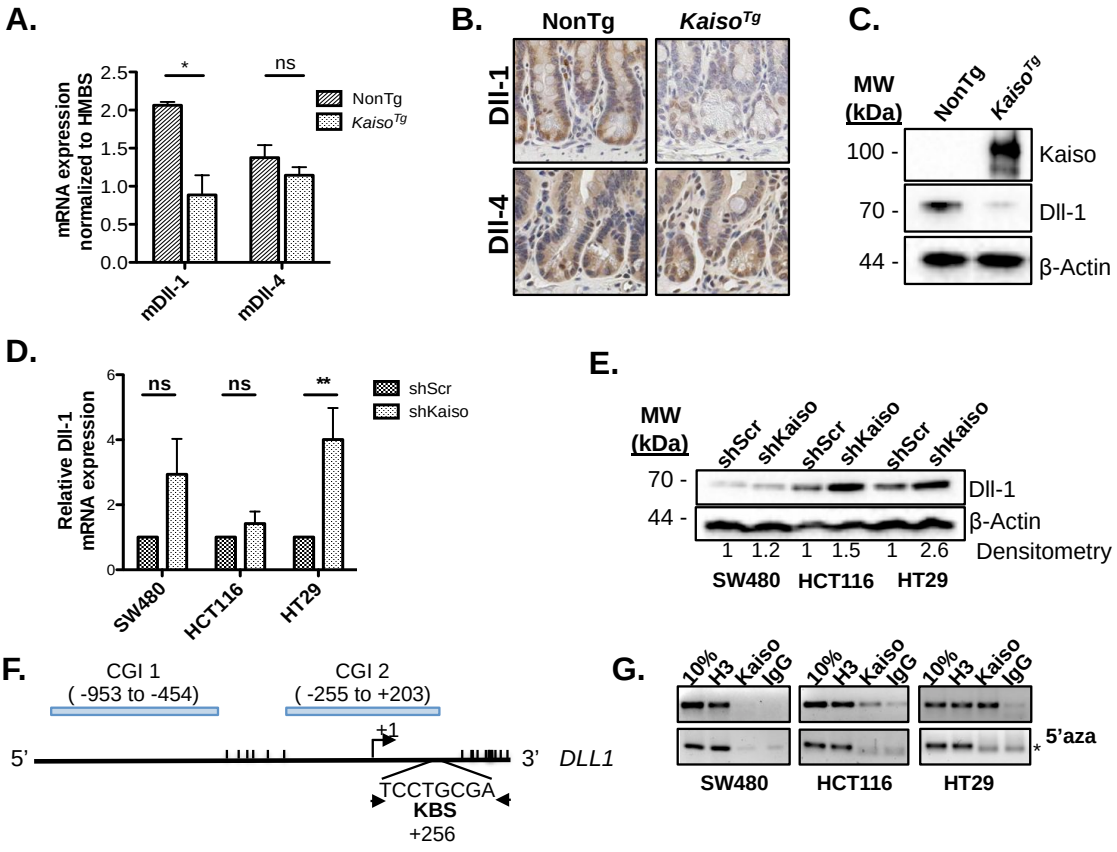
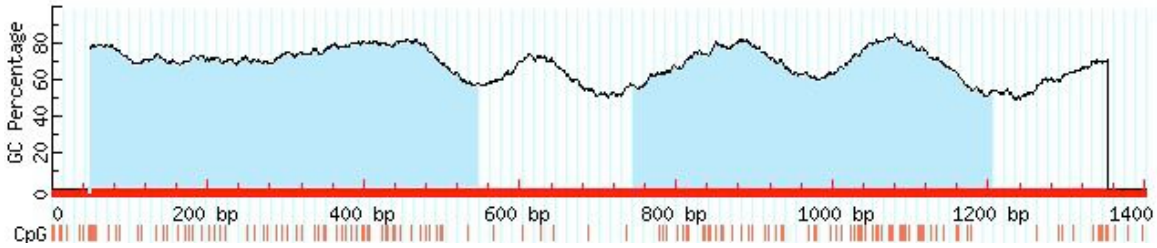

A.
mRNA expression normalized to HMBS
2.5
2.0
1.5
1.0
0.5
0.0
*
ns
NonTg
Kaiso^Tg
mDll-1
mDll-4
B.
NonTg
Kaiso^Tg
Dll-1
Dll-4
C.
MW (kDa)
NonTg
Kaiso^Tg
100 -
70 -
44 -
Kaiso
Dll-1
β-Actin
D.
Relative Dll-1 mRNA expression
6
4
2
0
ns
ns
**
shScr
shKaiso
SW480
HCT116
HT29
E.
MW (kDa)
shScr
shKaiso
shScr
shKaiso
shScr
shKaiso
70 -
44 -
Dll-1
β-Actin
1 1.2 1 1.5 1 2.6
Densitometry
SW480 HCT116 HT29
F.
CGI 1
(-953 to -454)
CGI 2
(-255 to +203)
+1
5'
3'
DLL1
TCCTGCGA
KBS
+256
G.
10%
H3
Kaiso
IgG
10%
H3
Kaiso
IgG
10%
H3
Kaiso
IgG
5'aza
*
SW480
HCT116
HT29

FIGURE 3.6 CpG island prediction of the *DLL1* promoter. The *DLL1* promoter spanning -1000 to + 400 bp of the TSS was analyzed for putative CpG islands. This region is GC-rich and contains two potential CpG islands at -953 to -454 bp and -255 to +203 bp of the TSS (+1), respectively.


GC Percentage
0
20
40
60
80
0
200 bp
400 bp
600 bp
800 bp
1000 bp
1200 bp
1400
CpG

The *NOTCH1* promoter contains three core KBS sites at positions -1599 bp, -1464 bp and -341 bp of the TSS. However, endogenous Kaiso binding was not observed at any of these loci in the colon cancer cell lines tested (data not shown), despite the presence of multiple CpG dinucleotides in the region analyzed.

3.3.5 Kaiso promotes expression of the Notch ligand Jagged-1.

Unlike Dll-1 and Dll-4, the Notch ligand Jagged-1 is dispensable for determining secretory cell fate decisions in the intestines (Pellegrinet *et al.,* 2011). However mounting evidence has highlighted an important role for Jagged-1 in Notch-mediated colon carcinogenesis and progression (Arcaroli *et al.,* 2016, Ba *et al.,* 2012, Dai *et al.,* 2014, Gao *et al.,* 2011, Guilmeau *et al.,* 2010, Reedijk *et al.,* 2008, Rodilla *et al.,* 2009, Sugiyama *et al.,* 2016). Given that Kaiso promotes polyp formation in the $Apc^{Min/+}$ mouse model of colon cancer and reduces overall survival of $Apc^{Min/+}$ mice (Pierre *et al.,* 2015b, Prokhortchouk *et al.,* 2006), we were prompted to examine Jagged-1 expression and tissue localization in our $Kaiso^{Tg}$ mice. Using immunofluorescence staining, we observed Jagged-1 positive cells primarily in the crypts of both NonTg and $Kaiso^{Tg}$ mice (Fig. 3.7 A). Notably, Kaiso-overexpression led to enhanced Jagged-1 expression, and an increase in the number of positively stained cells compared to NonTg counterparts (Fig. 3.7 A). Consistent with this finding, loss of Kaiso in stable Kaiso-depleted SW480 cells resulted in reduced Jagged-1 transcript and protein levels compared to control cells (Fig. 3.7 B, C). Notably, HCT116 and HT29 cells do not express significant levels of Jagged-1 mRNA or protein, and thus the effects of Kaiso depletion in these cell lines were negligible (data not shown). Collectively, these data show that, unlike its effects on Notch1 and Dll-1

expression, Kaiso promotes Jagged-1 expression in intestinal cells.

Since we observed a positive correlation between Jagged-1 and Kaiso expression in our studies, and Kaiso has also been found to activate expression of some target genes (Bassey-Archibong *et al.*, 2016, Koh *et al.*, 2014, Rodova *et al.*, 2004), we investigated whether *JAG1* might also be a putative Kaiso target. We searched a region spanning -1000 to +2500 bp of the TSS for putative KBS sites and identified at least five core KBS sites. However we focused on the -200 bp to +2500 bp region since this region had a high frequency of putative KBS sites, and also showed binding at 2 loci on the UCSC ENCODE Database (Rosenbloom *et al.*, 2013). Five putative CGIs and four core KBS sites (i.e. CTGCNA) were identified in the -200 to +2500 region of the JAG1 promoter at positions -6 bp (KBS-A), +2017 bp (KBS-B), +2188 bp and +2251 bp (collectively, KBS-C) relative to the TSS (Fig. 3.7 D and Fig. 3.8). Primers were designed to PCR amplify each locus, however given the proximity of the +2188 and +2251 KBSs to each other, primers were designed to encompass both KBSs. Kaiso associated weakly with KBS-A in SW480 and HCT116 cells, but not in HT29 cells (data not shown), and no association between Kaiso and KBS-C was detected in any of the cell lines tested (Fig. 3.7 E). However, strong endogenous Kaiso binding was detected at the KBS-B locus in all three cell lines, which was abrogated upon de-methylation with 5'-aza-cytidine (Fig. 3.7 E). Notably, KBS-B and -C both occur outside the CGIs but are both surrounded by several CpG dinucleotides. Thus similar to the *DLL1* KBS, Kaiso associates with the *JAG1* KBS-B locus in a methylation-dependent manner.

FIGURE 3.7 Kaiso promotes the expression of Jagged-1. (A) Immunofluorescence staining revealed enhanced Jagged-1 expression and increased numbers of Jagged-1 positive cells (yellow arrowheads), as quantified in the associated graph, in *Kaiso*^{Tg} crypts. The crypt boundary is indicated with a dotted white line. (**B)** *Jagged-1* mRNA and **(C)** protein levels are reduced in stable Kaiso-depleted SW480 cells, as determined by qRT-PCR and western blot, respectively. **(D)** Schematic representation of the human *JAG1* promoter from -200 bp to +2500 bp of the transcription start site (+1). Arrowheads indicate PCR-amplified regions. Vertical lines represent CpG dinucleotides. CpG Islands (CGI) 1-5 are located -112 to +114, +165 to +540, +547 to +824, +853 to +1458, and +1615 to +1886, respectively. **(E)** Kaiso associates with KBS-B, but not KBS-C of the *JAG1* promoter. 5'aza-treatement led to loss of Kaiso binding to KBS-B. Asterisk (*) denotes primer dimers. Statistical significance determined by student's t-test. Error bars are SEM, * $p<0.05$.

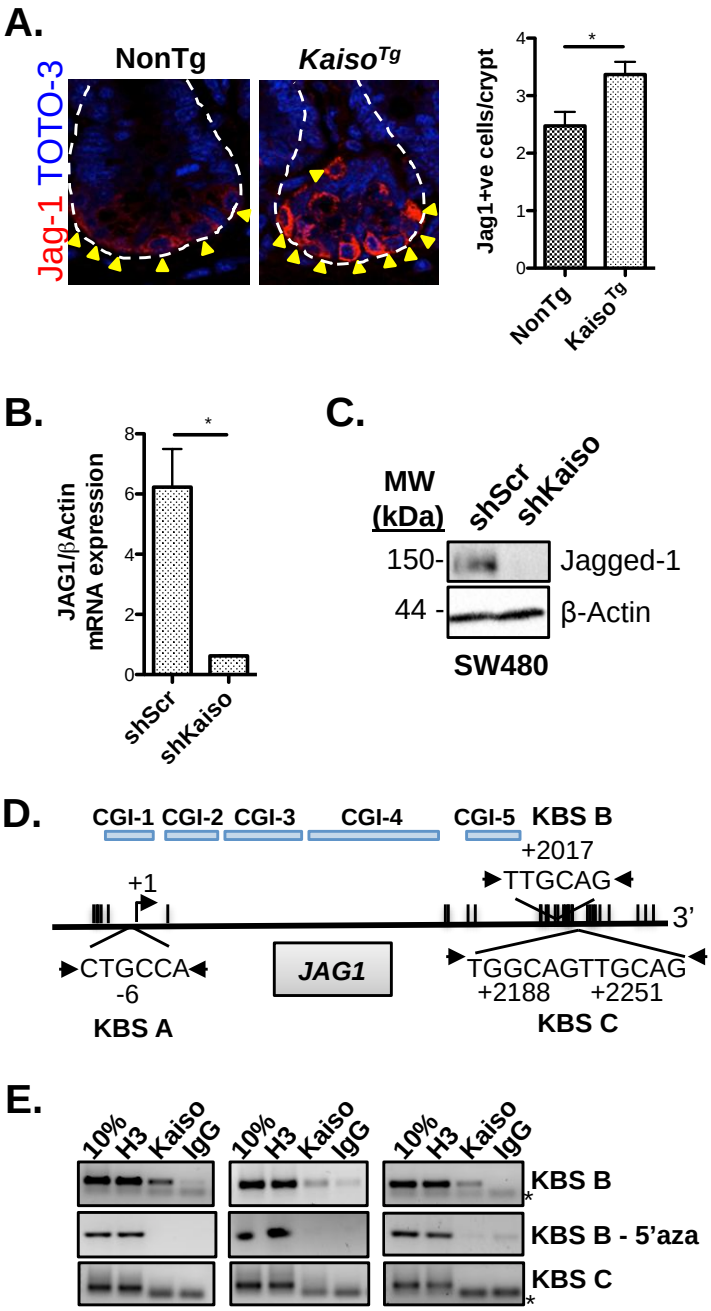
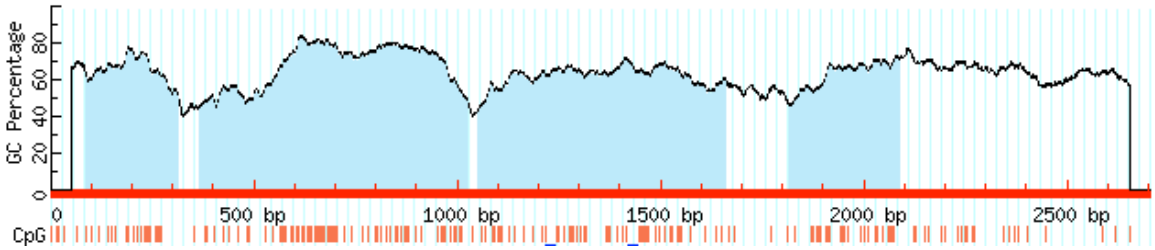

A.
NonTg
Kaiso^Tg
Jag-1 TOTO-3
Jag1+ve cells/crypt
NonTg
Kaiso^Tg
B.
JAG1/βActin mRNA expression
shScr
shKaiso
C.
MW (kDa)
shScr
shKaiso
150-
Jagged-1
44 -
β-Actin
SW480
D.
CGI-1 CGI-2 CGI-3 CGI-4 CGI-5 KBS B
+2017
TTGCAG
+1
3'
CTGCCA
-6
JAG1
TGGCAGTTGCAG
+2188 +2251
KBS A
KBS C
E.
10% H3 Kaiso IgG
KBS B
KBS B - 5'aza
KBS C
SW480
HCT116
HT29

FIGURE 3.8 CpG island prediction of the minimal *JAG1* promoter. The *JAG1* gene spanning -200 to +2500 bp of the TSS was analyzed for putative CpG islands. This GC-rich region harbors 5 potential CpG islands.


GC Percentage
0
20
40
60
80
0
500 bp
1000 bp
1500 bp
2000 bp
2500 bp
CpG

In summary, our data demonstrate that Kaiso-mediated regulation of Notch signaling in intestinal tissues occurs via regulation of multiple Notch pathway components. Indeed, our finding that Kaiso associates with the *DLL1* and *JAG1* promoters in a methylation-dependent manner implicates them as putative Kaiso target genes, and suggests that this may be one mechanism by which Kaiso regulates Notch signaling in intestinal cells. Remarkably, Kaiso appears to repress Dll-1 expression, which plays an important role in intestinal cell differentiation, but activates Jagged-1 expression, which is dispensable for cell fate determination.

3.4 DISCUSSION

The highly conserved Notch signaling pathway is fundamental for several biological processes, including binary cell fate decisions in the intestine where it functions to inhibit secretory cell differentiation, while promoting the absorptive cell fate, reviewed in (Crosnier *et al.*, 2006, de Santa Barbara *et al.*, 2003, Noah *et al.*, 2011). Herein we demonstrate that the POZ-ZF transcription factor Kaiso, whose overexpression enhances intestinal polyp formation in $Apc^{Min/+}$ mice (Pierre *et al.*, 2015b) and drives spontaneous intestinal inflammation, regulates the Notch pathway and secretory cell fates in intestinal tissues (Chaudhary *et al.*, 2013).

During our initial characterization of 12-mo. old $Kaiso^{Tg}$ mice, we noticed that $Kaiso^{Tg}$ mice exhibited an increase in neutrophil-specific intestinal inflammation (Chaudhary *et al.*, 2013) (and our unpublished data) and a Notch-depletion phenotype (i.e. increased numbers of secretory cell types). By examining subclinical 3-mo. old mice in this study, we found that younger $Kaiso^{Tg}$ mice do not display widespread signs of

chronic inflammation, but still phenocopy loss of Notch signaling. This finding suggests that the Notch-depletion phenotype in *Kaiso*^{Tg} mice precedes intestinal inflammation (Fig. 3.1) and may in fact play a role in the pathology of Kaiso-induced chronic inflammation at later ages. Similar findings were observed by Obata *et al..*, who demonstrated that Notch-signaling inhibition via IEC-specific deletion of *Rbpj* (RBP-J$^{IEC/\Delta}$) resulted in spontaneous inflammation in the colons of these mice (Obata *et al.,* 2012). Similar to *Kaiso*^{Tg}, RBP-J$^{IEC/\Delta}$ mice exhibited increased numbers of secretory cells throughout the intestine, as well as augmented neutrophil infiltration in inflamed regions (Obata *et al.,* 2012).

Notch pathway activation culminates in the expression of the Hes transcription factor family, which function to suppress the secretory cell lineage in the intestinal epithelium (Schröder & Gossler 2002, Ueo *et al.,* 2012). While Hes1-deficiency is sufficient to drive increased secretory cell numbers in immature mice, this effect is counteracted by Hes3 and Hes5 expression after 2 months of age (Ueo *et al.,* 2012). Indeed, combined loss of Hes1/3/5 resulted in augmented secretory cell numbers compared to loss of Hes1 alone in 2-mo. old mice (Ueo *et al.,* 2012). Thus, our hypothesis that Kaiso promotes secretory cell differentiation via Notch pathway inhibition is further strengthened by the observation that 3-mo. old *Kaiso*^{Tg} mice exhibit reduced expression of both Hes1 and Hes5 compared to age-matched NonTg siblings (Fig 3.3). However, qRT-PCR analyses revealed only a modest reduction in mRNA levels of both transcription factors, suggesting that Notch signaling is not completely abolished in *Kaiso*^{Tg} epithelium. This possibility is consistent with our observation that

only Dll-1 but not Dll-4, is reduced in *Kaiso*^{Tg} mice.

Using tamoxifen-inducible mouse models, Pellegrinet *et al.* demonstrated that *Dll-1* ablation was sufficient to cause a mild increase in goblet cells, while loss of *Dll-4* alone did not affect goblet cell numbers (Pellegrinet *et al.,* 2011). This study was subsequently substantiated by Stamataki and colleagues, who showed that inducible *Dll-1* knock-out led to an increase in enteroendocrine and Paneth cell numbers (Stamataki *et al.,* 2011). Notably however, genetic loss of both *Dll-1* and *Dll-4* produces a more striking phenotype, where the intestinal epithelium undergoes post-mitotic goblet cell conversion (Pellegrinet *et al.,* 2011). Moreover, while Dll-1 is able to fully rescue loss of *Dll-4*, Dll-4 is only partially able to rescue loss of Dll-1. Thus, the finding that Dll-1, and not Dll-4, is reduced in *Kaiso*^{Tg} mice compared to their NonTg siblings (Fig. 3.5), is consistent with these previous reports (Pellegrinet *et al.,* 2011, Stamataki *et al.,* 2011), and may explain why 3-mo. old mice exhibit a relatively modest secretory cell phenotype compared to other mouse models of Notch inhibition (Pellegrinet *et al.,* 2011, Riccio *et al.,* 2008, van Es *et al.,* 2005, VanDussen *et al.,* 2012). Moreover, although we also observed a reduction in Notch1 expression (Fig. 3.4), the functional redundancy of Notch1 and Notch2 in the intestine (Riccio *et al.,* 2008) suggests that the Notch-depletion phenotype caused by constitutive Kaiso overexpression is due to either a reduction in Dll-1 alone (Pellegrinet *et al.,* 2011), or a combination of both Notch-1 and Dll-1 inhibition. Nevertheless, our findings support a role for Kaiso in secretory cell fate decisions through suppression of Notch signaling

Although Jagged-1 is dispensable for cell differentiation in the intestines

(Pellegrinet *et al.*, 2011), increasing evidence supports a role for Jagged-1 in colon cancer (Arcaroli *et al.*, 2016, Dai *et al.*, 2014, Gao *et al.*, 2011, Rodilla *et al.*, 2009, Sugiyama *et al.*, 2016). While the enhanced Jagged-1 positivity in *Kaiso*^{Tg} mice was unexpected (Fig. 3.7), the data is consistent with our previous findings that Kaiso potentiates *Apc*$^{Min/+}$-mediated tumourigenesis (Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006), and raises the possibility that one mechanism by which Kaiso promotes intestinal tumourigenesis is via Jagged-1 activation.

Several previous studies have demonstrated that Kaiso is capable of binding to DNA using various mechanisms: either in a sequence-specific manner (via the KBS), via meCpG dinucleotides, or a combination of both (Bassey-Archibong *et al.*, 2016, Daniel *et al.*, 2002, Donaldson *et al.*, 2012, Pierre *et al.*, 2015a, Prokhortchouk *et al.*, 2001a, Prokhortchouk *et al.*, 2001b, Spring *et al.*, 2005). The observation that Kaiso's association with the *DLL1* and *JAG1* promoters is abolished upon demethylation with 5'-aza-cytidine suggests that Kaiso primarily binds to these regions via meCpG, and not KBS-specific, binding mechanisms. While extensive characterization of Kaiso's binding mechanisms to the *DLL1* and *JAG1* promoters is beyond the scope of this study, it is possible that the presence of a KBS may act to increase the specificity of Kaiso's association with meCpGs. Indeed, we reported such a phenomenon with the *CCND1* promoter, where Kaiso was found to associate with the +69 KBS in a methylation-dependent manner. Notably however, mutation of the core KBS nucleotides surrounding the meCpG weakened Kaiso's association with the *CCND1* +69 KBS site, suggesting that the presence of the KBS acts to strengthen and/or increase the specificity of binding to

meCpG sites (Donaldson *et al.*, 2012). Intriguingly, Kaiso did not associate with *JAG1* KBS-C or the *NOTCH1* KBS loci despite the presence of surrounding CpG dinucleotides, a phenomenon that underscores the complexity of Kaiso-mediated transcriptional regulation of its target genes.

While Kaiso overexpression was found to promote the formation of secretory cell types, contrary to our hypothesis, we did not observe a significant change in the number of goblet cells in *Kaiso*$^{-/y}$ mice (Fig. 3.2). During their characterization of *Kaiso*$^{-/y}$ mice, Prokhortchouk and colleagues also reported that loss of Kaiso did not produce gross morphological defects, nor did it cause significant alterations to Kaiso-target gene expression (Prokhortchouk *et al.*, 2006). Given that the Kaiso-like proteins, ZBTB4 and ZBTB38 also bind DNA sequences similar to the KBS (Filion *et al.*, 2006, Sasai *et al.*, 2010), it is possible that the lack of a secretory cell phenotype in the *Kaiso*$^{-/y}$ mice is due to functional redundancy conferred by these proteins, though this remains to be determined empirically. Nevertheless, the finding that Kaiso overexpression exerts distinct effects on Notch1, Dll-1 and Jagged-1 may still be physiologically relevant, since Kaiso expression is elevated in several diseases, including breast (Bassey-Archibong *et al.*, 2016, Vermeulen *et al.*, 2012), prostate (Jones *et al.*, 2012), and colon cancer (Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006), and in some cases of Crohn's disease (our unpublished data).

3.5 CONCLUSION

In conclusion, this study describes at least one mechanism by which Kaiso regulates Notch-mediated intestinal homeostasis. Specifically, we demonstrate that Kaiso inhibits

the expression of Notch1 and the Notch ligand Dll-1, but enhances the expression of Jagged-1. We postulate that Kaiso-mediated repression of Dll-1 is sufficient to promote the increase in secretory cells observed in our mice. While Kaiso-mediated activation of Jagged-1 in the intestinal epithelium likely does not contribute to the secretory cell phenotype, it is possible that the Kaiso/Jagged-1 interaction contributes to colon cancer progression.

CHAPTER 4 KAISO LOWERS ITS DEFENSES

Unraveling Kaiso's role in intestinal inflammation

PREFACE

This chapter describes the work that resulted in the following manuscript in preparation: **Shaiya C. Robinson***, Roopali Chaudhary*, Rodrigo Jimenez-Saiz, Lyndsay G.A. Rayner, Yulika Yoshida-Montezuma, Manel Jordana and Juliet M. Daniel. **Kaiso-induced intestinal inflammation is accompanied by misregulated intestinal repair and faulty cell adhesion.** *Co-first author.

Summary: This study provides a more in-depth analysis of the mechanisms governing Kaiso-driven intestinal inflammation. We found that *Kaiso*^{Tg} mice exhibit neutrophil-dependent inflammation and associated tissue damage, including the formation of crypt abscesses, fused and blunted villi and thickened *submucosa*. To pinpoint the event(s) contributing to Kaiso-induced inflammation, analyses were performed on subclinical (12-week old) and diseased (24-week old) *Kaiso*^{Tg} line A mice. Our findings demonstrate that, similar to human IBD, Kaiso-induced inflammation is multi-factorial and likely arises due to a combination of pathogenic neutrophil recruitment, altered cell-adhesion, and altered intestinal epithelial repair mechanisms.

***Author contributions*:** Dr. Roopali Chaudhary generated the data in Fig. 4.1 B and C, which shows that *Kaiso*^{Tg} mice exhibit histological signs of chronic inflammation. She

also generated Fig. 4.2A, which demonstrates the approximate age at which mice develop inflammation, and Fig. 4.3, which shows reduced barrier integrity upon inflammation in *Kaiso*^{Tg} mice. Dr. Chaudhary also provided technical assistance with BrdU injections of mice in Fig. 4.6B. Dr. Rodrigo Jimenez-Saiz performed flow cytometry analyses in Fig. 4.1D and F, which shows that neutrophils are increased in *Kaiso*^{Tg} mice as early as 14 weeks of age. Dr. Manel Jordana provided many of the reagents used in this study. I performed IHC analyses in Fig.1A showing elevated Kaiso expression in CD intestinal tissues; Fig. 4.2A, demonstrating increased levels of the neutrophil chemokine, MIP-2; and Fig. 4.4 & 4.5 showing expression and subcellular localization of cell adhesion proteins. I also performed the experiments in Fig 4.6, which examines the repair mechanisms in *Kaiso*^{Tg} mice. Dr. Chaudhary, Dr. Daniel and myself co-wrote the manuscript, and contributed equally to the conceptualization, critical analysis and revision of the manuscript.

4.1 ABSTRACT

Chronic intestinal inflammation contributes to pathologies such as inflammatory bowel disease (IBD) and colon cancer. While the precise etiology remains controversial, IBD is believed to manifest as a result of various factors. We previously reported that intestinal-specific overexpression of the transcription factor Kaiso results in an intestinal inflammatory response; however the cause of this inflammation is unknown. To elucidate the underlying mechanism(s) of the Kaiso-mediated intestinal inflammatory phenotype, we evaluated two independent Kaiso transgenic (*Kaiso*^{Tg}) mouse lines that express varying levels of Kaiso. Histological analyses of *Kaiso*^{Tg} mice revealed intestinal tissue damage including thickening of the mucosa, intestinal "lesions" and crypt abscesses, reminiscent of IBD pathology. Moreover, we found that higher Kaiso levels induced an intestinal neutrophilic response as early as 12 weeks, which worsened as the mice age. Notably, the Kaiso-induced intestinal inflammation correlated with a leaky intestinal barrier and misregulation of apical junctional proteins. Interestingly, Kaiso overexpression resulted in reduced proliferation and enhanced migration of intestinal epithelial cells prior to the onset of inflammation. Together, these data suggest that Kaiso overexpression regulates intestinal epithelial cell integrity and function, dysregulation of which lead to a chronic inflammatory phenotype.

4.2 INTRODUCTION

Inflammatory **b**owel **d**isease (IBD) is an umbrella term used to describe two disorders of the intestinal tract that are characterized by chronic inflammation (Maloy & Powrie 2011, Saleh & Trinchieri 2011): **C**rohn's **d**isease (CD), which affects both the small and large intestine, and **u**lcerative **c**olitis (UC), which is restricted to the large intestine (Xavier & Podolsky 2007). Currently there is no cure for IBD, and hence both UC and CD are primarily managed by alleviation of the symptoms (Saleh & Trinchieri 2011). Thus, a better understanding of the underlying molecular and/or genetic factors that contribute to IBD will enhance and facilitate development of better treatment options for this disease.

While the etiology of IBD remains elusive, various mouse models have demonstrated that IBD pathogenesis is multifactorial, with defects in intestinal permeability and intestinal repair mechanisms playing crucial roles. Multi-protein apical adhesion complexes, formed of tight junctions (TJs) and adherens junctions (AJs), establish the barrier at the apical ends of epithelial cells (Landy *et al.,* 2016, Poritz *et al.,* 2007, Vaziri *et al.,* 2012). Compromised intestinal barrier integrity, associated with loss of TJ proteins (e.g. ZO-1, claudins) and AJ proteins (e.g. E-cadherin, $p120^{ctn}$), has been implicated in a leaky intestinal barrier and subsequent intestinal inflammation. Our recent characterization of the novel transgenic mouse overexpressing the transcription factor Kaiso ($Kaiso^{Tg}$) in the intestines, revealed that at 12-months of age, $Kaiso^{Tg}$ mice displayed phenotypes consistent with chronic intestinal inflammation, namely, villus blunting, increased **m**yelo**p**er**o**xidase (MPO) levels, and enhanced neutrophil infiltration (Chaudhary *et al.,* 2013). Kaiso belongs to the family of **Po**x virus and **z**inc finger (POZ)

zinc **f**inger (POZ-ZF) transcription factors, and, like other members of this protein family, has roles in vertebrate development and disease (Bassey-Archibong *et al.,* 2016, Bassey-Archibong *et al.*, 2017b, Dai *et al.,* 2011, Iioka *et al.,* 2009, Jones *et al.,* 2014a, Kim *et al.*, 2004, Lopes *et al.*, 2008, Park *et al.,* 2005, Pierre *et al.,* 2015b, Prokhortchouk *et al.,* 2006, Wang *et al.,* 2016). While our study was the first to implicate Kaiso in intestinal inflammation, Kaiso's mechanism of action in inflammation had not yet been elucidated. We thus sought to further characterize *Kaiso*^{Tg} mice to gain insight into the sequence of events leading to the Kaiso-induced inflammatory phenotype. To this end, we first examined two independent mouse transgenic lines with differing Kaiso expression levels, (**v**illin **K**aiso line A, VKA – moderate Kaiso, and VKE – high Kaiso) and corresponding differences in the severity of intestinal inflammation. Notably, the higher Kaiso-expressing line (VKE) developed earlier and more extensive inflammation than the moderate Kaiso-expressing line (VKA), suggesting that high Kaiso expression increases the susceptibility to inflammation. To elucidate the predisposing event(s) (or factors) that contribute to Kaiso-induced inflammation, subclinical (12-week old) *Kaiso*^{Tg} mice were assessed for changes in the expression and subcellular localization of cell adhesion molecules, as well as for defects in intestinal repair processes. Notably, changes in ZO-1, claudin-2 and E-cadherin were observed in diseased (24-week old) mice, while subclinical (12-week old) mice exhibited only a change in E-cadherin. Additionally, subclinical *Kaiso*^{Tg} mice exhibited misregulated epithelial repair processes prior to the onset of intestinal inflammation. Our findings suggest that Kaiso overexpression leads to altered epithelial repair mechanisms and misregulated expression of cell adhesion

proteins, which we propose creates an environment that fosters or supports an inflammatory condition.

4.3 RESULTS

4.3.1 Kaiso expression is increased in inflammatory bowel disease.

Our finding that intestinal-specific Kaiso overexpression results in spontaneous chronic inflammation in 12-month old mice (Chaudhary *et al.*, 2013), prompted us to examine the physiological relevance of this phenomenon. We first assessed Kaiso expression and subcellular localization in a human colon **t**issue **m**icro**a**rray (TMA) comprised of normal, IBD and adenocarcinoma intestinal tissues using **i**mmuno**h**isto**c**hemistry (IHC). While Kaiso localized to the cytoplasm and nucleus of both normal and IBD tissues, we detected increased Kaiso expression in IBD intestinal tissues compared to normal tissues (Fig 4.1 A), supporting the notion that Kaiso is a physiologically relevant participant in the development of chronic intestinal inflammation.

4.3.2 Kaiso-induced intestinal inflammation leads to long-term tissue damage.

To further characterize the Kaiso inflammatory phenotype, two **v**illin-***K****aiso***^{Tg} (VK) mouse lines were examined: the moderate Kaiso-expressing Line A (VKA) and the high Kaiso-expressing Line E (VKE) (Chaudhary *et al.*, 2013). Both VKA and VKE mice displayed a thickened *muscularis externa* at ~8 months of age (Fig. 4.1 B), and exhibited discontinuous “lesions” throughout the length of the small intestine separated by healthy villi. These lesions were comprised of blunted and fused villi, and elevated immune cell infiltration into the underlying *lamina propria* (Fig. 4.1 B).

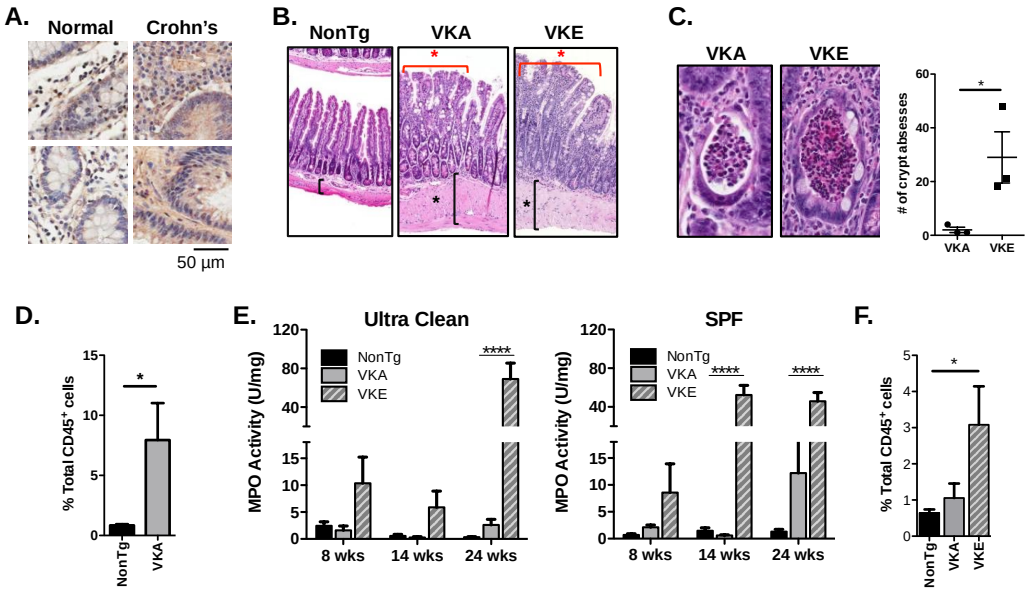
Among the discerning phenotypes associated with IBD, are crypt abscesses – the aggregation of polymorphonuclear leukocytes, especially neutrophils, in the crypt lumen (Le Berre *et al.*, 1995). By ~ 8 months of age, both *Kaiso*^{Tg} lines (n=3/genotype) formed crypt abscesses, although VKE mice developed significantly more crypt abscesses compared to VKA mice (Fig. 4.1 C). No crypt abscesses were detected in the intestines of age-matched non-transgenic (NonTg) littermates. Consistent with our previous findings (Chaudhary *et al.*, 2013, Pierre *et al.*, 2015b), together, these data highlight that intestinal-specific Kaiso overexpression drives intestinal inflammation.

4.3.3 High Kaiso expression induces neutrophil infiltration and activation.

Since *Kaiso*^{Tg} mice display chronic inflammation and tissue damage associated with increased immune cell infiltration, we next sought to identify the precise immune cell types invading the *lamina propria* using flow cytometry. The innate and adaptive immune cell population, including neutrophils, eosinophils, dendritic cells, natural killer cells, T and B cells, and tissue-resident macrophages, were assessed in 8-month old VKA mice. VKA mice showed a significant increase in neutrophils at 8 months of age (Fig. 4.1 D) with no changes in other leukocytes.

To determine if bacterial and pathogenic load from the environment influences the development of intestinal inflammation in *Kaiso*^{Tg} mice, MPO activity, a surrogate marker of activated neutrophils (Brazil *et al.*, 2013), was measured in 8- and 14-week old

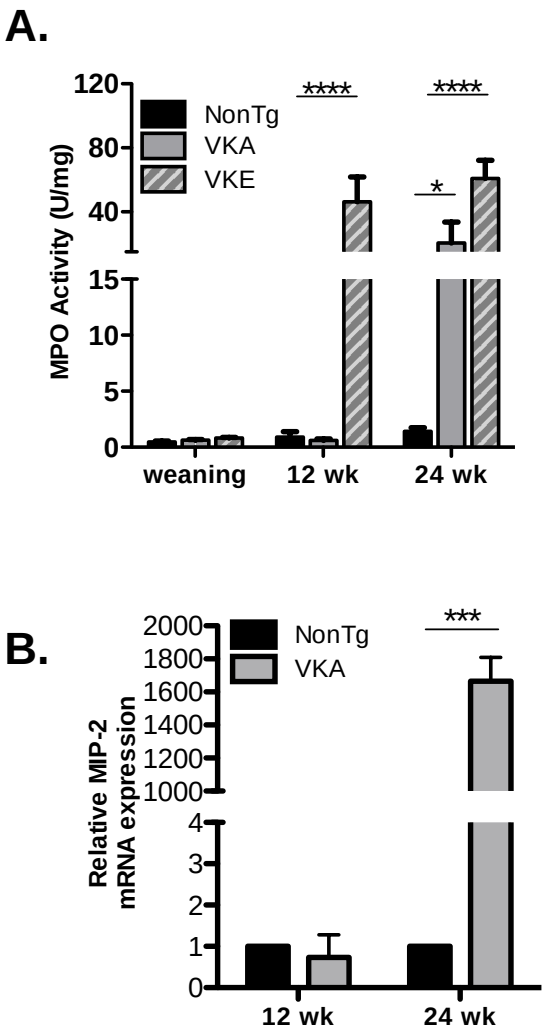
FIGURE 4.1 *Kaiso*^{Tg} mice display hallmarks of chronic inflammation. (A) Inflammation in Crohn's disease patients correlates with higher Kaiso expression levels. **(B)** *Kaiso*^{Tg} mice display discontinuous lesions through the small intestine (red asterisks) and thickening of the *muscularis externa* (black asterisks) compared to the NonTg siblings. **(C)** Line A (VKA) show fewer crypt abscesses at 8 months of age compared to Line E (VKE). **(D)** At 8 months, VKA mice exhibit a higher neutrophil population compared to their NonTg siblings, with a slight decrease in macrophages. **(E)** Early age MPO analysis on age matched VKA and VKE mice show an increase in inflammation in VKE mice compared to VKA and NonTg mice at 14 weeks in SPF (n=5-7 per genotype per age). **(F)** At 14 weeks, VKE mice show a significant increase in neutrophils compared to age-matched NonTg mice and VKA mice.


A.
Normal
Crohn's
50 µm
B.
NonTg
VKA
VKE
C.
VKA
VKE
# of crypt absesses
D.
% Total CD45+ cells
NonTg
VKA
E.
Ultra Clean
SPF
NonTg
VKA
VKE
MPO Activity (U/mg)
8 wks
14 wks
24 wks
F.
% Total CD45+ cells
NonTg
VKA
VKE

mice housed in two conditions: an ultraclean room (low bacterial and pathogenic load), or the common specific pathogen free (SPF) room (Fig. 4.1 E). While there was no difference in the neutrophil activity of VKA mice in either housing condition, VKE mice presented with elevated MPO activity at 8 weeks under both housing conditions, which significantly increased at 14 weeks of age under SPF conditions (Fig. 4.1 E). The early manifestation of MPO activity in VKE mice suggests that high Kaiso expression enhances susceptibility to inflammation, which is influenced by the bacterial load in the intestines.

We next compared the neutrophil population in the *lamina propria* at 14 weeks when VKE, and not VKA, mice have active inflammation. Similar to 8-month old VKA mice, 14-week old VKE mice displayed significantly increased neutrophil infiltration into the *lamina propria*, while VKA showed a slight but non-significant increase ($p = 0.06$) (Fig. 4.1 F). Together, these data suggest that high Kaiso expression induces an early onset of intestinal inflammation that is neutrophil-specific and environment-dependent. To determine the onset of the neutrophil-specific inflammation, in both VKA and VKE mice, neutrophil activity was assayed at various time points: 3- (weaning), 12- and 24-weeks of age (Fig. 4.2 A). Neither VKA nor VKE mice exhibited inflammation upon weaning, although VKE mice displayed significant neutrophil activity by 12 weeks of age. While neutrophil activity was significantly elevated in both VKA and VKE mice by 24 weeks, VKE mice exhibited significantly more MPO activity than their VKA counterparts (Fig. 4.2 A).

FIGURE 4.2 *Kaiso*^{Tg} mice develop inflammation with age. (A) Kaiso transgenics do not show inflammation upon weaning. However, Line E (VKE) mice develop inflammation rapidly, while Line A (VKA) mice develop inflammation at 24-weeks of age. **(B)** Expression levels of the neutrophil attracting cytokine, MIP-2, are increased at the inflammatory stage in VKA mice compared to NonTg mice.

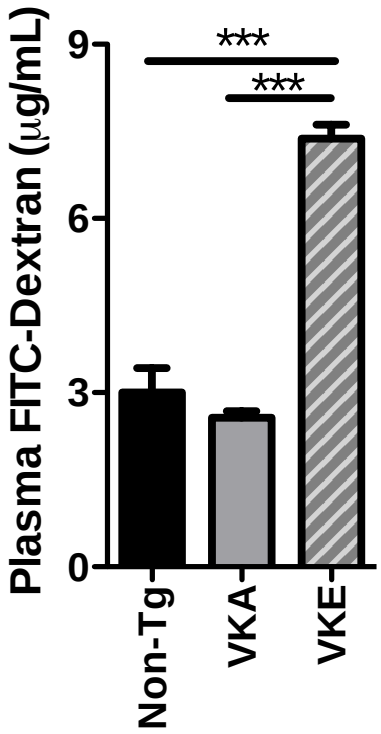

A.
MPO Activity (U/mg)
120
80
40
15
10
5
0
NonTg
VKA
VKE
****
****
*
weaning
12 wk
24 wk
B.
Relative MIP-2 mRNA expression
2000
1800
1600
1400
1200
1000
4
3
2
1
0
NonTg
VKA
***
12 wk
24 wk

The potent neutrophil chemokine, macrophage inflammatory protein-2 (MIP-2), is secreted by various cell types, including intestinal epithelial cells (IECs) (Deweindt *et al.*, 1995, Ohtsuka *et al.*, 2001). As a first step in evaluating the underlying mechanism(s) of Kaiso-induced inflammation, we examined mRNA expression levels of MIP-2 in age-matched NonTg and *Kaiso*^{Tg} IECs. To determine if there was a change in MIP-2 levels prior to, and after, inflammation, comparisons were focused on subclinical 12-week old VKA mice, and diseased 24-week old VKA mice (Fig. 4.2 A). VKE mice were not examined since they exhibit enhanced MPO activity as early as 8-weeks, and significantly elevated MPO levels by 12-weeks of age (Fig. 4.1 E & 4.2A). mRNA from IECs was isolated from age-matched VKA and NonTg littermates (n=6/genotype), and qRT-PCR for *MIP-2* was performed. Consistent with MPO activity data (Fig. 4.2 A), diseased VKA mice have increased *MIP-2* expression at 24 weeks, but not at 12-weeks, compared to their age-matched NonTg counterparts (Fig. 4.2 B). Taken together, these data indicated that Kaiso overexpression produces increased neutrophil recruitment and activity within the intestines.

4.3.4 Kaiso^{Tg} mice exhibit hallmarks of a defective intestinal epithelial barrier upon inflammation.

Intestinal inflammation is often associated with a compromised intestinal epithelial barrier and an increase in intestinal permeability (Pastorelli *et al.*, 2013). In light of our findings that Kaiso overexpression promotes intestinal inflammation, we next interrogated the integrity of the intestinal epithelial barrier of *Kaiso*^{Tg} mice. Mice were

FIGURE 4.3 Kaiso-induced inflammation is associated with a barrier defect. Inflamed intestinal tissues of 14-week old VKE mice exhibit significantly increased permeability to 4 kDa FITC-dextran, compared to the non-inflamed tissues of age-matched VKA and NonTg mice.


Plasma FITC-Dextran (μg/mL)
9
6
3
0
***
***
Non-Tg
VKA
VKE

administered 4 kDa FITC-dextran by intra-gastric oral gavage, and plasma FITC-dextran was measured 2 hours post-administration. At 14-weeks of age, a significant increase in plasma FITC-dextran was detected in VKE mice, hinting at the enhanced gut permeability due to a defective intestinal epithelial barrier (Fig. 4.3). Notably, subclinical age-matched VKA mice did not exhibit any permeability changes, implying that the severe inflammation and intestinal barrier defect observed in VKE mice are due to higher Kaiso expression.

Loss of the Kaiso binding partner and E-cadherin co-factor, $p120^{ctn}$, results in spontaneous inflammation in mice (Smalley-Freed *et al.*, 2010, Smalley-Freed *et al.*, 2011). Since our group and others recently showed that Kaiso-depletion causes de-repression of epithelial proteins such as E-cadherin and ZO-1 in breast and prostate cancer cell lines (Bassey-Archibong *et al.*, 2016, Jones *et al.*, 2012), we postulated that Kaiso overexpression may modulate adhesion protein expression in the intestinal epithelium. To test this hypothesis, the expression and subellular localization of several cell adhesion proteins were compared in subclinical and diseased VKA mice relative to their NonTg counterparts, at 12- and 24-weeks of age, respectively. Using IHC, we first examined the expression and localization of tight junction proteins, ZO-1 and Claudin-2. In 12-week old NonTg mice, ZO-1 localized to the apical membrane in IECs of both the villus and crypt, and is visible as distinct punctae between neighboring cells in the villus (Fig. 4.4 A). Subclinical 12-week old VKA mice still exhibited apically localized ZO-1 in both the villus and crypt, but distinct ZO-1 punctae between neighboring villus cells were not observed (Fig. 4.4 A). At 24-weeks of age, NonTg mice exhibited more intense

staining of apically localized ZO-1. Intriguingly, by 24-weeks of age, VKA mice exhibited moderately enhanced ZO-1 localization at the apical membrane compared to age-matched NonTg siblings (Fig. 4.4 A).

We next examined the subcellular localization of the pore-forming claudin, Claudin-2 by IHC. Apically localized Claudin-2 was detected primarily in the crypts of both NonTg and VKA mice at 12- and 24-weeks of age (Fig. 4.4 B). While localization of Claudin-2 appeared relatively unchanged in the epithelium of 12-week old VKA mice compared to age-matched NonTg mice, the crypts of 24-week old VKA mice exhibited slightly enhanced Claudin-2 at the apical membrane compared to their age-matched NonTg counterparts (Fig. 4.4B).

IHC was also performed to assess the subcellular localization of E-cadherin, $p120^{ctn}$ and β-catenin. All three adherens junction proteins localized to the lateral membrane in both genotypes, both prior to (12-weeks) and after (24-weeks) inflammation (Fig. 4.5). No detectable change in $p120^{ctn}$ or β-catenin localization was observed between VKA and NonTg mice at either time point, in either the villi or crypts (Fig 4.5 B, C). However, 12-week old VKA mice displayed reduced E-cadherin at the lateral cell membrane in both villi and crypts compared to age-matched NonTg littermates (Fig. 4.5 A). A similar trend was observed in the villi, but not the crypts, of 24-week old VKA mice relative to their NonTg counterparts. Since only E-cadherin localization was perturbed prior to measurable inflammation (i.e. in 12-week old mice), these data suggest that reduced E-cadherin at the lateral membrane may contribute to subsequent intestinal inflammation as mice age.

FIGURE 4.4 Comparison of tight junction-associated proteins in subclinical and diseased *Kaiso*^{Tg} mice. **(A)** ZO-1 localizes to the apical surface in both NonTg and VKA mice at both time points, however, ZO-1 punctae present in the villi of 12-week old NonTg mice is absent in age-matched VKA mice. No change in ZO-1 subcellular localization is observed at 24-week of age, however slightly enhanced ZO-1 staining was observed in the villi of 24-week old VKA mice. **(B)** Diseased VKA mice exhibit enhanced apical Claudin-2 localization in the crypts compared to age-matched NonTg mice. No change in Claudin-2 localization was observed in 12-week old VKA mice relative to their NonTg counterparts.

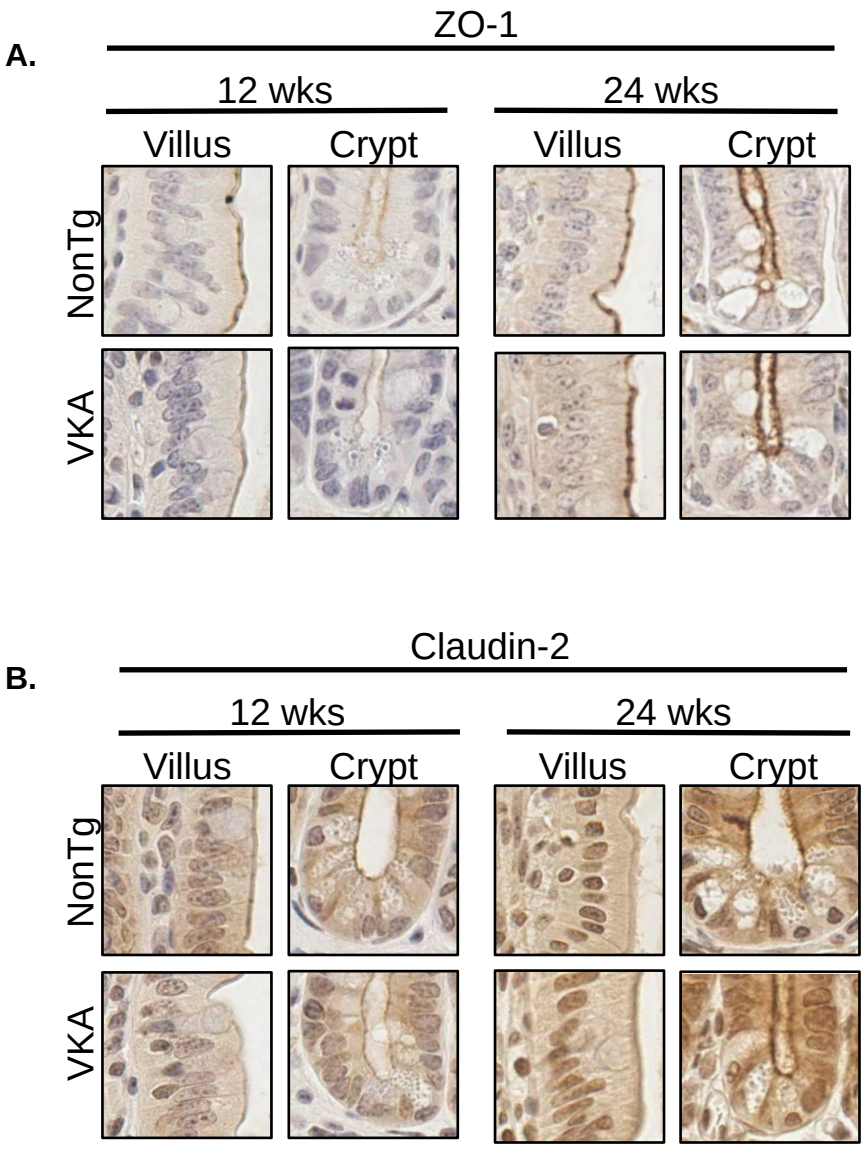
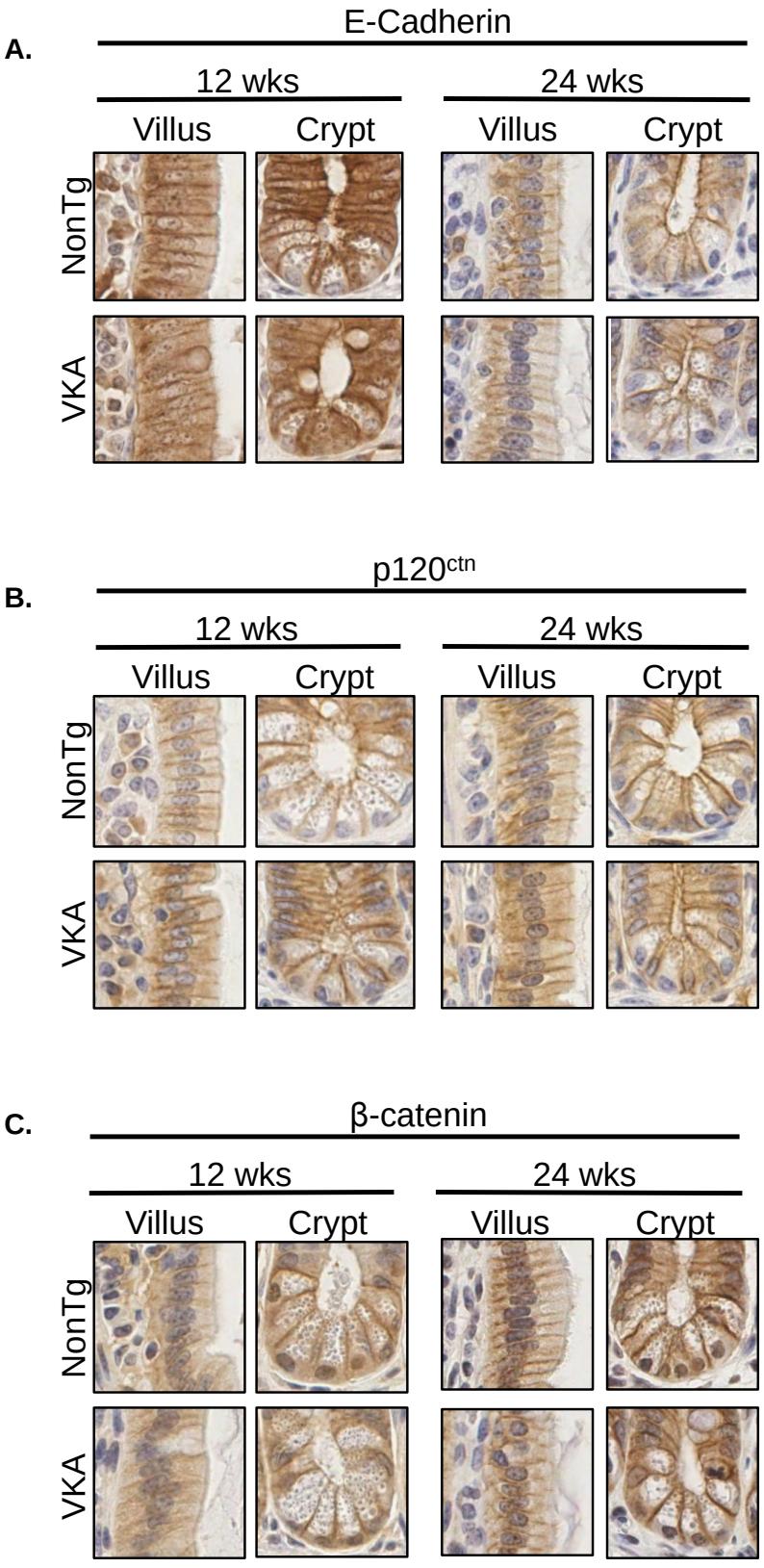

A.
ZO-1
12 wks
24 wks
Villus
Crypt
Villus
Crypt
NonTg
VKA
B.
Claudin-2
12 wks
24 wks
Villus
Crypt
Villus
Crypt
NonTg
VKA

FIGURE 4.5 Comparison of adherens junction-associated proteins in subclinical and diseased *Kaiso*^{Tg} mice. IHC of **(A)** E-cadherin, **(B)** p120^{ctn} and **(C)** β-catenin in 12- and 24-week old mice. **(A)** 12- and 24-week old *Kaiso*^{Tg} mice display a modest reduction in laterally-localized E-cadherin in both villi and crypts, compared to age-matched NonTg mice. **(B, C)** Kaiso overexpression did not alter the expression or subcellular localization of either p120^{ctn} or β-catenin at either time point.


A.
E-Cadherin
12 wks
24 wks
Villus
Crypt
Villus
Crypt
NonTg
VKA
B.
p120ctn
12 wks
24 wks
Villus
Crypt
Villus
Crypt
NonTg
VKA
C.
β-catenin
12 wks
24 wks
Villus
Crypt
Villus
Crypt
NonTg
VKA

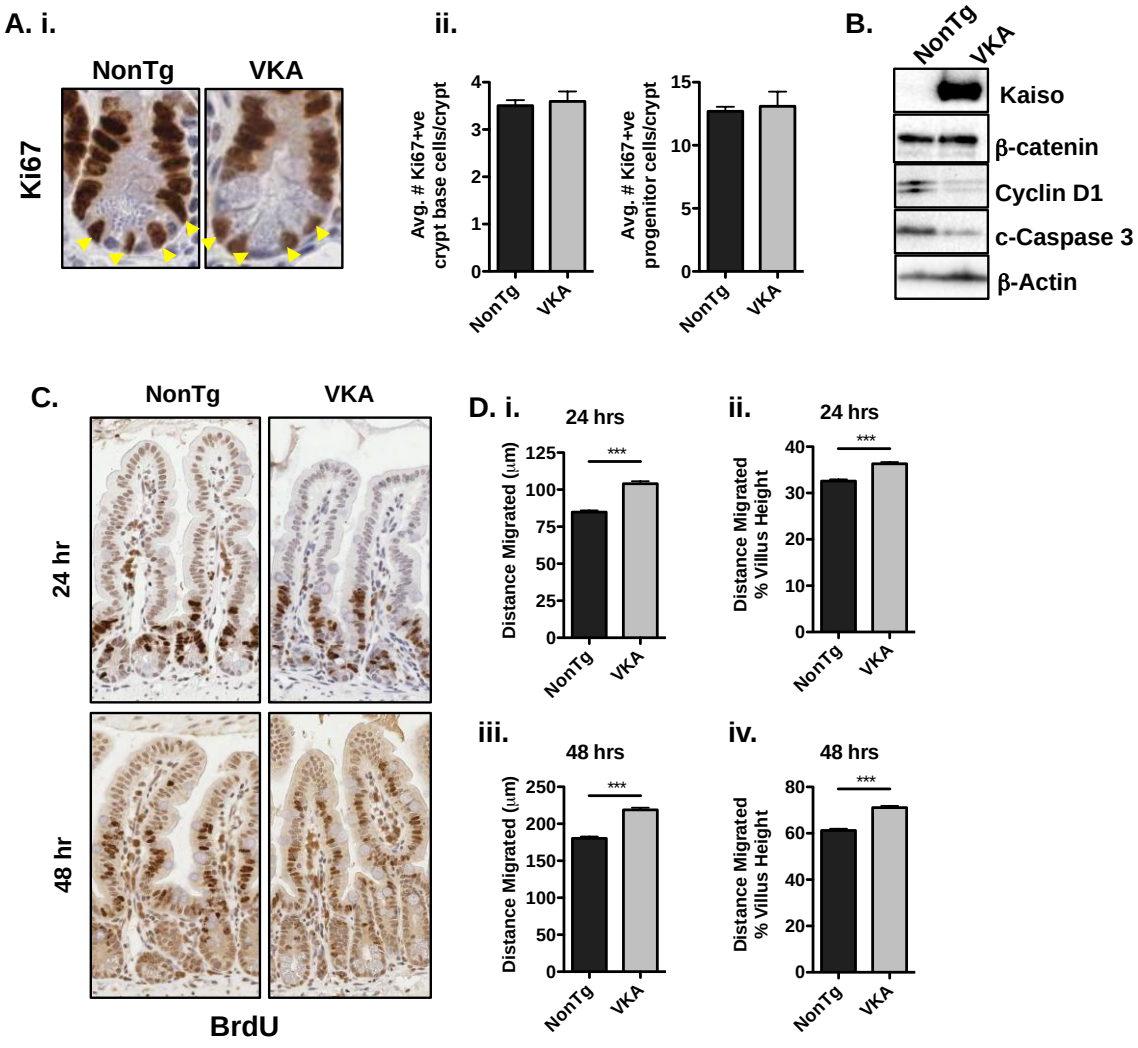
4.3.5 Kaiso overexpression results in altered intestinal epithelial cell repair processes prior to inflammation onset.

Given our recent finding that Notch signalling is impaired by Kaiso overexpression **(Chapter 3, Robinson et al., 2017b)**, coupled with previous studies linking Notch signalling inhibition to defective intestinal renewal and subsequent neutrophil-specific inflammation (Obata *et al.*, 2012), we were prompted to investigate whether intestinal repair mechanisms were altered in subclinical VKA mice. Intestinal renewal processes is achieved by the delicate balance between generation of new cells by proliferation, and their migration up the crypt-villus axis, followed by their subsequent apoptosis once cells reach the villus tip (Pastorelli *et al.*, 2013). We therefore examined proliferation in 12-week old VKA and NonTg mice as a first step in determining whether the regenerative capacity of the intestinal epithelium was perturbed in subclinical VKA mice. To this end, intestines were stained for the proliferation marker, Ki67, by IHC. Positively-labelled crypt base cells, were quantified separately from the total number of Ki67 positive cells. However, Kaiso overexpression did not result in a change in the number of Ki67-positive crypt base, or progenitor cells (Fig. 4.6 A). We next compared the protein expression levels of cell proliferation and apoptosis markers from isolated IECs of 12-week old NonTg and VKE or VKA mice. Consistent with previous findings (Chaudhary *et al.*, 2013, Dai *et al.*, 2011, Donaldson *et al.*, 2012, Jiang *et al.*, 2012), 12-week old VKA mice displayed diminished Cyclin D1 protein expression relative to their NonTg siblings (Fig. 4.6 B). However this is unlikely due to changes in canonical Wnt signalling, since the Wnt effector, β-catenin was unchanged in 12-week old VKA mice. Examination of the apoptotic marker, cleaved Caspase 3 (c-Caspase 3) revealed reduced expression in

VKA mice prior to inflammation onset (Fig 4.6 B). While the Ki67 index was unchanged by Kaiso-overexpression, the finding that subclinical VKA mice exhibited altered Cyclin D1 and c-Caspase 3 expression indicate abnormal intestinal renewal processes in 12-week old VKA mice, independently of Wnt/β-catenin signalling.

Next, the migratory capacity of the epithelium of 12-week old VKA mice was assessed using a BrdU migration assay. To ascertain whether IEC migration was affected prior to inflammation onset (which is detected by 24 weeks of age), subclinical 12-week old VKA and age-matched NonTg mice were intraperitoneally injected with BrdU and euthanized 24- and 48-hours post-injection. Since differentiated IECs do not proliferate, the location of BrdU-retaining cells along the crypt-villus axis has been used as a measure of cell migration (Fevr *et al.*, 2007). The distance travelled by BrdU-positive cells was quantified from ~ 300 villi from 3 mice/genotype (Fig. 4.6 C). Surprisingly, by 24-hours post-injection, we observed significant increase in the migration of BrdU-retaining VKA intestinal cells (104.0 ± 1.66 μm) compared to NonTg intestinal cells (84.89 ± 1.14 μm, Fig. 4.6 D). A similar trend was observed at the 48-hour time-point, as BrdU-retaining VKA intestinal cells had migrated significantly further (218.9 ± 2.97 μm) compared to those of their NonTg siblings (180.3 ± 2.60 μm, Fig. 4.6 D). Altogether, these data demonstrate that the regenerative capacity of the *Kaiso*^{Tg} epithelium is perturbed prior to measurable signs of inflammation. Specifically, Kaiso overexpression slows overall proliferation and apoptosis, but enhances IEC migration, which together may ultimately affect the integrity and function of the intestinal epithelium.

FIGURE 4.6 Intestinal epithelial cell repair mechanisms are altered prior to the onset of inflammation. (A) VKA mice do not demonstrate a change in number of Ki67-positive cells **(B)** Proliferation marker Cyclin D1 and apoptotic marker cleaved (c-) Caspase 3 are both decreased in VKA mice, while β-catenin levels were unchanged. **(C)** VKA mice show increased migration of epithelial cells up the crypt-villus axis relative to their NonTg counterparts. Yellow arrowheads denote crypt base cells – presumably Lgr5 positive crypt base columnar stem cells.


A. i.
NonTg
VKA
Ki67
ii.
Avg. # Ki67+ve crypt base cells/crypt
Avg. # Ki67+ve progenitor cells/crypt
NonTg
VKA
B.
NonTg
VKA
Kaiso
β-catenin
Cyclin D1
c-Caspase 3
β-Actin
C.
NonTg
VKA
24 hr
48 hr
BrdU
D. i.
24 hrs
Distance Migrated (μm)
***
ii.
24 hrs
Distance Migrated % Villus Height
***
iii.
48 hrs
Distance Migrated (μm)
***
iv.
48 hrs
Distance Migrated % Villus Height
***

4.4 DISCUSSION

We previously demonstrated that intestinal-specific overexpression of the transcription factor Kaiso drives a chronic inflammatory phenotype in mice (Chaudhary *et al.*, 2013, Pierre *et al.*, 2015b), hinting that Kaiso may be important in IBD pathogenesis. The observation that Kaiso expression is elevated in intestinal tissues of patients with Crohn's disease (Fig. 4.1) supported the hypothesis that Kaiso potentiates inflammation and IBD disease progression, and necessitated a more detailed characterization of the mechanism(s) contributing to Kaiso-induced inflammatory phenotype.

Cell-cell adhesion is crucial to preventing the translocation of luminal antigens across the intestinal barrier, and several studies have attributed intestinal inflammation to a loss of apical junctional proteins, such as E-cadherin, reviewed in (Mehta *et al.*, 2015), $p120^{ctn}$ (Smalley-Freed *et al.*, 2010, Smalley-Freed *et al.*, 2011) and ZO-1 (Poritz *et al.*, 2007). We previously reported enhanced nuclear $p120^{ctn}$localization in 1 year-old VKA mice (Chaudhary *et al.*, 2013), which was consistent with other studies demonstrating intestinal inflammation in $p120^{ctn}$ null tissues (Smalley-Freed *et al.*, 2010, Smalley-Freed *et al.*, 2011). However, the finding that 12- and 24-week old mice did not exhibit a shift in $p120^{ctn}$ localization (Fig. 4.5) suggests that altered localization of $p120^{ctn}$ develops over time. However, consistent with previous reports demonstrating Kaiso-mediated E-cadherin inhibition (Bassey-Archibong *et al.*, 2016, Jones *et al.*, 2014b, Jones *et al.*, 2012), we observed diminished E-cadherin localization at the lateral membrane of VKA mice. Given the essential role of E-cadherin in maintaining intestinal epithelial barrier

integrity (Mehta *et al.*, 2015), our findings indicate that VKA mice possess a weaker epithelial barrier than their NonTg littermates.

In addition to reduced localization of E-cadherin at the lateral membrane, *Kaiso*^{Tg} mice exhibited enhanced apical membrane staining of pore-forming Claudin-2 upon inflammation (Fig. 4.4). Increased Claudin-2 expression has been reported in both CD and UC and has been postulated to play a role in IBD-associated diarrhea (Luettig *et al.*, 2015). Thus, it is possible that increased Claudin-2 may also play a role in Kaiso-driven inflammation. However, given that Claudin-2 only permits translocation of small molecules (radii $\leq$ 4 Å), the observed permeability to 4kDa FITC-dextran in inflamed *Kaiso*^{Tg} intestines (Fig. 4.3) is likely not due to the enhanced membrane localization of Claudin-2; instead, it is possible that persistent neutrophil recruitment in *Kaiso*^{Tg} mice may play a role.

While neutrophil recruitment into the intestine plays a protective role in intestinal homeostasis, continued neutrophil recruitment and migration across the epithelial barrier has been shown to contribute to the pathogenesis of colitis (Brazil *et al.*, 2013, Kucharzik *et al.*, 2001). Neutrophil-mediated lesions, such as crypt abscesses, have also been postulated to contribute to intestinal leakiness, as neutrophil transmigration has been shown to create gaps in the epithelial lining (Kucharzik *et al.*, 2001, Nusrat *et al.*, 1997). The observed dose-dependent increase in MPO activity, enhanced transcript levels of the neutrophil chemoattractant, *MIP-2* (Fig. 4.2), and the formation of crypt abscesses (Fig 4.1) in diseased *Kaiso*^{Tg} mice support the notion that pathogenic neutrophil recruitment contributes to inflammation caused by Kaiso overexpression.

The finding that subclinical 12-week old VKA mice exhibited altered IEC migration and proliferation (Fig. 4.6) hints that misregulated intestinal repair mechanisms may play a role in predisposing *Kaiso*^{Tg} mice to subsequent inflammation. While some studies suggest that proliferation in the crypt propels IEC migration (Parker *et al.*, 2017), others indicate that proliferation and migration occur independently, and that active collective cell migration drives the movement of cells up the crypt-villus axis, reviewed in (Iizuka & Konno 2011, Sturm & Dignass 2008). Our findings are consistent with the latter hypothesis, which supports uncoupled intestinal proliferation and migration. The accelerated IEC migration in *Kaiso*^{Tg} mice may be explained by the recent finding that Kaiso promotes expression of TGFβR-I and -II (Bassey-Archibong *et al.,* 2016), which are well known for their pro-migratory roles (Moustakas & Heldin 2016), and/or via the observed reduction in E-cadherin at the lateral membrane (Fig. 4.5).

Altered renewal processes of *Kaiso*^{Tg} IECs may be due to Kaiso's regulation of Cyclin D1 (Dai *et al.,* 2011, Donaldson *et al.,* 2012, Jiang *et al.,* 2012, Park *et al.*, 2005), p53 (Bassey-Archibong *et al.*, 2017b, Koh *et al.,* 2015, Koh *et al.*, 2014), and/or the Notch signalling pathway (Robinson *et al.,* 2017b), which is essential for ISC proliferation (Demitrack & Samuelson 2016). Regardless of the mechanism(s) responsible for the abnormal repair processes, our findings raise the possibility that IECs migrate more rapidly in *Kaiso*^{Tg} mice than can be replaced by proliferation.

In conclusion, we report that Kaiso-induced intestinal inflammation involves perturbation of intestinal epithelial cell integrity and function, dysregulation of which leads to a chronic inflammatory phenotype. We propose a working model of Kaiso-

mediated inflammation, whereby reduced membrane localization of E-cadherin, pathogenic neutrophil recruitment, and abnormal epithelial repair processes render the epithelium susceptible to inflammation. This is further exacerbated by altered membrane expression of Claudin-2 as mice age, and together these perturbations ultimately contribute to chronic inflammation in the $Kaiso^{Tg}$ intestine.

CHAPTER 5 KAISO & ZNF131: A TUG OF WAR?

Elucidating the roles of Kaiso and Znf131 on Cyclin D1 regulation in intestinal cells

PREFACE

This chapter describes the work that resulted in the following manuscript, which was published on September 4th 2017 in *Biomedical and Biophysical Research Communications*. The manuscript has been re-formatted to maintain consistency with other chapters. Significant changes include omission of experimental methods and reference list, inclusion of the Pearson correlation scatter plot in Figure 4 (which had been omitted in the published article), and re-formatting of the in-text citations.

Shaiya C. Robinson, Nickett S. Donaldson, Anna Dvorkin-Gheva, Joseph Longo, Lloyd He, and Juliet M. Daniel. 2017. **The POZ-ZF transcription factor Znf131 is implicated as a regulator of Kaiso-mediated biological processes.** *Biochemical and Biophysical Research Communications.* 2017. pii: S0006-291X(17)31765-5. doi: 10.1016/j.bbrc.2017.09.007.

***Summary*:** In light of our previous finding that Kaiso and Znf131 interact via their POZ domains, we posited that Znf131 would co-regulate Kaiso target genes. In this work, we demonstrated that Znf131 indirectly associates with several Kaiso target genes (i.e. *ID2, MTA2, MMP7, WNT11* and *CCND1*) at the KBS. We also found that Znf131 promotes the expression of Cyclin D1, which is attenuated upon Kaiso overexpression. Notably, Kaiso appears to be indirectly regulating Znf131 expression itself, as it does not associate with the

KBS site in the proximal *ZNF131* promoter. However, the presence of three E-box sequences in the *ZNF131* promoter raises the possibility that Kaiso inhibits Znf131 via its regulation of c-myc. Finally, we found that Kaiso and Znf131 expression levels are elevated in colorectal tumours, and both proteins positively correlate with CRC. These findings led to the working hypothesis that Znf131 and Kaiso interact to regulate *CCND1* and other Kaiso-mediated processes.

Author Contributions: Dr. Nickett S. Donaldson pioneered the Znf131 studies during her tenure as a Ph.D. candidate, and initiated many of the experiments that lead to this manuscript (N.S. Donaldson, Ph.D. thesis). She performed the ChIP in Fig. 5.1A and the EMSA in Fig. 5.1B. Joseph Longo performed IHC of Znf131 in Fig. 5.2A, showing Znf131 expression in the intestinal crypts of NonTg mice, which is absent in $Kaiso^{Tg}$ mice. Dr. Anna Dvorkin-Gheva performed the computational analyses in Fig. 5.4, which show a positive correlation of Kaiso and Znf131 in colorectal cancer. I performed all other experiments (Figs 5.1 C-F; 5.2 B-E; and 5.3) and analyzed the data.

In addition to the experiments outlined above, I also wrote the manuscript and assembled all the figures therein. Dr. Daniel, Dr. Donaldson and myself conceptualized the experiments, and Dr. Daniel and I contributed equally to the critical assessment and revisions of the manuscript.

5.1 ABSTRACT

Znf131 belongs to the family of POZ-ZF transcription factors, but, in contrast to most other characterized POZ-ZF proteins that function as transcriptional repressors, Znf131 acts as a transcriptional activator. Znf131 heterodimerizes with the POZ-ZF protein Kaiso, which itself represses a subset of canonical Wnt target genes, including the cell cycle regulator Cyclin D1. Herein, we report a possible role for Znf131 in Kaiso-mediated processes. Notably, we found that Znf131 associates with several Kaiso target gene promoters, including that of *CCND1*. ChIP analysis revealed that Znf131 indirectly associates with the *CCND1* promoter in HCT116 and MCF7 cells via a region that encompasses the previously characterized +69 Kaiso Binding Site, hinting that the Znf131/Kaiso heterodimer may co-regulate Cyclin D1 expression. We also demonstrate that Kaiso inhibits Znf131 expression, raising the possibility that Kaiso and Znf131 act to fine-tune target gene expression. Together, our findings implicate Znf131 as a co-regulator of Kaiso-mediated biological processes.

5.2 INTRODUCTION

The **Po**x virus and **z**inc finger (POZ) **z**inc **f**inger (ZF) family of transcription factors is a rapidly growing family of proteins that are characterized by an N-terminal POZ domain, which mediates protein-protein interactions, and a C-terminal DNA-binding domain comprised of multiple zinc fingers (Kelly & Daniel 2006). While best known for their roles in vertebrate development, mounting evidence demonstrates that POZ-ZF proteins also play crucial roles in tumourigenesis (Kelly & Daniel 2006). For example, the well-characterized POZ-ZF proteins PLZF and BCL-6 are implicated in promyelocytic leukemia and B-cell lymphoma, respectively (Chang *et al.*, 1996, Chen *et al.*, 1994), while the more recently characterized POZ-ZF protein Kaiso is linked to several cancers including pancreatic (Jones *et al.*, 2016), colon (Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006), prostate (Jones *et al.*, 2012) and breast (Bassey-Archibong *et al.*, 2017a, Bassey-Archibong *et al.*, 2016, Vermeulen *et al.*, 2012). Kaiso was initially identified as a binding partner of the Armadillo protein and Src kinase substrate $p120^{ctn}$ (Daniel & Reynolds 1999, Kim *et al.*, 2002), and like most POZ-ZF proteins, Kaiso acts primarily as a transcriptional repressor (Barrett *et al.*, 2012, Donaldson *et al.*, 2012, Filion *et al.*, 2006, Lopes *et al.*, 2008, Pierre *et al.*, 2015a, Ruzov *et al.*, 2004, Spring *et al.*, 2005). During our characterization of Kaiso, we identified the minimally characterized POZ-ZF transcription factor Znf131 as a Kaiso interaction partner (Donaldson *et al.*, 2010, Trappe *et al.*, 2002).

In vitro, Znf131 recognizes and binds the **Z**nf131 **B**inding **E**lement (ZBE), a 12-bp palindromic sequence that is separated by a variable number of intervening

nucleotides (GTCGCR-N_X-YGCGAC; R-purine, Y-pyrimidine, N-any nucleotide). In contrast to most characterized POZ-ZF transcription factors, artificial promoter-reporter assays revealed that Znf131 functions as a transcriptional activator. Notably, we have previously shown that Znf131's transcriptional activity can be attenuated by Kaiso co-expression (Donaldson *et al.*, 2010). To-date, no *bona fide* Znf131 target genes have been identified. However, since Znf131 and Kaiso interact via their POZ domains (Donaldson *et al.*, 2010), we hypothesized that Znf131 may co-regulate Kaiso target genes, and thus play a role in Kaiso-mediated biological processes. Herein, we report that Znf131 associates with a subset of Kaiso target genes at the **K**aiso **B**inding **S**ite (KBS). We also found that ectopic Znf131 expression activated a minimal Cyclin D1 (*CCND1)* promoter that we previously showed was repressed by Kaiso (Donaldson *et al.*, 2012). Interestingly, Znf131-mediated activation of the *CCND1* promoter was attenuated upon Kaiso co-expression in a dose-dependent manner. Additionally we report an inverse correlation between Znf131 and Kaiso expression in intestinal cells, but a direct correlation in **c**olo**r**ectal **c**ancer (CRC) tumours. Together, our findings are the first to implicate Znf131 in the co-regulation of Kaiso target genes, suggesting that Znf131 imposes upon Kaiso-mediated homeostasis.

5.3 RESULTS

5.3.1 Znf131 associates with several Kaiso target genes, including CCND1.

The finding that Znf131 and Kaiso interact via their POZ domains (Donaldson *et al.*, 2010) prompted us to examine whether Znf131 might also associate with, and co-

regulate, Kaiso target genes. To this end, ChIP of HCT116 cell lysates was performed using Kaiso- and Znf131-specific antibodies. We found that both Kaiso and Znf131 co-occupied the *MTA2*, *ID2*, *MMP7*, *WNT11* and *CCND1* promoters, all of which have been implicated in tumourigenesis and/or Wnt signaling (Gialeli *et al.*, 2011, Kumar & Wang 2016, Musgrove *et al.*, 2011, Nair *et al.*, 2014, Said *et al.*, 2014, Uysal-Onganer & Kypta 2012) (Fig. 5.1 A). These results suggest that Znf131 might synergize or antagonize Kaiso-mediated regulation of these target genes.

Of the Kaiso target genes assayed, Kaiso-mediated regulation of Cyclin D1 *(CCND1)* has been the most extensively characterized (Dai *et al.*, 2011, Donaldson *et al.*, 2012, Jiang *et al.*, 2012, Park *et al.*, 2005). Our group previously showed that Kaiso associated with the +69 *CCND1* KBS site using both methylation-dependent and sequence-specific mechanisms (Donaldson *et al.*, 2012). Thus to determine whether Znf131 associated specifically with the +69 *CCND1* KBS, EMSA was performed using purified GST-Znf131 proteins incubated with either non-methylated or methylated oligos corresponding to this region. As expected (Donaldson *et al.*, 2012), full-length Kaiso and the ΔPOZ- and ZF-Kaiso truncated proteins bound to the +69 *CCND1* KBS oligo in a methylation-dependent manner (Fig. 5.1 B). However, the GST-Znf131 proteins did not bind either the methylated or non-methylated +69 *CCND1* KBS oligos (Fig. 5.1 B), indicating that Znf131 does not directly associate with this region.

Since Znf131 did not appear to bind directly to *CCND1*-derived oligos *in vitro*, we postulated that Znf131 was associating indirectly with the *CCND1* promoter via Kaiso. Thus we performed ChIP of the +69 *CCND1* KBS from stable Kaiso-depleted (sh-

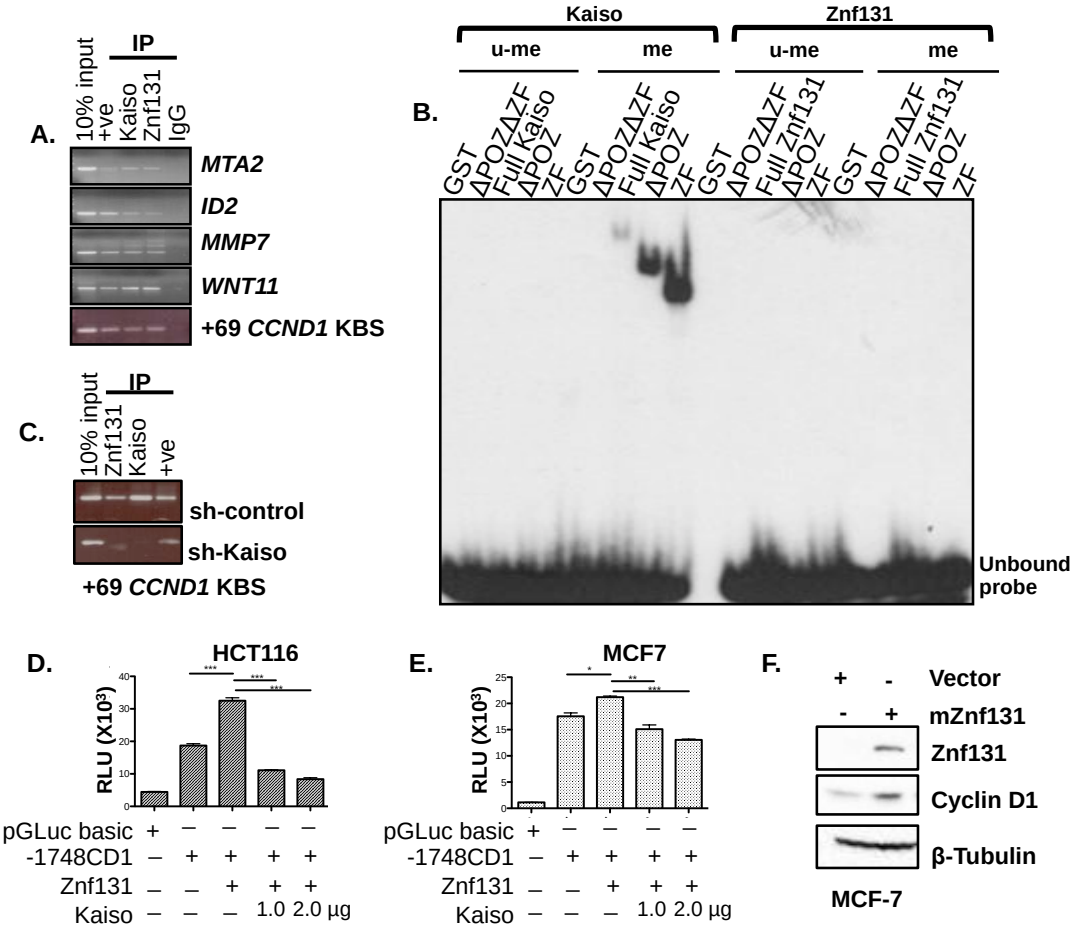
Kaiso) or control (sh-scrambled) HCT116 cells using Kaiso- or Znf131-specific antibodies. Consistent with our hypothesis, Znf131 only associated with the endogenous *CCND1* promoter in control HCT116 cells that expressed Kaiso, but not in stable Kaiso-depleted HCT116 cells (Fig. 5.1 C). The faint band observed in the Znf131 IP lane is non-specific DNA that is smaller than the expected *CCND1* promoter fragment. Together, these findings suggest that Znf131 indirectly associates with the *CCND1* promoter via its binding partner Kaiso.

5.3.2 Znf131 positively regulates Cyclin D1 expression.

Since Znf131 indirectly associates with the *CCND1* promoter, we next determined whether Znf131 could transcriptionally regulate a minimal *CCND1* promoter-reporter construct (pGLuc-*1748CD1*), previously shown to be negatively regulated by Kaiso (Donaldson *et al.*, 2012). To this end, MCF7 and HCT116 cells were co-transfected with pGLuc-*1748CD1,* pCS2+ MT-mZnf131, and increasing amounts of pcDNA3-Kaiso. Znf131 expression transactivated the minimal -*1748CD1* promoter, which was attenuated upon co-expression of Kaiso (Fig. 5.1 D, E). Notably, this promoter construct lacks the Znf131 binding element (ZBE; GTCGCR-Nx-YGCGAC), further supporting the hypothesis that Znf131 activates Cyclin D1 via its association with Kaiso. This finding also suggests that heterodimerization between Znf131 and Kaiso may alter the transcriptional activity of both proteins.

Given that Znf131 activated the *CCND1* promoter-reporter construct, we next ascertained the effects of Znf131 on Cyclin D1 protein expression levels. Since *Cyclin D1* is a target gene of the Wnt/β-catenin signaling pathway, only MCF7 cells were used

FIGURE 5.1 Kaiso expression attenuates Znf131 regulation of a minimal *CCND1* promoter in HCT116 cells. (A) ChIP-PCR analysis revealed that Kaiso and Znf131 associate with various Kaiso target genes in HCT116 cells, including the +69 KBS site of the *CCND1* promoter. **(B)** EMSA analyses revealed that purified Znf131 does not associate with the +69 *CCND1* KBS site, irrespective of methylation status (me – methylated; u-me – unmethylated). **(C)** ChIP-PCR analysis of stable HCT116 sh-control and sh-Kaiso cells revealed that Znf131 only associates with the +69 KBS in the presence of Kaiso. Luciferase assays in HCT116 **(D)** and MCF7 **(E)** cells demonstrated that Znf131 activates a minimal *CCND1* promoter, while co-expression with Kaiso resulted in attenuation of Znf131-mediated transactivation. Error bars represent SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. RLU – Relative light units. **(F)** Transient Znf131 overexpression in synchronized MCF7 cells resulted in increased Cyclin D1 protein levels.


A.
IP
10% input
+ve
Kaiso
Znf131
IgG
MTA2
ID2
MMP7
WNT11
+69 CCND1 KBS
B.
Kaiso
Znf131
u-me
me
u-me
me
GST
ΔPOZΔZF
Full Kaiso
ΔPOZ
ZF
GST
ΔPOZΔZF
Full Kaiso
ΔPOZ
ZF
GST
ΔPOZΔZF
Full Znf131
ΔPOZ
ZF
GST
ΔPOZΔZF
Full Znf131
ΔPOZ
ZF
Unbound probe
C.
IP
10% input
Znf131
Kaiso
+ve
sh-control
sh-Kaiso
+69 CCND1 KBS
D.
HCT116
RLU (X10³)
pGLuc basic + – – – –
-1748CD1 – + + + +
Znf131 – – + + +
Kaiso – – – 1.0 2.0 µg
E.
MCF7
RLU (X10³)
pGLuc basic + – – – –
-1748CD1 – + + + +
Znf131 – – + + +
Kaiso – – – 1.0 2.0 µg
F.
+ - Vector
- + mZnf131
Znf131
Cyclin D1
β-Tubulin
MCF-7

for this experiment, as HCT116 cells exhibit constitutively active Wnt/β-catenin signaling (Ilyas *et al.,* 1997, Morin *et al.,* 1997). MCF7 cells were transiently transfected with pCS2+MT-mZnf131 and serum-starved for 24 hours post-transfection to synchronize the cells at G0. Release into the cell cycle was achieved upon addition of 10% serum, and protein was isolated after 6 hours. Western blot analysis showed increased Cyclin D1 protein levels in cells transfected with Znf131 compared to vector-transfected cells (Fig. 5.1 F). Collectively these data support a role for Znf131 in positively regulating Cyclin D1 expression.

5.3.3 Kaiso inhibits Znf131 expression in intestinal cells.

The antagonistic relationship between Kaiso and Znf131 raised the possibility that perhaps Kaiso inhibits Znf131 in a negative feedback loop. To address this, Znf131 expression was examined in our *Kaiso*^{Tg} mice, which constitutively overexpress Kaiso in the intestinal epithelium (Chaudhary *et al.,* 2013). Znf131 was localized at the base of intestinal crypts of non-transgenic (NonTg) mice, but was absent in the epithelium of *Kaiso*^{Tg} littermates (Fig. 5.2 A). This finding was supported by both western blot and qRT-PCR analyses of isolated intestinal epithelial cells, where Znf131 protein and mRNA expression were significantly reduced in *Kaiso*^{Tg} IECs (Fig. 5.2 B, C). Conversely, stable RNAi-mediated knockdown of Kaiso in HCT116 and HT29 cell lines revealed enhanced Znf131 protein and mRNA expression relative to control cell lines (Fig. 5.2 D, E).

The decreased Znf131 expression observed in Kaiso-overexpressing tissues led us to ascertain whether Kaiso might inhibit Znf131 expression by associating with the

ZNF131 gene promoter. Thus, a region spanning -1000 to +100 bp of the transcription start site was interrogated for KBS sequences and the presence of CpG dinucleotides. We identified a core KBS (CTGCCA) at position -222 bp (Fig. 5.3 A) and two CpG islands (CGI). Importantly, the -222 core KBS was positioned in close proximity to a CpG dinucleotide and CGI-2, hinting at the possibility that Kaiso might associate with this region in a methylation-dependent manner, akin to some of its other target genes (Bassey-Archibong *et al.*, 2016, Donaldson *et al.*, 2012). To determine whether Kaiso associated with this site, ChIP was performed on both HCT116 and HT29 cell lysates using the Kaiso-specific 6F monoclonal antibody (Daniel *et al.*, 2001), or with antibodies against Histone H3 or non-specific IgG, as positive and negative controls, respectively. PCR was performed using primers flanking the KBS and part of CGI-2. However, no Kaiso binding was observed at this locus in either cell line (Fig. 5.3 B). Together, these data indicate that although Kaiso and Znf131 expression are inversely correlated in intestinal cells, and while the *ZNF131* promoter possesses a core KBS, Kaiso's effects on Znf131 may be independent of *ZNF131* promoter regulation.

5.3.4 Znf131 is positively correlated with Kaiso in colon cancer.

Several studies have implicated Kaiso in various types of cancer, including colon cancer (Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006). To gain insight into the relationship between Kaiso (encoded by *ZBTB33*) and Znf131 in colon cancer, we examined the expression levels of both transcription factors in the publically available CRC dataset, GSE39582. Consistent with previous reports (Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006), *Kaiso* mRNA is elevated in CRC tumours relative to normal tissues ($p<0.005$;

FIGURE 5.2 Kaiso expression attenuates Znf131 expression in intestinal cells. Intestinal tissue and epithelial cells (IECs) were harvested from both NonTg and *Kaiso*^{Tg} mice, and analyzed by **(A)** IHC, **(B)** western blot and **(C)** qRT-PCR. Kaiso overexpression resulted in reduced Znf131 transcript and protein levels *in vivo*. **(D&E)** Stable Kaiso depletion in HCT116 and HT29 cells resulted in increased Znf131 protein and transcript levels, as determined by **(D)** western blot (gamma changes employed) and **(E)** qRT-PCR, respectively. Error bars represent SEM. *$p < 0.05$, **$p < 0.01$.

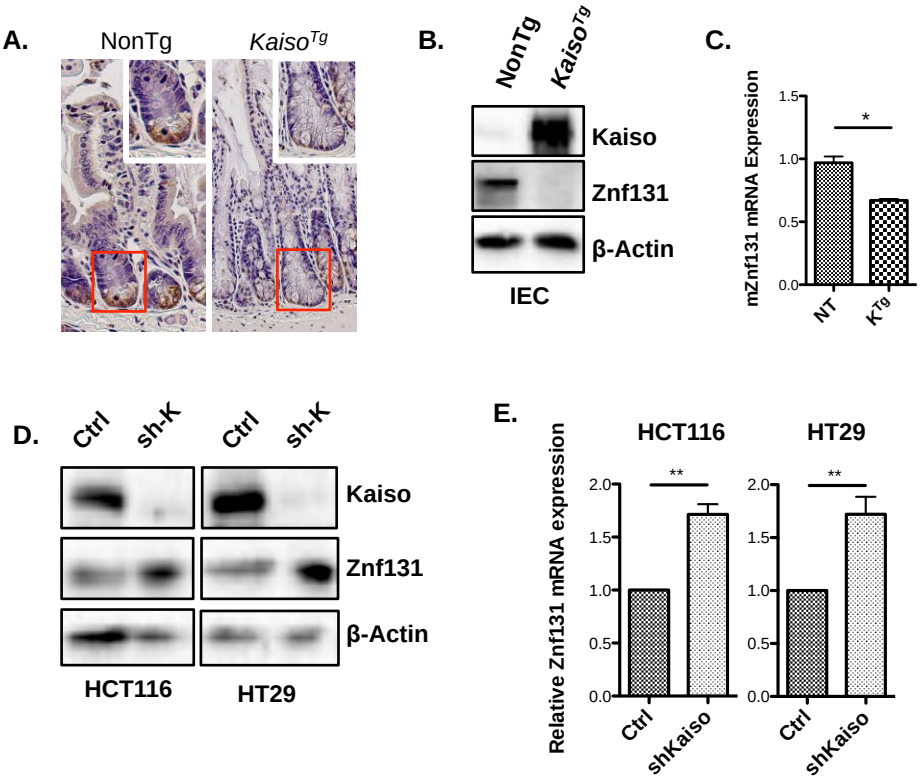

A.
NonTg
KaisoTg
B.
NonTg
KaisoTg
Kaiso
Znf131
β-Actin
IEC
C.
mZnf131 mRNA Expression
1.5
1.0
0.5
0.0
*
NT
KTg
D.
Ctrl
sh-K
Ctrl
sh-K
Kaiso
Znf131
β-Actin
HCT116
HT29
E.
HCT116
HT29
Relative Znf131 mRNA expression
2.0
1.5
1.0
0.5
0.0
**
**
Ctrl
shKaiso
Ctrl
shKaiso

Figure 5.3. Kaiso does not associate with the ZNF131 promoter in intestinal cells. **(A)** Schematic representation of the *ZNF131* gene promoter, which contains two CpG islands (CGIs) and several CpG dinucleotides (black vertical lines). Arrowheads denote region amplified by PCR. **(B)** ChIP-PCR of HCT116 and HT29 cells revealed that Kaiso does not associate with the core KBS in the *ZNF131* promoter.

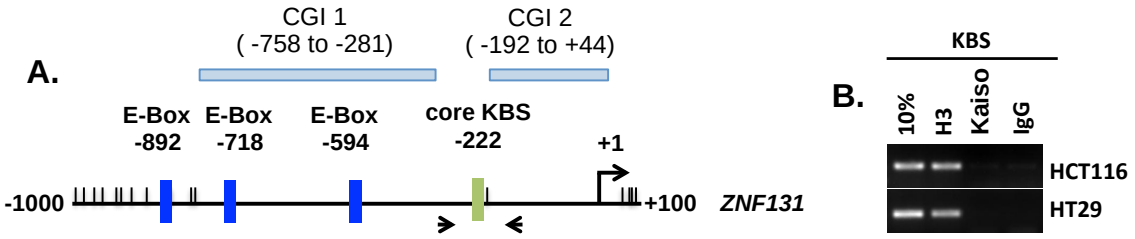

A.
CGI 1
(-758 to -281)
CGI 2
(-192 to +44)
E-Box
-892
E-Box
-718
E-Box
-594
core KBS
-222
+1
-1000
+100
ZNF131
B.
KBS
10%
H3
Kaiso
IgG
HCT116
HT29

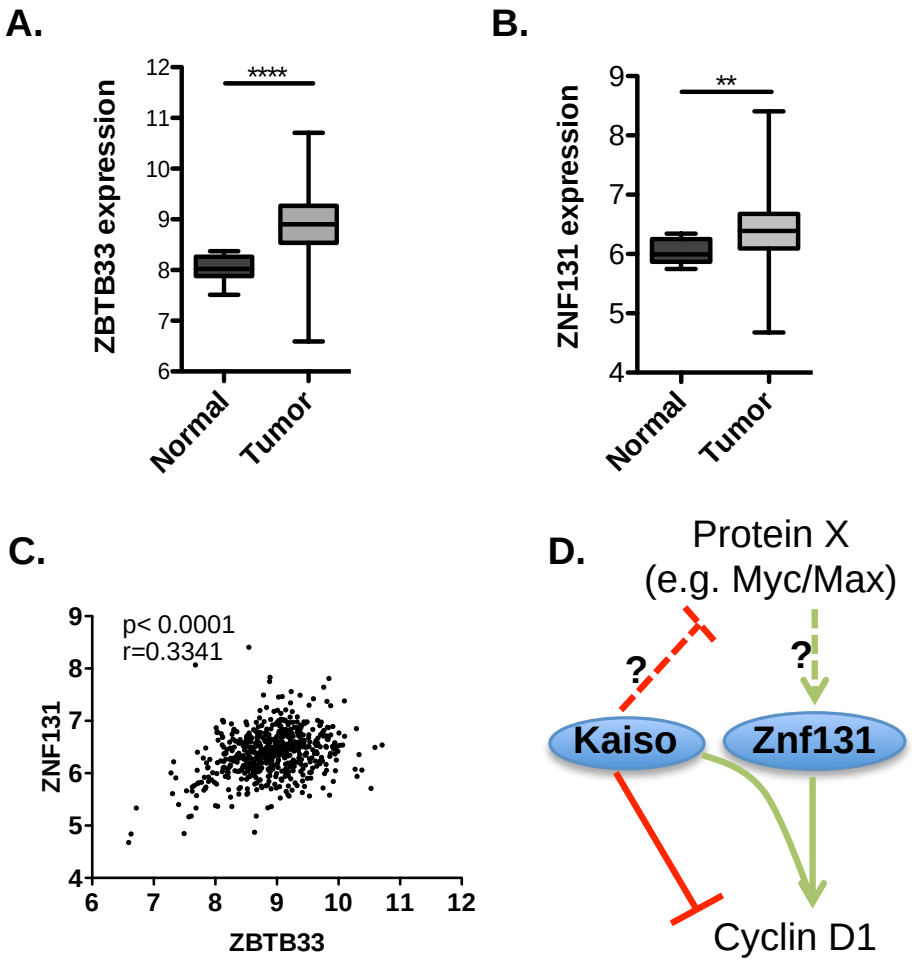
Fig. 5.4 A). Intriguingly, *Znf131* mRNA expression levels were also significantly elevated in CRC compared to normal intestinal tissues ($p<0.0005$; Fig. 5.4 B). In agreement with these findings, Pearson correlation analysis revealed a weak but significant positive correlation between *Kaiso* and *Znf131* in CRC (Fig. 5.4 C, $p<0.0001$). These data hint at a possible synergy between Kaiso and Znf131 in colorectal tumourigenesis.

5.4 DISCUSSION

In this study, we report that Znf131 associates with several Kaiso target genes, implicating it in Kaiso-mediated biological processes. Since Znf131 did not directly associate with core KBSs of the *CCND1* promoter (Fig. 5.1), we postulated that Znf131 might be recruited to the +69 core KBS via its physical association with Kaiso. Indeed, the finding that Znf131's association with the +69 KBS was abolished upon Kaiso depletion (Fig. 5.1) supports the notion that Znf131 indirectly binds to this site via Kaiso.

Depending on their protein-binding partner, transcription factors may either repress or activate target gene expression. Interestingly, in contrast to Kaiso, whose ectopic expression repressed a minimal *CCND1* promoter-reporter construct (Donaldson *et al.*, 2012), overexpression of Znf131 resulted in its transactivation. This was consistent with our previous report that Znf131 transactivated an artificial promoter-reporter construct, *4XZBE*-luciferase, but was attenuated by Kaiso overexpression (Donaldson *et al.*, 2010). Importantly, the *-1748CD1* and *4XZBE*-luciferase constructs lack ZBE (Fig. 5.1) and KBS sequences (Donaldson *et al.*, 2010), respectively. This suggests that

Figure 5.4. Kaiso and Znf131 are positively correlated in colon cancer. (A) Kaiso (*ZBTB33*) and **(B)** Znf131 (*ZNF131*) expression levels were analyzed in the publically available GSE39582 colon cancer dataset. Transcript levels for both *Znf131* and *Kaiso* were significantly increased in colon tumours relative to normal intestinal tissues. ** $p < 0.005$, *** $p < 0.0005$. **(D)** Working model depicting the hypothesized interaction between Kaiso and Znf131. Alone, Kaiso represses Cyclin D1 (Dai et al., 2011; Donaldson et al., 2010; Jiang et al., 2012; Park et al., 2005), but Kaiso/Znf131 heterocomplexes result in Cyclin D1 activation. Kaiso indirectly suppresses Znf131 expression by a currently unknown mechanism, which may function to prevent over-activation of Cyclin D1 by Kaiso/Znf131 complexes. **Figure has been modified by inclusion of the Pearson correlation analysis (panel 5.4 C).*


A.
ZBTB33 expression
****
Normal
Tumor
B.
ZNF131 expression
**
Normal
Tumor
C.
p< 0.0001
r=0.3341
ZNF131
ZBTB33
D.
Protein X
(e.g. Myc/Max)
?
?
Kaiso
Znf131
Cyclin D1

protein-protein interactions between Kaiso and Znf131 result in altered transcriptional activity via their respective DNA binding elements.

The finding that Kaiso and Znf131 expression were inversely correlated in intestinal cells suggested that the level of engagement between Kaiso and Znf131 tightly regulates target gene expression. Interestingly, we observed a significant positive correlation between both transcription factors in human colon tumours (Fig. 5.4). However, a positive correlation between Znf131 and Kaiso in CRC tumours is consistent with our hypothesis that a Kaiso/Znf131 protein complex positively regulates Cyclin D1 (Fig. 5.4 D), which would enhance CRC tumourigenesis.

While Kaiso did not associate with the -222 putative core KBS in the *ZNF131* promoter in CRC cell lines, the presence of 3 E-box sequences in the region analyzed (Fig. 5.3) raises the possibility that Znf131 is regulated by Myc – a proto-oncogene that is over-expressed in various cancers, including CRC (Lee *et al.*, 2015). Publically available ChIP-sequencing data from the ENCODE database revealed binding of Myc/Max complexes to the *ZNF131* promoter (Rosenbloom *et al.*, 2013), but full characterization of Znf131 as a putative Myc target gene is beyond the scope of this study. However given that Myc is highly regulated by various signaling pathways, including Wnt and Notch signaling (He *et al.*, 1998, Palomero *et al.*, 2006, Weng *et al.*, 2006), coupled with recent findings linking Kaiso to both pathways (Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006, Robinson *et al.*, 2017b, Zhao *et al.*, 2016), it is possible that Kaiso indirectly regulates Znf131 via either pathway.

Future studies are required to fully unravel the molecular mechanisms dictating Kaiso's regulation of Znf131. However, the findings presented here hint at a role for Znf131 in the regulation of Kaiso-mediated biological processes. This may also have implications for Kaiso-mediated pathologies, such as colon (Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006), breast (Bassey-Archibong *et al.*, 2016, Bassey-Archibong *et al.*, 2017b, Jones *et al.*, 2014b, Vermeulen *et al.*, 2012) and prostate cancer (Wang *et al.*, 2016), as well as chronic intestinal inflammation (Chaudhary *et al.*, 2013).

CHAPTER 6 DISCUSSION

Much of what is known about Kaiso in mammalian development has been elucidated in the last decade, and a growing number of these studies have interrogated Kaiso's role in intestinal homeostasis (Chaudhary *et al.*, 2013, Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006, Robinson *et al.*, 2017b, Shumskaya *et al.*, 2015). The Prokhortchouk group was the first to initiate murine *in vivo* studies using their novel Kaiso knockout mouse model (Prokhortchouk *et al.*, 2006). While *Kaiso*$^{-/y}$ mice did not develop any gross morphological defects, *Kaiso*$^{-/y}$*;Apc*$^{Min/+}$ mice formed fewer intestinal polyps, and lived significantly longer than their *Apc*$^{Min/+}$ counterparts (Prokhortchouk *et al.*, 2006). This was in contrast to studies in *Xenopus* and cultured cells (Dai *et al.*, 2011, Donaldson *et al.*, 2012, Jiang *et al.*, 2012, Park *et al.*, 2005, Spring *et al.*, 2005), as it suggested that Kaiso potentiates the canonical Wnt signalling pathway in the intestinal epithelium. However, our group subsequently corroborated the findings of Prokhortchouk *et al.*, when we found that intestinal-specific overexpression of Kaiso in *Apc*$^{Min/+}$ mice resulted in significantly reduced lifespan, and the formation of more, albeit smaller, intestinal polyps (Pierre *et al.*, 2015b).

While several studies have highlighted Kaiso as a critical regulator of the canonical Wnt signalling pathway, we were the first to implicate Kaiso as a pro-inflammatory-mediator and potential negative regulator of Notch signalling (Chaudhary *et al.*, 2013). Specifically, our characterization of 12-month old *Kaiso*^{Tg} mice revealed that intestinal-specific Kaiso-overexpression produced two striking phenotypes: 1) *Kaiso*^{Tg} mice exhibited increased intestinal inflammation, and 2) *Kaiso*^{Tg} intestines displayed an

increase in the number of secretory cell types, a hallmark phenotype of Notch signalling inhibition (Chaudhary *et al.,* 2013). Since initial characterizations of *Kaiso*^{Tg} were performed on already diseased 12 month-old mice, it was unclear when and how mice developed inflammation; whether the inflammation and secretory cell hyperplasia occurred together or independently; and how Kaiso regulated the Notch signalling pathway. The findings presented in this thesis addressed these outstanding questions and provided greater insight into Kaiso-mediated intestinal homeostasis.

6.1 Defining Kaiso's role in the Notch signalling pathway in intestinal cells.

6.1.1 Kaiso^{Tg} mice exhibit a modest increase in secretory cell types due to Notch pathway inhibition.

The reduced expression of Hes1 and Hes5 supported the hypothesis that Kaiso overexpression inhibits Notch signalling. However, since both Hes transcription factors were only marginally reduced in *Kaiso*^{Tg} mice, this suggested that Kaiso overexpression does not fully attenuate Notch signalling in the intestine – a notion supported by the relatively mild secretory cell phenotype in *Kaiso*^{Tg} mice compared to other mouse models of Notch inhibition. For example, *Rbp-J* deletion (van Es *et al.,* 2005, VanDussen *et al.,* 2012), combined deletion of *Notch1* and *-2* (Riccio *et al.,* 2008), *Hes1, -3,* and *-5* (Ueo *et al.,* 2012), and mice treated with the γ-secretase inhibitor, DBZ, (van Es *et al.,* 2005) have all resulted in a loss of proliferation and post-mitotic conversion of IECs into secretory cells, especially goblet cells. In contrast, *Kaiso*^{Tg} mice did not exhibit a complete loss of proliferation, as demonstrated by positive staining for the proliferation marker, Ki67, in the crypts (Chaudhary *et al.,* 2013, Pierre *et al.,* 2015b, and **Chapter 4**).

While 3 month-old *Kaiso*^{Tg} mice exhibited reduced *Notch1* expression, given that *Notch2* expression levels were not significantly changed in our mice **(Chapter 3, Robinson *et al.*, 2017b)**, and Notch1 and Notch2 function redundantly in the adult intestine (Riccio *et al.*, 2008), the observed secretory cell hyperplasia in *Kaiso*^{Tg}'s was likely not caused by *Notch1* inhibition alone. However, the secretory cell hyperplasia in 3 month-old *Kaiso*^{Tg} mice could be explained by the sole reduction in Dll-1 in *Kaiso*^{Tg} mice **(Chapter 3, Robinson *et al.*, 2017b)**, and is consistent with previous findings (Pellegrinet *et al.*, 2011, Stamataki *et al.*, 2011). In their 2011 study, Pellegrinet *et al.* found that while Dll-1 can fully compensate for the loss of Dll-4, Dll-4 can only partially compensate for Dll-1 loss – indicating that cell fate determination is governed primarily by Dll-1-mediated Notch pathway activation (Pellegrinet *et al.*, 2011). Indeed, while *Dll-1*$^{-/-}$ mice exhibited a modest increase in the number of secretory cells (Pellegrinet *et al.*, 2011, Stamataki *et al.*, 2011), *Dll-1/Dll-4* double knockout mice exhibited significant secretory cell hyperplasia, and a complete loss of Lgr5-positive ISCs, Ki67-positivity, and Hes1 immunoreactivity (Pellegrinet *et al.*, 2011). In agreement with the previously highlighted studies, *Kaiso*^{Tg} mice exhibited a relatively mild secretory cell phenotype and did not exhibit altered Dll-4 levels, suggesting that Dll-4 acts to partially compensate for Dll-1 loss in *Kaiso*^{Tg} mice.

6.1.2 A possible role for Kaiso in Jagged-1-mediated colon cancer progression?

An unexpected observation from this study was that Kaiso promoted rather than repressed the expression of the Notch ligand, Jagged-1, in the intestinal epithelium **(Chapter 3, Robinson *et al.*, 2017b**). Several groups have demonstrated that Jagged-1 is

a canonical Wnt target gene (Estrach *et al.,* 2006, Katoh 2006, Rodilla *et al.,* 2009), however canonical Wnt signalling was not significantly elevated in the epithelium of 3 month-old mice (**Chapter 4**). This finding, together with the observations that (i) stable Kaiso-depletion was associated with reduced Jagged-1 expression in SW480 cells, and (ii) Kaiso-associated with the *JAG1* promoter **(Chapter 3, Robinson *et al.,* 2017b)**, supports the notion that Jagged-1 induction in the intestinal epithelium was caused, at least in part, by Kaiso-mediated transcriptional activation.

Jagged-1 does not contribute to intestinal cell fate decisions *in vivo* (Pellegrinet *et al.*, 2011), however several studies have implicated a complex role for Jagged-1 in colon cancer progression (Dai *et al.,* 2014, Fender *et al.,* 2015, Gao *et al.,* 2011, Guilmeau *et al.,* 2010, Reedijk *et al.,* 2008, Sugiyama *et al.,* 2016). For example, Rodilla *et al.* observed elevated active Notch signalling in $Apc^{Min/+}$ tumours, which was sufficiently attenuated by single-copy loss of Jagged-1 – $Apc^{Min/+};Jag1^{+/\Delta}$ mice presented with smaller intestinal polyps, which also accompanied by reduced Hes1 expression (Rodilla *et al.,* 2009). The positive correlation between Kaiso and Jagged-1 expression in intestinal epithelium **(Chapter 3, Robinson *et al.,* 2017b)** prompts the question, is Kaiso involved in Jagged-1 mediated tumourigenesis?

We previously reported that $Kaiso^{Tg};Apc^{Min/+}$ mice exhibit increased polyps compared to $Apc^{Min/+}$ siblings, and factors such as elevated inflammation and Wnt signalling have been proposed to contribute to this phenotype (Pierre *et al.,* 2015b). However our finding that Kaiso promotes Jagged-1 in intestinal cells adds an additional layer of complexity to Kaiso-mediated tumourigenesis **(Chapter 3, Robinson *et al.,***

2017b). Similar to 3 month-old *Kaiso*^{Tg} mice, preliminary experiments of 3 month-old *Kaiso*^{Tg};*Apc*$^{Min/+}$ revealed an increase in the number of Jagged-1 positive cells in non-tumour tissue compared to *Apc*$^{Min/+}$ siblings (Appendix A1.1 A). Intriguingly however, the polyps of *Kaiso*^{Tg};*Apc*$^{Min/+}$ mice exhibited reduced Jagged-1, Hes1, and Hes5 staining compared to their *Apc*$^{Min/+}$ littermates (Appendix Fig. A1.1 B, C), which together hints at reduced Jagged-1-mediated Notch signalling in Kaiso-driven intestinal tumours. While we recognize that other factors, such as increased apoptosis, may contribute to the smaller polyp phenotype (Koh *et al.*, 2014, Pierre *et al.*, 2015b), it is possible that loss of Jagged-1 expression in *Kaiso*^{Tg};*Apc*$^{Min/+}$ polyps may also play a role. Still, an important outstanding issue for future studies would be characterization of the molecular mechanism(s) responsible for the paradoxical phenomenon of increased, but smaller, polyps in *Kaiso*^{Tg};*Apc*$^{Min/+}$ mice.

Given that Jagged-1 and Kaiso have independently been implicated in CRC progression (Fender *et al.*, 2015, Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006, Reedijk *et al.*, 2008, Rodilla *et al.*, 2009, Sugiyama *et al.*, 2016), and since Kaiso was found to promote Jagged-1 expression **(Chapter 3, Robinson *et al.*, 2017b)**, we interrogated the prognostic significance of the Kaiso/Jagged-1 interaction by examining the overall survival of patients with tumours exhibiting positive or inverse patterns of Kaiso and Jagged-1 expression. In spite of their respective pro-tumourigenic effects, no correlation in overall CRC survival was observed in patients exhibiting positive, or inverse patterns of Jagged-1 and Kaiso expression (Appendix Fig. A1.2), indicating that combined Kaiso/Jagged-1 expression is not a useful prognostic indicator. Nevertheless, it is still

possible that the Kaiso/Jagged-1 axis plays a role in CRC progression and warrants further investigation.

6.2 Mechanistic insight into Kaiso-induced intestinal inflammation.

6.2.1 Kaiso^{Tg} mice exhibit altered expression of E-cadherin, Claudin-2 and ZO-1 upon inflammation.

The observed reduction in E-cadherin at the lateral membrane of subclinical 12-week old mice implied that decreased E-cadherin may be a contributing factor to subsequent intestinal inflammation (**Chapter 4**). Indeed, several reports have demonstrated reduced E-cadherin expression in both UC and CD, reviewed in (Mehta *et al.*, 2015), which is strengthened by the identification of E-cadherin as an IBD-susceptibility locus (Barrett *et al.*, 2009, McGovern *et al.*, 2010, Muise *et al.*, 2009). Previous findings from our group and others have demonstrated that Kaiso directly associates with, and transcriptionally inhibits, the E-cadherin (*CDH1*) gene promoter (Bassey-Archibong *et al.*, 2016, Buck-Koehntop *et al.*, 2012, Jones *et al.*, 2014b, Jones *et al.*, 2012). Whether Kaiso's transcriptional effects on E-cadherin gene expression also extend to intestinal cells remains to be determined empirically. However, the inverse correlation between Kaiso and E-cadherin in VKA mice, hints that the reduction in E-cadherin may arise due to Kaiso's direct effects on the *CDH1* promoter.

In addition to diminished membrane-associated E-cadherin, VKA mice also exhibited increased apically localized Claudin-2 upon inflammation (i.e. at 24-weeks of age; **Chapter 4**). Claudin-2 is among the family of Claudin gatekeepers of paracellular permselectivity, which is defined as the selective permeability of molecules based on

electrical resistance, size and charge, reviewed in Anderson & Van Itallie 2009). A number of studies have reported that "leaky" epithelia exhibit increased Claudin-2 expression, and enhanced Claudin-2 has been described in both CD and UC, reviewed in (Landy *et al.*, 2016). The increased Claudin-2 in *Kaiso*^{Tg} mice would suggest increased cation flux across the intestinal epithelium, which would have significant implications for other IBD-associated phenotypes, such as loose stools/diarrhea and dehydration (Rosenthal *et al.*, 2010). These phenotypes were not examined in this thesis, however the use of *Kaiso*^{Tg} mice as a *bona fide* murine model of IBD warrants their examination.

The findings summarized and interpreted here provide insight behind the development of Kaiso-induced inflammation. While the data imply that *Kaiso*^{Tg} mice possess a compromised intestinal barrier, this thesis has not definitively addressed whether or not these mice exhibit a true barrier defect. Experiments examining the ultrastructure of apical cell adhesion complexes (e.g. transmission and scanning electron microscopy) in *Kaiso*^{Tg} mice are needed to verify this possibility. While enhanced Claudin-2 at the apical membrane might suggest increased solute flux, it would not explain the increased permeability to 4 kDa FITC-dextran in *Kaiso*^{Tg} mice (**Chapter 4)**, since Claudin-2 selectively permits passage of molecules with radii smaller than 4 Å (Rosenthal *et al.*, 2010). However, it is conceivable that diminished E-cadherin sufficiently weakens cell-cell adhesion to increase the likelihood of mechanical barrier disruption caused by pathogenic neutrophil recruitment. We explore this possibility in greater detail in **Section 6.2.2** below.

6.2.2 Friend or foe? Kaiso^{Tg} mice exhibit neutrophil-specific intestinal inflammation.

Several observations from the current study support the notion that Kaiso-overexpression drives neutrophil-specific intestinal inflammation: (i) Diseased VKA and VKE mice exhibited the formation of crypt abscesses – the luminal accumulation of neutrophils at crypt surface (Gewirtz *et al.*, 2002, Kucharzik *et al.*, 2001); (ii) Inflamed *Kaiso*^{Tg} tissues were enriched for neutrophils and not other immune cell types; and (iii) transgenic mice exhibited enhanced MPO activity, which was coincident with inflammation onset (**Chapter 4**). The finding that *Kaiso*^{Tg} mice exhibit a strong neutrophil response, coupled with the observation that other immune cell types were not recruited in inflamed *Kaiso*^{Tg} intestinal tissues, raises the possibility that the tissue damage observed in our mice is driven by persistent neutrophil recruitment.

During an acute immune response, tissue-resident monocytes secrete chemokines to recruit neutrophils to the site of infection. Circulating neutrophils traverse the vasculature and, upon reaching the site of infection, release an arsenal of antimicrobial enzymes, such as MPO (Fournier & Parkos 2012). Neutrophil apoptosis precedes a second wave of monocyte recruitment, which persists over several days of inflammation resolution (Ortega-Gómez *et al.*, 2013). However, persistent neutrophil recruitment has been shown to play a more pathogenic role. Continued transepithelial migration of neutrophils has reported to compromise the integrity of the intestinal barrier (Kucharzik *et al.*, 2001), leading to increased permeability and the formation of crypt abscesses (Fournier & Parkos 2012), supporting the hypothesis that persistent neutrophil recruitment contributes to Kaiso-induced inflammation.

IL-8 is a potent neutrophil activator and chemokine produced by monocytes and other cell types, including IECs (Eckmann *et al.*, 1993). We found no change in the murine IL-8 homologue, MIP-2, in subclinical *Kaiso*^{Tg} mice, and a marked increase in MIP-2 levels in diseased mice. Previous studies have shown that MIP-2 activity is modulated by MMP7 – a metalloproteinase produced by Paneth cells (Swee *et al.*, 2008). Moreover, *MMP7*$^{-/-}$ mice have been shown to exhibit delayed neutrophil recruitment in response to DSS-mediated colonic injury (Swee *et al.*, 2008). Since Paneth cells and MMP7 staining are increased in *Kaiso*^{Tg} mice **(Chapter 3, Robinson *et al.*, 2017b,** and Pierre *et al.*, 2015b), it is possible that MMP7-mediated activation of MIP-2 contributes to neutrophil recruitment in *Kaiso*^{Tg} intestines, raising the possibility that Notch-pathway inhibition plays a role in the pathogenesis of Kaiso-induced inflammation (**Section 6.3**).

As mentioned previously, the formation of crypt abscesses in diseased *Kaiso*^{Tg} mice is a hallmark phenotype of chronic transmigration across the intestinal epithelium (Gewirtz *et al.*, 2002, Kucharzik *et al.*, 2001). While elevated MIP-2 levels might trigger the recruitment of neutrophils into the intestinal epithelium, chronic transepithelial migration of neutrophils could contribute to the compromised barrier integrity and increased permeability to 4 kDa FITC-dextran observed in diseased *Kaiso*^{Tg} mice (**Chapter 4**). Other groups have shown that neutrophil transmigration across T84 IEC cell monolayers reduces transepithelial resistance and increases permeability (Nusrat *et al.*, 1997, Sumagin *et al.*, 2014). Furthermore, patients with UC exhibit breaks in the intestinal barrier at regions exhibiting neutrophil transmigration (Nusrat *et al.*, 1997). Thus, taken together, the findings from our study in Chapter 4 and those highlighted here

suggest a pathogenic role for neutrophils in the development of Kaiso-induced inflammation.

6.3 Kaiso: the nexus of Notch signalling and intestinal inflammation?

Emerging studies have implicated Notch signalling in the genesis of intestinal inflammation, although the precise role of Notch in this process remains controversial (Fazio & Ricciardiello 2016). Below, we highlight a pro-inflammatory role for Notch signalling in the intestinal epithelium, and discuss a possible role for Kaiso at the center of both processes.

6.3.1 A possible connection between Kaiso, Notch signalling and Inflammation.

As a first step in determining whether secretory cell hyperplasia in *Kaiso*^{Tg} mice occurred independently of chronic inflammation, 3 month-old *Kaiso*^{Tg} mice were assessed for phenotypes of hallmark of widespread chronic inflammation. In contrast to 12 month-old *Kaiso*^{Tg} mice (Chaudhary *et al.,* 2013), 3 month-old transgenic mice did not show a change in MPO activity (a marker for activated neutrophils), nor was there evidence of widespread inflammation-related tissue damage (e.g. thickened *submucosa*, fused villi etc.). Notably however, 3 month-old *Kaiso*^{Tg} mice still showed a significant increase in the number of secretory cells – i.e. goblet, enteroendocrine and Paneth cells **(Chapter 3, Robinson *et al.,* 2017b)**. While we previously reported that some 3 month-old *Kaiso*^{Tg} mice developed localized foci consistent with inflammation-related tissue damage (Pierre *et al.,* 2015b), these mice did not exhibit increased MPO activity (**Chapters 3 & 4**). Moreover, the observed secretory cell hyperplasia occurred along the length of the small

intestine in areas void of tissue damage. Together, these data support the notion that the Kaiso-induced increase in secretory cells (and hence Kaiso-mediated Notch inhibition) occurred independently of subsequent chronic intestinal inflammation.

While all mouse models of Notch signalling inhibition report an increase of one or more secretory cell types (Sancho *et al.*, 2015), two other reports have highlighted the development of spontaneous intestinal inflammation akin to *Kaiso*^{Tg} mice (**Chapter 4**, Guilmeau *et al.*, 2008, Obata *et al.*, 2012). In their study, Obata and colleagues found that IEC-specific-deletion of the Notch pathway transcription factor, *Rbp-J* (*Rbp-J*$^{\Delta IEC}$) in 6-week old mice, produced an increase in all three secretory cell types. Importantly, by 5-6.5 months of age, *Rbp-J*$^{\Delta IEC}$ mice spontaneously developed colitis (i.e. colonic inflammation), as evidenced by various phenotypes, including a thickened colonic *submucosa*, mucus accumulation, rectal prolapse and increased neutrophil recruitment into inflamed regions (Obata *et al.*, 2012). Similarly, intestinal-specific deletion of **p**rotein ***O*-fu**cosyl**t**ransferase 1 (Pofut1), an enzyme involved in glycosylation of Notch ligands and hence required for downstream Notch signalling (Okajima *et al.*, 2003, Wang *et al.*, 2001), led to an increase in secretory cells and subsequent chronic intestinal inflammation in mice (Guilmeau *et al.*, 2008).

The precise mechanism of inflammation due to loss of Notch signalling is currently unknown, although a defective intestinal barrier may play a role. Indeed, both *Rbp-J*$^{\Delta IEC}$ and *Kaiso*^{Tg} mice developed a compromised intestinal epithelial barrier, as measured by FITC-dextran permeability assays (**Chapter 4** and Obata *et al.*, 2012). Notably however, whereas FITC-dextran permeability assays revealed a barrier defect in

subclinical *Rbp-J*$^{\Delta IEC}$ mice by ~3 months of age (Obata *et al.*, 2012), subclinical 3 month-old line A *Kaiso*^{Tg} mice did not show a difference in intestinal permeability (**Chapter 4**). This might be due to differences in study design (rectally versus orally administered FITC-dextran in *Rbp-J*$^{\Delta IEC}$ and *Kaiso*^{Tg}, respectively), or the extent of Notch pathway inhibition between the two mouse models.

Since *Kaiso*^{Tg} mice exhibit an increase in goblet cells **(Chapter 3, Robinson *et al.*, 2017b)**, another possible contributor of chronic inflammation in *Kaiso*^{Tg} mice is the excessive trapping of bacteria in the mucous layer. Mucins secreted by goblet cells form a mucous layer which acts as the first line of defence against microbial infection in the GI tract (Pelaseyed *et al.*, 2014). Indeed, loss of the mucous layer in *Muc2* $^{-/-}$ mice permits direct contact between luminal microbes and the surface intestinal epithelium, ultimately resulting in intestinal inflammation (Van der Sluis *et al.*, 2006, Wenzel *et al.*, 2014). Consistent with this finding, a reduction of goblet cells has also been reported in both CD and UC (Gersemann *et al.*, 2009, Gersemann *et al.*, 2011).

However, the mucous layer may be a double-edged sword, as excessive mucous production and impaired mucous clearance have been shown to contribute to intestinal disease, reviewed in (Johansson *et al.*, 2013) and (van der Doef *et al.*, 2011). In fact, Guilmeau *et al.* found that, in addition to an increase in secretory cell types, *Pofut1* knockout mice also exhibited an increase in trapped luminal microbes and bacterial translocation (Guilmeau *et al.*, 2008). Thus, it is conceivable that the increased goblet cells in *Kaiso*^{Tg} mice **(Chapter 3, Robinson *et al.*, 2017b)** may cause enhanced Mucin2 secretion that impairs the movement and/or clearance of mucous-associated flora.

6.3.2 Subclinical Kaiso^{Tg} mice exhibit altered intestinal epithelial repair mechanisms.

In addition to its role in mediating cell fate decisions, Notch signalling is also critical for epithelial renewal by modulating ISC proliferation, a process essential for repair of intestinal damage, reviewed in (Demitrack & Samuelson 2016). Other mouse models that show concomitant Notch depletion and inflammation demonstrated altered intestinal repair mechanisms (Guilmeau *et al.,* 2008, Obata *et al.,* 2012). Hence, the finding that Kaiso inhibits Notch signalling in the intestinal epithelium inspired studies to examine whether a similar phenomenon occurred in *Kaiso^{Tg}* mice prior to inflammation onset.

Among the distinguishing features of the subclinical VKA mice was the reduced expression of Cyclin D1 and cleaved-Caspase 3 in 3 month-old VKA mice relative to age-matched NonTg mice, hinting that the overall epithelial turnover process is delayed by Kaiso-overexpression. While we previously reported no change in Ki67 positivity in 3-month old mice (Pierre *et al.*, 2015b), we assessed Ki67 positivity in presumed crypt base columnar stem cells, which are regulated by Notch signalling (Srinivasan *et al.,* 2016, VanDussen *et al.*, 2012). Curiously, there was no change in the total number of Ki67+ve cells, or presumed ISC found in the bottom 1/4 of the crypt (**Chapter 4**). A similar phenomenon was also reported for *Pofut1$^{F/F}$* mice, where loss of Pofut1 in the small intestine led to reduced Cyclin D1 and cleaved Caspase 3 protein expression, but no change in the total number Ki67+ve cells (Guilmeau *et al.,* 2008).

Ki67 is a general proliferation marker that labels cells at all stages of the cell cycle (Scholzen & Gerdes 2000). Thus, use of Ki67 may not have been the most effective approach to assay for proliferation in *Kaiso^{Tg}* mice, which do not exhibit complete cell

cycle arrest (Chaudhary *et al.*, 2013, Pierre *et al.*, 2015b). An alternative, and currently ongoing, approach would be to assess the rate of BrdU incorporation in age-matched *Kaiso*^{Tg} and NonTg mice. Given the reduced expression of Cyclin D1 in *Kaiso*^{Tg} mice, it is possible that transgenic mice would exhibit slower BrdU incorporation than their NonTg counterparts.

The observed reduction of Cyclin D1 in *Kaiso*^{Tg} mice may occur via Notch pathway disruption (Riccio *et al.*, 2008), direct inhibition by Kaiso (Dai *et al.*, 2011, Donaldson *et al.*, 2012, Jiang *et al.*, 2012, Park *et al.*, 2005), or both – although delineating these possibilities would necessitate rescuing Notch pathway activity in *Kaiso*^{Tg} mice. Nevertheless, the finding that subclinical *Kaiso*^{Tg} mice exhibit reduced Cyclin D1 expression supports the notion that proliferation is altered by Kaiso-overexpression.

In addition to proliferation and apoptosis, another key process for intestinal renewal is cell migration (Crosnier *et al.*, 2006). Since we observed decreased Cyclin D1 expression in 12-week old VKA mice, we initially expected a concomitant delay in IEC migration. Unexpectedly however, subclinical *Kaiso*^{Tg} mice displayed increased cell migration along the crypt-villus axis **(Chapter 4)**, suggesting that proliferation and migration in the *Kaiso*^{Tg} intestinal epithelium was uncoupled.

Intestinal epithelial restitution is a process that describes the resealing of wounds caused by damage to GI mucosa independently of ISC proliferation, reviewed in (Sturm & Dignass 2008). Importantly, E-cadherin and TGFβ signalling – both previously shown to be regulated by Kaiso (Bassey-Archibong *et al.*, 2016, Buck-Koehntop *et al.*, 2012,

Jones *et al.*, 2014b, Jones *et al.*, 2012) – are involved in the process of intestinal epithelial restitution (Sturm & Dignass 2008). Although Kaiso's transcriptional regulation of E-cadherin and TGFβR-I and -RII in intestinal cells still remains to be determined, it is possible that Kaiso mediates cell migration in the intestinal epithelium via direct suppression of E-cadherin, and/or TGFβ pathway activation.

To the best of our knowledge, no other study has reported accelerated IEC migration and slowed intestinal proliferation. Nevertheless, it is possible that the observed abnormal intestinal repair mechanisms in subclinical *Kaiso*^{Tg} mice play a role in promoting intestinal inflammation as mice age.

6.4 Kaiso-mediated transcriptional regulation dictates homeostatic outcomes in intestinal cells.

6.4.1 Kaiso exhibits methylation-dependent binding to the DLL1 and JAG1 promoters in intestinal cells.

Our finding that Kaiso misexpression altered transcript and protein levels of Dll-1 and Jagged-1 prompted further investigation into whether both Notch ligands were putative Kaiso target genes. Kaiso has been shown to associate with a sequence-specific consensus (TCCTGCNA) (Daniel *et al.*, 2002), meCpGs (Prokhortchouk *et al.*, 2001a) or a palindromic sequence, TCTCGCGAGA (Raghav *et al.*, 2012). However, while the *DLL1* and *JAG1* promoters both contained KBS and CpG dinucleotide sites **(Chapter 3, Robinson *et al.*, 2017b)**, the palindromic sequence was not present in either promoter in the regions analyzed.

Although the exact mechanism of Kaiso binding to the *DLL1* and *JAG1* promoters was beyond the scope of this study, we postulate that Kaiso associates with both gene

promoters using both methylation-dependent and sequence-specific binding mechanisms, as we previously demonstrated with the *CCND1* promoter (Donaldson *et al.,* 2012). Relying on both methylation and sequence-specific binding would significantly increase the specificity with which Kaiso associates with its target genes. However, to confirm this empirically, mutational analyses of the *DLL1* and *JAG1* promoters, together with minimal promoter assays, are necessary and should be examined in future studies.

Intriguingly, although Kaiso associated with the *JAG1* promoter in a methylation-dependent manner, Jagged-1 transcript and protein levels correlated positively with Kaiso expression, suggesting that Kaiso activates Jagged-1 expression **(Chapter 3, Robinson *et al.*, 2017b)**. This was an unexpected finding since meCpG's are well known for their role in gene repression, reviewed in (Bird 2002). However, CpG methylation has also been reported to contribute to transactivation. For example, methylation of the **c**AMP **r**esponse **e**lement (CRE) has been shown to result in transactivation by **C**CAAT-**e**nhancer-**b**inding **p**rotein alpha (C/EBP-α; Rishi *et al.,* 2010), and DNA methylation has been associated with activation of the FoxA2 gene (Bahar Halpern *et al.,* 2014). Similarly, we recently demonstrated that Kaiso promotes the expression of TGFβR1, and associates with its promoter using KBS-specific and meCpG-dependent mechanisms (Bassey-Archibong *et al.,* 2016). Therefore, it is possible that Kaiso exerts similar methylation-dependent transactivational properties at the *JAG1* locus.

6.4.2 The POZ-ZF transcription factor, Znf131, is implicated in the co-regulation of Kaiso target genes.

Our finding that Znf131 indirectly associated with Kaiso target genes at the KBS **(Chapter 5, Robinson *et al.*, 2017a)**, implicates Znf131 as a regulator of Kaiso target genes, and, by extension, Kaiso-mediated biological processes. This phenomenon (i.e. a transcription factor co-regulating gene expression dependent on its interaction with another transcription factor) has been reported before. For example, Myc/Max heterodimers activate gene expression, whereas Myc/Miz1 complexes result in transcriptional repression, reviewed in (Eilers & Eisenman 2008) and (Wanzel *et al.*, 2003). While mechanisms governing Myc/Miz1-mediated trans-repression have been reported (Brenner *et al.*, 2005), the molecular mechanism(s) governing the transcriptional activities of Kaiso/Znf131 complexes are still unknown and should be considered for future investigation.

In light of the observation that Kaiso and Znf131 exert opposing effects on the *CCND1* minimal promoter **(Chapter 5, Robinson *et al.*, 2017a)**, it is tempting to speculate that the levels of Znf131 and Kaiso act to tightly control/fine-tune gene expression. We propose that elevated levels of Znf131 activate *Cyclin D1* expression, which is attenuated as Kaiso levels increase. To prevent constitutive target gene regulation, we propose that Kaiso inhibits Znf131-mediated gene activation by suppressing Znf131 expression **(Chapter 5, Robinson *et al.*, 2017a)**. Indeed, *Kaiso*^{Tg} mice exhibit significantly reduced Znf131 levels, though ChIP data implied that this may not be caused by Kaiso-mediated transcriptional repression of the *ZNF131* promoter. Given the presence of E-box sequences in the *ZNF131* promoter and the reported binding

of Myc to the *ZNF131* promoter, as determined by the publically available ChIP-seq ENCODE database (Rosenbloom *et al.,* 2013), it would be interesting to determine whether Kaiso indirectly regulates Znf131 via regulation of cMyc, which is also a direct target of the Wnt and Notch signalling pathways (Palomero *et al.,* 2006).

While these studies provided some insight into the relationship between Kaiso and Znf131, there are still many unanswered questions. Nevertheless, these findings hint that the level of engagement between Znf131 and Kaiso may alter biological outcomes.

6.5 Future Directions

6.5.1 Taking Kaiso's role in Notch-mediated intestinal homeostasis one step further

6.5.1.1 Does Notch-inhibition play a role in Kaiso-mediated intestinal inflammation?

To test the hypothesis that Notch-depletion plays a central role in Kaiso-induced inflammation, it would be necessary to rescue Notch signalling in *Kaiso*^{Tg} mice and determine whether chronic inflammation is still persists. While previous studies have employed the RosaNotch/VilCre transgenic mouse to overexpress NICD in the intestinal epithelium, neonatal lethality (Fre *et al.,* 2005) would necessitate the use of an inducible NICD transgenic mouse. Given that the Kaiso transgene is also driven by the Villin promoter (Chaudhary *et al.,* 2013), inducible intestinal-specific NICD overexpression would be better accomplished using the Lgr5-Cre^{ERT2} driver (Barker *et al.,* 2007), which would allow for tamoxifen-inducible expression of NICD in CBC cells of the small intestine and colon. If Notch pathway activation in diseased *Kaiso*^{Tg} mice (i.e. > 6 months old for VKA or > 3 months old for VKE) results in attenuated inflammation (as measured

by MPO activity, crypt abscesses etc.), it would support the hypothesis that Kaiso-mediated inflammation is driven, at least in part, by a loss of Notch signalling.

6.5.1.2 Assessing cell differentiation and stem cell maintenance in Kaiso^{Tg} mice

In addition to possibly highlighting a role for Notch pathway inhibition in Kaiso-mediated inflammation, use of the Lgr5-Cre^{ERT2} mouse would also provide valuable insight into Kaiso-mediated cell differentiation and stem cell proliferation. Downstream of the cre-recombinase coding sequence, the Lgr5-Cre^{ERT2} mouse also contains the coding sequence for GFP under the control of an internal ribosomal entry site (Barker *et al.*, 2007). Thus, these mice have been used for lineage tracing experiments, where GFP+ve cells represent cell types derived from Lgr5+ve ISCs (Barker *et al.*, 2007). Since Lgr5+ve CBC cells give rise to all cell types in the intestinal epithelium, the progeny of a cross between *Kaiso^{Tg}* and Lgr5-Cre^{ERT2} mice would provide greater insight into Kaiso-mediated cell differentiation. Importantly, *Kaiso^{Tg}*;Lgr5-Cre^{ERT2} mice would also enlighten Kaiso's role in ISC maintenance, and whether Kaiso-mediated inflammation also impacts the stem cell compartment. This would have particularly important implications for intestinal renewal capabilities, especially in diseased *Kaiso^{Tg}* mice.

6.5.1.3 Elucidating the Kaiso/Jagged-1 axis in colon cancer progression

Both Kaiso and Jagged-1 have been independently implicated CRC progression (Dai *et al.*, 2014, Gao *et al.*, 2011, Guilmeau *et al.*, 2010, Pierre *et al.*, 2015b, Prokhortchouk *et al.*, 2006, Reedijk *et al.*, 2008). Given the positive correlation between Kaiso and Jagged-1 in intestinal cells **(Chapter 3, Robinson *et al.*, 2017b)**, future studies should examine

whether Kaiso-mediated regulation of Jagged-1 contributes to the development and/or progression of CRC. This can be addressed using *in vitro* approaches to rescue Jagged-1 expression in Kaiso-depleted cells and assaying for cell migration, invasion and proliferation. This can, and should, be complemented by *in vivo* assays such as crossing *Kaiso*^{Tg}*;Apc*$^{Min/+}$ with *Jag1*$^{+/\Delta}$ mice and examining tumour progression in the resulting progeny.

An unexpected observation of the *Kaiso*^{Tg}*;Apc*$^{Min/+}$ mice was that the increased Jagged-1 expression observed in non-tumour tissue was not maintained in the polyps (Appendix Figure A1.1). Since Kaiso bound to the *JAG1* promoter in a methylation-dependent manner **(Chapter 3, Robinson *et al.*, 2017b)**, future studies should also include comparative methylomics of normal and tumour tissue (via laser capture microdissection) of both *Kaiso*^{Tg}*;Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ mice. Not only will such a study shed light on Kaiso-mediated regulation of *JAG1* in tumour tissues, but it will also provide significant insight into Kaiso-mediated transcriptional regulation of various other target genes in colon cancer.

6.5.2 Examining a possible connection between Kaiso, Wnt, Notch and Hedgehog signalling in the intestinal epithelium

In order to obtain a complete and comprehensive understanding of Kaiso's role in intestinal development and diseases, it is necessary to consider the various pathways that are also required for homeostasis. One such pathway, which was not examined in this work, is the highly conserved **H**edge**h**og (Hh) signalling pathway. Of the Hh ligands, **I**ndian Hh (Ihh) is the predominant ligand expressed by IECs in the adult intestinal

epithelium. Ihh signals to responsive cells in the underlying mesenchyme, including smooth muscle precursor and differentiated cells, as well as myoblast-like cells and pericytes (Büller *et al.,* 2012).

Communication between the epithelium and mesenchyme via Hh signalling has been shown to play a pivotal role in regulating the size of intestinal crypts; mediating IEC proliferation and regeneration; and dictating immune responses in the *lamina propria* (Büller *et al.,* 2012). It is therefore not surprising that Hh signalling converges upon other pathways, including Wnt and Notch signalling, that also regulate the aforementioned cellular processes. Indeed, under homeostatic conditions Hh signalling is known to oppose canonical Wnt signals in the intestine (Büller *et al.,* 2012), and a role for *Ihh* has been implicated in the severity of CRC (Büller *et al.,* 2015, Gerling *et al.*, 2016). Given Kaiso's known roles in intestinal homeostasis **(Chapter 3, Robinson *et al.*, 2017b)**, inflammation (**Chapter 4** and Chaudhary *et al.,* 2013), and CRC (Pierre *et al.,* 2015b, Prokhortchouk *et al.,* 2006), studies elucidating a possible link between Kaiso and Hh signalling in intestinal homeostasis and disease warrant future investigation.

6.5.3 Is Kaiso-mediated inflammation driven by pathogenic neutrophil recruitment?

Part of our proposed working hypothesis is that pathogenic neutrophil recruitment drives intestinal inflammation and associated tissue damage in *Kaiso*^{Tg} mice (Figure 6.1). One way to address this directly would be to attenuate neutrophil recruitment via neutralizing antibodies against the neutrophil chemokine, MIP-2, and ascertain whether inflammation persists. Neutralizing antibodies have been used to alleviate inflammation in IBD patients, reviewed in (Cohen & Sachar 2017), and a similar approach has been used in other

mouse models of colitis, including SAMP1/YitFc **(s**enescence **a**ccelerated **m**ice senescence-**p**rone, YitFc substrain) and DSS-treated mice (Marini *et al.,* 2003, Xiao *et al.*, 2016).

Lastly, we previously postulated that Kaiso-mediated intestinal inflammation drove tumourigenesis in $Apc^{Min/+}$ mice (Pierre *et al.,* 2015b). While Kaiso-mediated tumourigenesis is likely multi-factorial, neutralization of neutrophil-attracting chemokines should at least partially ameliorate tumourigenesis in $Kaiso^{Tg}$;$Apc^{Min/+}$ mice. Ongoing studies are currently investigating whether Kaiso overexpression promotes azoxymethane-induced colon cancer progression. Given the pathogenic role of neutrophils in Kaiso-induced inflammation (**Chapter 4** and Chaudhary *et al.,* 2013), concurrent studies depleting neutrophil recruitment in AOM-treated mice would further support the notion that neutrophil-driven inflammation drives Kaiso-mediated tumourigenesis.

6.5.4 How does Znf131 factor into Kaiso-mediated intestinal homeostasis?

In spite of recent advances (Han *et al.,* 2008, Iguchi *et al.,* 2015, Oh & Chung 2012, Oh & Chung 2013a, Oh & Chung 2013b, Oh & Chung 2013c, Varier *et al.,* 2016), Znf131's biological role and the implications of its engagement with Kaiso in cellular processes remain largely elusive. The observation that Znf131 localized to intestinal crypts and was absent in $Kaiso^{Tg}$ epithelia **(Chapter 5**, **Robinson *et al.*, 2017a)**, together with our recent finding that Kaiso inhibits Notch signalling in the intestines **(Chapter 3, Robinson *et al.*, 2017b)**, raises the possibility that perhaps Znf131 is a downstream target of Notch signalling. One approach to determine Znf131's role in intestinal homeostasis, would be

to exploit the conditional Znf131 knockout mouse (Iguchi *et al.*, 2015). By mating *Znf131*$^{flox/flox}$ with a VilCre driver, one could determine whether intestinal-specific loss of Znf131 phenocopies loss of Notch signalling, and, by extension, Kaiso-overexpression. Since *Kaiso*^{Tg} mice already exhibit reduced Znf131 expression, it would be interesting to determine whether Znf131-overexpression would rescue Kaiso-mediated secretory cell hyperplasia and inflammation. Unfortunately, such a Znf131 transgenic mouse does not currently exist. However, the interplay between Kaiso and Znf131 on Notch signalling can be elucidated *in vitro* by creating double knock-down and over-expression CRC cell lines.

6.6 The overall significance of Kaiso as a Notch pathway and pro-inflammatory mediator.

6.6.1 Kaiso as a Notch pathway mediator

Notch signalling is a highly conserved pathway across species, and is central to various developmental and homeostatic processes, reviewed in (Guruharsha *et al.*, 2012) and (Hansson *et al.*, 2004). This study is the first to definitively demonstrate a role for Kaiso in Notch-mediated intestinal homeostasis and the first to elucidate a mechanism by which Kaiso regulates Notch signalling in intestinal cells. Given the emerging role of Notch signalling in diseases such as CRC and IBD, our findings suggest that Kaiso may also play a crucial role in these processes.

Aside from a role in intestinal homeostasis, Kaiso's regulation of Notch signalling may also extend to other tissues in which Notch is a central homeostatic mediator. Among many other developmental programs, Notch signalling has well-characterized

roles in neurogenesis, reviewed in (Imayoshi *et al.*, 2010), and other groups have reported high Kaiso expression levels in the neural tissues of developing and adult vertebrates (Della Ragione *et al.*, 2006, Kim *et al.*, 2002, Martin Caballero *et al.*, 2009, Yao *et al.*, 2010). Interestingly, recent findings have demonstrated cross-talk between Kaiso and canonical Wnt signalling in neuronal differentiation (Zhao *et al.*, 2016). Thus it is possible that the highlighted role for Kaiso in Notch-mediated intestinal homeostasis is conserved across other tissues, including those of the neural lineage.

6.6.2 Kaiso as a pro-inflammatory mediator.

This thesis has also offered substantially detailed insight into Kaiso's role in intestinal diseases, particularly IBD. The role of Notch signalling in IBD remains controversial, and it is possible that Notch signalling is a double-edge sword in IBD progression. However, our study supports the notion that Notch pathway inhibition plays a pathogenic role in IBD, and that Kaiso may be a key mediator in this process. Indeed, IHC analysis revealed enhanced Kaiso expression in Crohn's disease intestinal tissues compared to normal tissues (**Chapter 4**). Whether Kaiso upregulation is a cause or consequence of intestinal inflammation remains to be determined, since preliminary studies demonstrated that chemically-induced colitis also results in increased Kaiso expression (R. Chaudhary, Ph.D. Thesis, 2015). Nevertheless, the finding that Kaiso is elevated in inflamed intestinal tissues supports the use of our *Kaiso*^{Tg} mice as a relevant IBD mouse model.

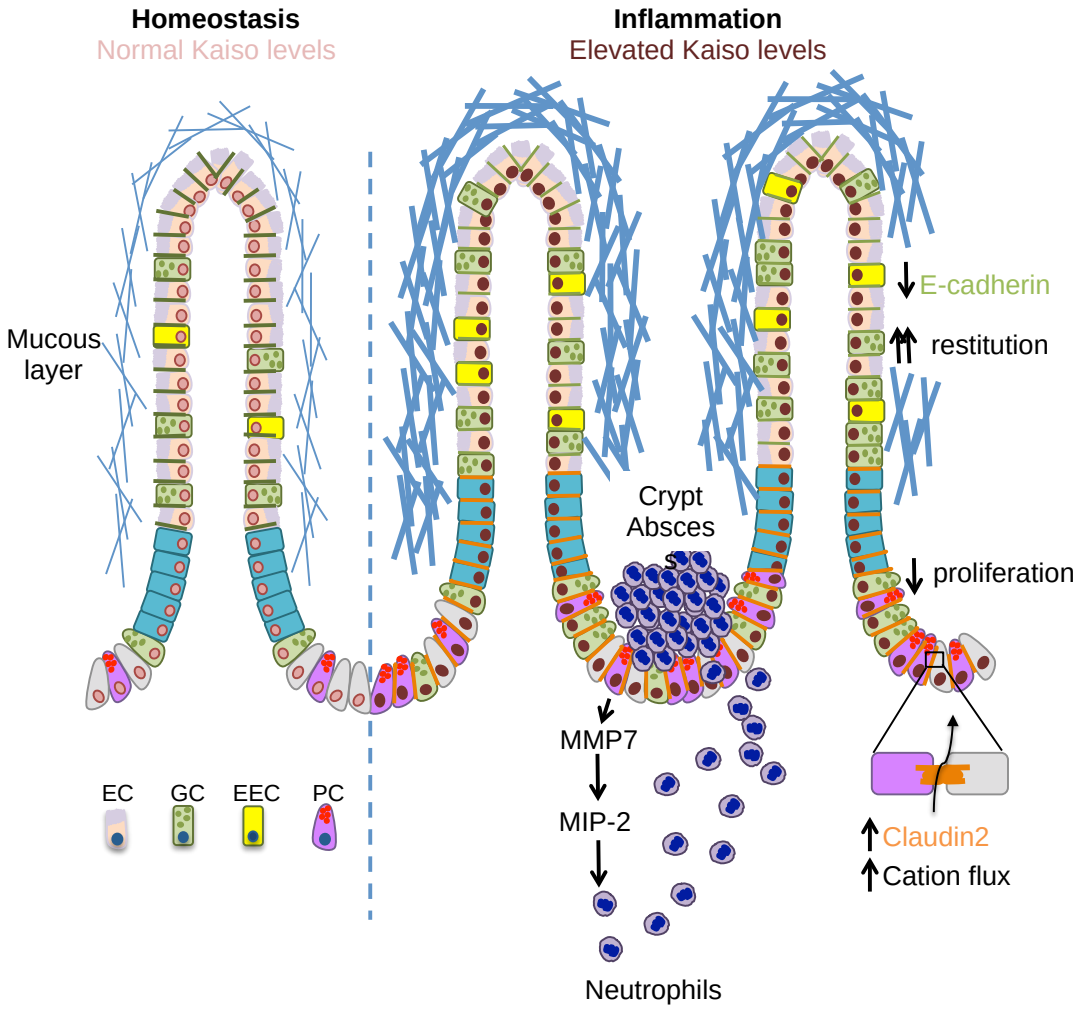
We found that *Kaiso*^{Tg} mice exhibit neutrophil-specific intestinal inflammation (**Chapter 4**), and previously posited that this may have a significant impact on CRC progression in mice (Pierre *et al.*, 2015b). Indeed, IBD sufferers are more likely to

develop colon cancer than non-IBD persons. Thus, as studies progress to validating Kaiso as a therapeutic option for IBD, our findings suggest that targeting Kaiso may have the mutual benefit of treating IBD and attenuating CRC progression.

6.7 Putting the puzzle together – a summary & working model of Kaiso-mediated intestinal inflammation.

Findings from our group and others have highlighted that Kaiso plays a critical role in intestinal homeostasis, and the pioneering studies of *Kaiso*^{Tg} mice provided the framework upon which this thesis is built (Chaudhary *et al.*, 2013). Based on our findings, we put forth a working model that proposes a multifactorial progression to Kaiso-induced inflammation and places Kaiso-mediated Notch inhibition as a significant event in the induction of subsequent intestinal inflammation (Figure 6.1). According to our model, the increase in goblet cells due to Notch inhibition leads to increased mucin secretion, slowing the clearance of potentially harmful bacteria. The increase in Paneth cells would lead to enhanced MMP7 production, thus resulting in increased MIP-2 activation and subsequent neutrophil recruitment. Persistent MIP-2 activity triggers ongoing neutrophil recruitment and transmigration, producing the observed tissue damage and permeability defects. These phenotypes, together with the misexpression of cell adhesion proteins (such as E-cadherin and Claudin-2), and altered epithelial repair mechanisms, all contribute to the onset of intestinal inflammation. While a plethora of questions still remain unanswered and warrant further investigation, these findings suggest that Kaiso plays an important and pathogenic role in the progression of IBD.

FIGURE 6.1 Working model of Kaiso's role in intestinal homeostasis. We propose a multifactorial model of Kaiso-mediated intestinal inflammation. KaisoTg mice exhibit impaired Notch signalling, leading to a disproportionate number of secretory cell types. The increase in goblet cells results in greater mucous production, which may cause increased bacterial trapping and/or delayed bacterial clearance. The increase in Paneth cells would result in a greater number of MMP7 positive cells, which could lead to augmented neutrophil recruitment via MIP2 activation. Pathogenic neutrophil recruitment triggers intestinal tissue damage, and thus exacerbates inflammation. Kaiso overexpression also results in increased Claudin-2, which is responsible for paracellular permeability. Lastly, Kaiso overexpression causes abnormal intestinal repair mechanisms, including restitution and proliferation. EC – enterocyte (*absorptive cells*), *Secretory cell types:* GC – goblet cell, EEC – enteroendocrine cell, PC – Paneth cell


Homeostasis
Normal Kaiso levels
Inflammation
Elevated Kaiso levels
Mucous layer
EC
GC
EEC
PC
Crypt Absces
s
MMP7
MIP-2
Neutrophils
E-cadherin
restitution
proliferation
Claudin2
Cation flux

APPENDIX

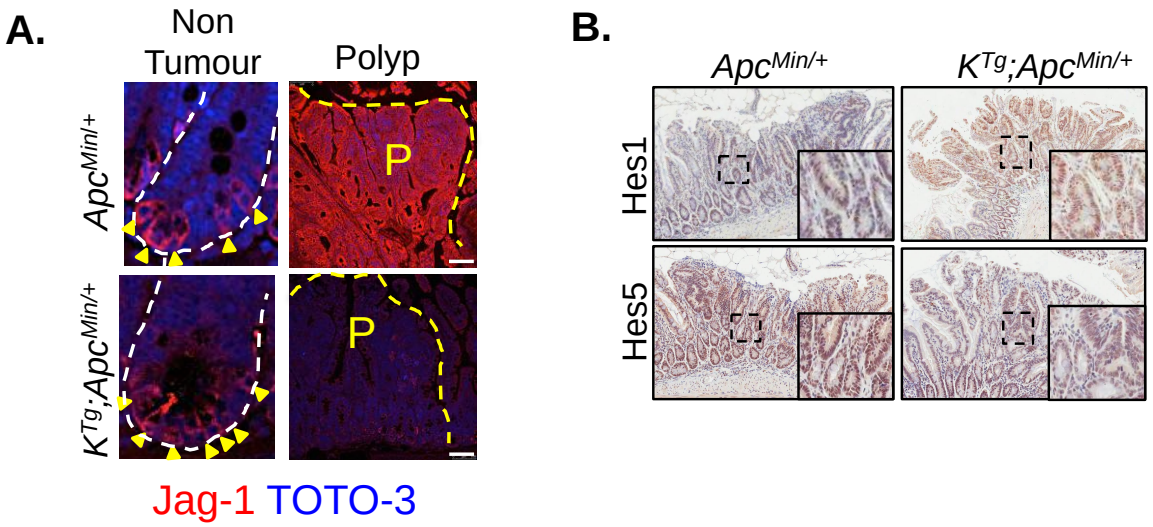


Figure A1.1. Kaiso-overexpression causes differential Jagged-1 regulation in normal and tumour tissue in the *Apc*$^{Min/+}$ mouse model of colon cancer. (A) Jagged-1 immunofluorescence staining of *Apc*$^{Min/+}$, and *Kaiso*^{Tg}*;Apc*$^{Min/+}$ (*K*^{Tg}*;Apc*$^{Min/+}$) polyps and adjacent non-tumour tissue. Nuclei were counterstained with TOTO-3. Crypt boundary is indicated with a dotted white line. Polyp boundary (P) is marked with a dotted yellow line. **(B)** IHC analysis reveals reduced Hes1 and Hes5 staining in *K*^{Tg}*;Apc*$^{Min/+}$ mice compared to A*pc*$^{Min/+}$ siblings.

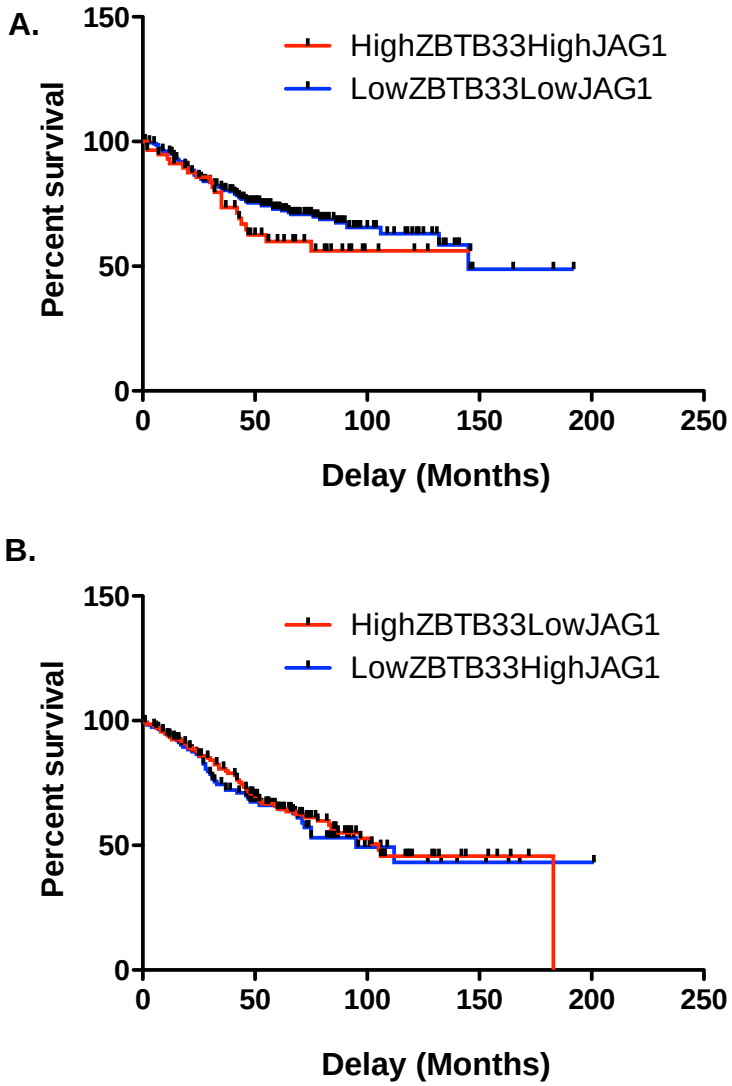


Figure A1.2. Combined Kaiso and Jagged-1 expression in CRC tumours does not correlate with patient survival. Kaplan-Meier survival curves examining the **(A)** direct and **(B)** inverse relationship between Kaiso (ZBTB33) and JAG1 expression on overall CRC survival using the publically available colon cancer Gene Expression Omnibus dataset, GSE39582. Analyses were performed by Dr. Anna Dvorkin-Gheva. Statistical significance determined using Log-Rank test.

A2. Elucidating a role for Kaiso in the regulation of PPARγ: a preliminary study

Preface

Summary: It is well known that PPARγ is the master regulator of adipocyte differentiation, and has been implicated in various other processes, including inflammation (Christodoulides & Vidal-Puig 2010, Martin 2010). Previous findings have hinted at a role for Kaiso in fat cell differentiation, as it was shown that Kaiso-depletion in 3T3-L1 preadipocytes accelerated their terminal differentiation (Raghav *et al.,* 2012). Since both Kaiso and PPARγ have been linked to inflammation (**Chapter 4**, Chaudhary *et al.,* 2013, Martin 2010), the aim of this preliminary study was to investigate whether Kaiso-mediated regulation of PPARγ in intestinal cells contributed to the inflammation observed in *Kaiso*^{Tg} mice. Intriguingly, the preliminary findings reported here imply that Kaiso promotes PPARγ expression in IECs. Additionally, though Kaiso was not found to associate with the *PPARG* gene promoter, both Kaiso and PPARγ were found to interact in a protein complex. The biological significance of this protein interaction still remains to be elucidated.

A2.1 Introduction

A2.1.1 Peroxisome Proliferator-Activated Receptors

The **P**eroxisome **P**roliferator-**A**ctivated **R**eceptors (PPARs) belong to the family of ligand-activated nuclear receptor transcription factors (Berger & Moller 2002). PPARs are activated by natural and synthetic ligands such as fatty acid derivatives and **t**hia**z**oli**d**inediones (TZDs, e.g. rosiglitazone), respectively (Berger & Moller 2002). Functioning as obligate heterodimers, PPARs dimerize with **r**etinoid **X** **r**eceptor (RXR) to regulate gene expression. PPARs recognize and bind the **P**eroxisome **P**roliferator **R**esponse **E**lement (PPRE), a DNA consensus sequence consisting of two identical hexameric AGGTCA sequences separated by a single intervening nucleotide (Dreyer *et al.*, 1992). In the absence of ligand, co-repressor proteins such as NCoR and SMRT bind PPAR:RXR, thus maintaining the heterodimer in an inactive state (Watson *et al.,* 2012, Yu *et al.,* 2005). Ligand binding causes the release of co-repressor complexes from PPAR:RXR, recruitment and binding of co-activators which ultimately results in transcriptional activation of PPAR target genes (DiRenzo *et al.,* 1997, Zoete *et al.,* 2007).

The physiological roles for PPARs are diverse, as they have been shown to play important roles in cellular differentiation (Brun *et al.,* 1996), lipid metabolism (Wang *et al.*, 2003), insulin sensitivity (Kintscher & Law 2005) and inflammation (Peyrin-Biroulet *et al.,* 2010, Rousseaux *et al.,* 2005). To date, three PPAR isotypes have been identified – PPARα, -β/δ and -γ – each of them encoded by separate genes (Dreyer *et al.,* 1992).

A2.1.2 PPARγ is an inflammatory mediator

While best characterized for its roles in adipogenesis and insulin sensitivity, PPARγ is also known to play a significant role in modulating the inflammatory response. For instance, pro-inflammatory cytokines such as **t**umour **n**ecrosis **f**actor α (TNFα), IL-6 and IL-10 are trans-repressed by PPARγ via several different mechanisms (Berger & Moller 2002, Chawla 2010, Martin 2010). For instance, PPARγ may engage in a protein interaction with transcription factors, such as **n**uclear **f**actor-κB (NFκB), thus preventing them from binding to their respective DNA elements; sequester co-activator proteins required by inflammatory transcription factors; or it may inhibit signalling pathways (e.g. MAPK) that activate transcription factors involved in mediating inflammatory response (Li & Yang 2011, Martin 2010, Ricote & Glass 2007).

PPARγ's role in modulating inflammation has implications for the function of several different tissues but several studies have implicated PPARγ in mediating inflammation in the intestines (Martin 2010, Mohapatra *et al.,* 2010, Rousseaux *et al.,* 2005). For instance, depletion of PPARγ in murine IECs resulted in increased susceptibility to colonic inflammation when challenged with DSS, a chemical inducer of IBD (Bassaganya-Riera *et al.,* 2004, Mohapatra *et al.*, 2010). Interestingly, such mice exhibited greater leukocyte infiltration, weight loss and mucosal wall thickening (Bassaganya-Riera *et al.,* 2004, Mohapatra *et al.*, 2010). Furthermore, treatment of the IL-10-deficient mouse model of IBD with the PPARγ ligand, rosiglitazone, resulted in delayed onset of IBD and a reduction in proinflammatory cytokines, including TNFα (Lytle *et al.,* 2005).

Recently, it has been shown that Kaiso might be involved in regulating PPARγ expression (Raghav *et al.,* 2012). Specifically, ChIP-seq analyses revealed that Kaiso tethers the corepressor SMRT to promoter-proximal sites of active genes in 3T3-L1 preadipocytes. While *in vitro* analysis of protein-DNA interactions showed that Kaiso:SMRT complexes associate with the KBS, Kaiso was shown to primarily recruit SMRT via a sequence-specific methylation-dependent mechanism (Raghav *et al.,* 2012). Interestingly, depletion of both SMRT and Kaiso in 3T3-L1 resulted in enhanced adipogenesis compared to the shRNA control, and depletion of SMRT upregulated genes required for adipogenesis, including PPARγ (Raghav *et al.,* 2012). Taken together, these data hint at the exciting possibility that Kaiso might regulate PPARγ, and PPARγ-mediated processes.

A2.1.3 Rationale & Hypothesis

Kaiso has been implicated as a negative regulator of adipogenesis, a process that is governed primarily by PPARγ. Biological functions for PPARγ extend well beyond its roles in adipogenesis, and include processes such as inflammation. Interestingly, our intestinal specific Kaiso-overexpressing transgenic mice display phenotypes associated with IBD, including increased intestinal inflammation, anemia and reduced weight (**Chapter 4**, Chaudhary *et al.,* 2013, Pierre *et al.,* 2015b). Given that Kaiso negatively regulates adipogenesis, a PPARγ-mediated process, and PPARγ protects against inflammation, our **hypothesis is two-fold: 1. PPARγ is a Kaiso target gene and 2. Kaiso inhibits PPARγ-mediated anti-inflammatory effects**.

A2.2 Materials and Methods

Unless otherwise stated, methods were performed as described in Chapter 2

A2.2.3 Immunohistochemistry

Intestinal tissues were harvested from NonTg or *Kaiso*^{Tg} mice, fixed in 10% neutral-buffered formalin for 24 hours at 4°C and stored in 70% ethanol. IHC was performed as described in Chapter 2 with the following modifications: Antigen retrieval was accomplished by microwaving tissues in 1X sodium citrate (pH 6) for 2 min at high power, and then for 8 min at 40% power. Tissues were incubated with anti-PPARγ antibody (1:75, Millipore cat # 07-466) in a humidified chamber for at 4 ^{o}C overnight.

A2.2.4 Immunofluorescence (cultured cells)

HCT116 cells were seeded onto glass coverslips in 18-well dishes to such that they were ~ 70% confluent within 24 hours. Cells were washed twice with cold 1X PBS; pH7.4 and fixed with 4% paraformaldehyde (pH 7.2) for 10 minutes at RT. Cells were washed as described and permeabilized with PBST (1XPBS/0.3% Triton-X100) for 10 minutes at RT. Cells were washed as described and blocked with for 30 minutes at RT with 5% NGS, 1% BSA. anti-Kaiso rabbit pAb (1:1000; gift from Dr. Albert Reynolds) and anti-PPARγ mouse mAb (1:100 Santa Cruz # sc-7273) antibodies were incubated for 2 hours at RT and washed 5X with cold 1XPBS. Secondary Alexa-fluor antibodies (Life Technologies) and Hoescht nuclear stain were incubated for 1 hour at RT. After 3X 5-min washes with cold 1XPBS, coverslips were mounted using Aqua Polymount (Polysciences Inc.)**,** and imaged using a confocal microscope (Leica Microsystems).

A2.2.5 Reporter Assays

The PPRE X3-TK-luc reporter construct was purchased from Addgene (Plasmid #1015; deposited by Bruce Spiegelman), and the 3XPPRE sites were subsequently subcloned into pGLuc-TATA using HindIII and BamHI, as described in Chapter 2. Artificial promoter-reporter assays were also performed as described in Chapter 2. Briefly, 4 x 10^5 HCT116 cells were seeded into a 6-well dish. Twenty-four hours post-seeding, cells were co-transfected with 0.75 µg pGLuc-TATA or pGLuc-TATA-3XPPRE, 1.0 µg pcDNA3-FLAG-PPARγ and increasing amounts of either pRS-Kaiso or pcDNA3-Kaiso, using TurboFect Transfection Reagent (Thermo Fisher Scientific). Secreted *Gaussia* luciferase was assayed 24-hours post-transfection using BioLux *Gaussia* luciferase assay kit (New England Biolabs). Relative light units were normalized to β-galactosidase.

A2.2.6 Chromatin Immunoprecipitation

The primer sequences used for ChIP qRT-PCR are provided in **Table A2.1**.

A2.2.7 Reverse Transcription PCR

mRNA was isolated and converted to cDNA using the qScript cDNA synthesis kit, according to the manufacturers instructions (Quanta Biosciences). PCR was performed using the 2XFrogga PCR Mix (Frogga Bio). Primer sequences for hPPARy are listed in **Table A2.1**.

Table A2. 1 Primer sequences used in the PPARγ study and their corresponding annealing temperatures.

Assay	Target	Primer Sequence (5'- 3')	Annealing temperature
ChIP-qPCR	*Site 1*	F: CAAGTGGGAACATGTCAGAG R: ATTATCATCCGAGTGTTCGG	55 °C
	Site 2	F: CACGCCCAGCTAATTTTTGT R: TGTACAATTCAGGCCGGGTA	55 °C
	Site 3	F: CCTCAAGTCCAGGAGACCAG R: TCAGAGTTCCTTGCGTTTGT	55 °C
	Site 4	F: GCGAAGTCGCTGCTTTGT R: CCTAAACACCGAGGCGTAAA	55 °C
	-ve	F: AGGCAATGCAAATCAAAACC R: TAGTTCACTGCAGCCTCGAA	55 °C
qRT-PCR	*mPPARγ*	F: GTCACACTCTGACAGGAGCC R: TCACCGCTTCTTTCAAATCTTGT	55 °C
RT-PCR	*hPPARγ*	F: TCTCTCCGTAATGGAAGACC R: GCATTATGAGACATCCCCAC	53 °C

A2.2.8 In vitro differentiation of 3T3-L1 cells

$1X10^6$ 3T3-L1 preadipocytes were seeded in to 60 mm plates and grown to confluency. To determine the effects of Kaiso on 3T3-L1 differentiation, cells were transfected with 6 µg of pcDNA3.1-Kaiso-Kozak (or pcDNA3.1 empty as a control) for 24 hours. Untransfected 3T3-L1 cells we included as a positive control. The media was then replaced with Basal Media (DMEM, 10 %FBS, 1X antibiotic/antimycotic) for 3 days, then replaced every two days, as outlined in **Table A2.2**. At the end of differentiation, cells were either stained with Oil Red O or lysed for western blot analysis.

A2.2.9 Oil Red O staining

A stock solution of 0.5% Oil Red O (Sigma Aldrich) was prepared in 100% isopropanol, mixed overnight at room temperature and filter sterilized through a 0.2 µM filter. A 6:4

working stock of Oil Red O was prepared by mixing the stock solution with distilled water and incubating the mixture for 20 minutes at RT. The working stock was filtered through a 0.2 µM filter until fine particles were removed. To stain cells with Oil Red O, cells were washed twice with cold 1XPBS and fixed with 4% formaldehyde in 1XPBS for 1 hour at room temperature. This was followed by two cold 1XPBS washes and a 60% isopropanol wash for 5 minutes with rocking. Cells were allowed to dry and then incubated with 2 mL of the Oil Red O working stock solution for 2 hours at room temperature. Cells were then washed extensively with water until it ran clear, dried and then imaged.

Table A2. 2 Overview of protocol used to terminally differentiate 3T3-L1 preadipocytes

Day -1	Transient transfection	6 µg pcDNA3.1 Kaiso/Empty
Day 0-3	Basal Medium (BM)	DMEM 10%FBS 1XPSF
Day 3-5	Differentiation Medium I	BMI 0.5 mM IBMX 1 µg/mL insulin 1 µM dexamethasone + 2 µM Rosiglitazone OR 0.1% DMSO
Day 5-7	Differentiation Medium II	BMI 1 µg/mL insulin
Day 7-14	BM	-replaced every 2 days

A2.3 Results and Discussion

A2.3.1 Kaiso overexpression promotes PPARγ in intestinal cells

Since it was previously shown that Kaiso-depletion in 3T3-L1 preadipocytes enhanced their differentiation (Raghav *et al.,* 2012) and given that PPARγ is a master-regulator of adipogenesis (Christodoulides & Vidal-Puig 2010), we hypothesized that Kaiso acts to inhibit PPARγ. Not only would this explain Kaiso's role in adipogenesis, but given the protective role of PPARγ in intestinal inflammation (Martin 2010), it would also provide a potential mechanism by which Kaiso-overexpression promotes inflammation in mouse intestines. Thus, as a first step, the small intestines of *Kaiso*^{Tg} and NonTg mice were analyzed for PPARγ protein expression and subcellular localization by immunohistochemistry. As expected, PPARγ expression was observed in the cytoplasm and nucleus of both NonTg and *Kaiso*^{Tg} tissues. However, the tissues of *Kaiso*^{Tg} mice exhibited greater PPARγ staining compared to that of NonTg mice (Appendix Fig. A2. 1A). In agreement with this finding, qRT-PCR analysis demonstrated that *Kaiso*^{Tg} mice express more *Pparγ* transcript than NonTg mice (Appendix Fig. A2.1 B). We next wanted to ascertain the effects of Kaiso on PPARγ expression in our stable Kaiso-depleted HCT116 colon carcinoma cells. In agreement with *in vivo* findings, PPARγ protein and transcript levels were decreased in Kaiso-depleted HCT116 cells compared to the vector control (Appendix Fig. A2.1 C, D). Altogether, these data suggest that, in contrast to those by Raghav and colleagues (2012), Kaiso functions to promote PPARγ expression in the context of intestinal cells.

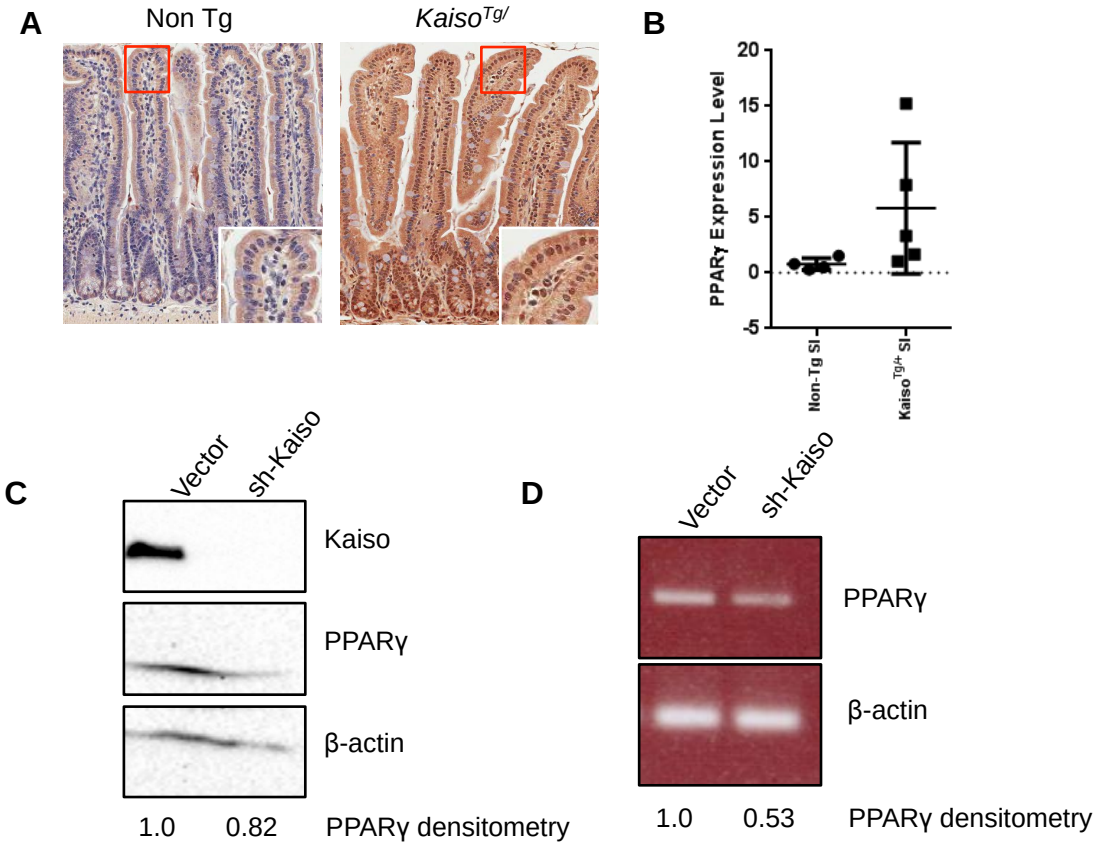


Figure A2.1. Kaiso promotes the expression of PPARγ in colon cells. A) Immunohistochemistry of PPARγ shows increased PPARγ staining in small intestines of *Kaiso*^{Tg} mice. **B)** qRT-PCR analysis (*performed by Dr. Roopali Chaudhary*) shows increased *PPARγ* mRNA expression in *Kaiso*^{Tg} small intestines compared to NonTg mice. Stable HC116 pRS-empty and pRS-Kaiso cells were analyzed by **C)** western blot and **D)** RT-PCR for PPARγ and Kaiso expression. Expression levels were quantified by densitometry and show reduced PPARγ mRNA expression in Kaiso-depleted cells compared to vector control.

A2.3.2 Kaiso does not interact with the PPARG proximal promoter region.

While Kaiso functions primarily as a transcriptional repressor (Kelly & Daniel 2006), it has also been shown to exhibit trans-activational properties (Bassey-Archibong *et al.*, 2016, Blattler *et al.*, 2013, Koh *et al.*, 2014, Rodova *et al.*, 2004). Since we observed that Kaiso positively regulates PPARγ expression, the *PPARG* gene was analyzed for full- and core-KBS sequences to determine whether Kaiso might directly regulate *PPARγ* expression. We searched a region spanning -3000bp to +1000bp surrounding the transcriptional start site for putative KBS sequences and found 5 putative core-KBSs and one putative full-KBS (Appendix Fig. A2.2 B). Chromatin from HCT116 cells was immunoprecipitated with Kaiso 6F, Histone H3 (positive control) or Non-specific IgG (negative control) antibodies, and purified DNA was quantified by qPCR. Interestingly however, Kaiso did not associate with any of the putative KBS sequences identified in our search (Appendix Fig. A2.2 B), hinting that Kaiso does not regulate *PPARγ* expression via binding to these sites.

Previous studies in 3T3-L1 preadipocytes have found that Kaiso:SMRT protein complexes bind to the consensus TCTCGCGAGA in promoter proximal regions in a methylation-dependent manner (Raghav *et al.*, 2012). However, we did not find this sequence in the *PPARG* proximal promoter region, nor was PPARG identified as a Kaiso:SMRT target in the ChIP-seq dataset by Raghav and colleagues (Raghav *et al.*, 2012). These data suggest that Kaiso likely does not directly regulate *PPARγ* transcription.

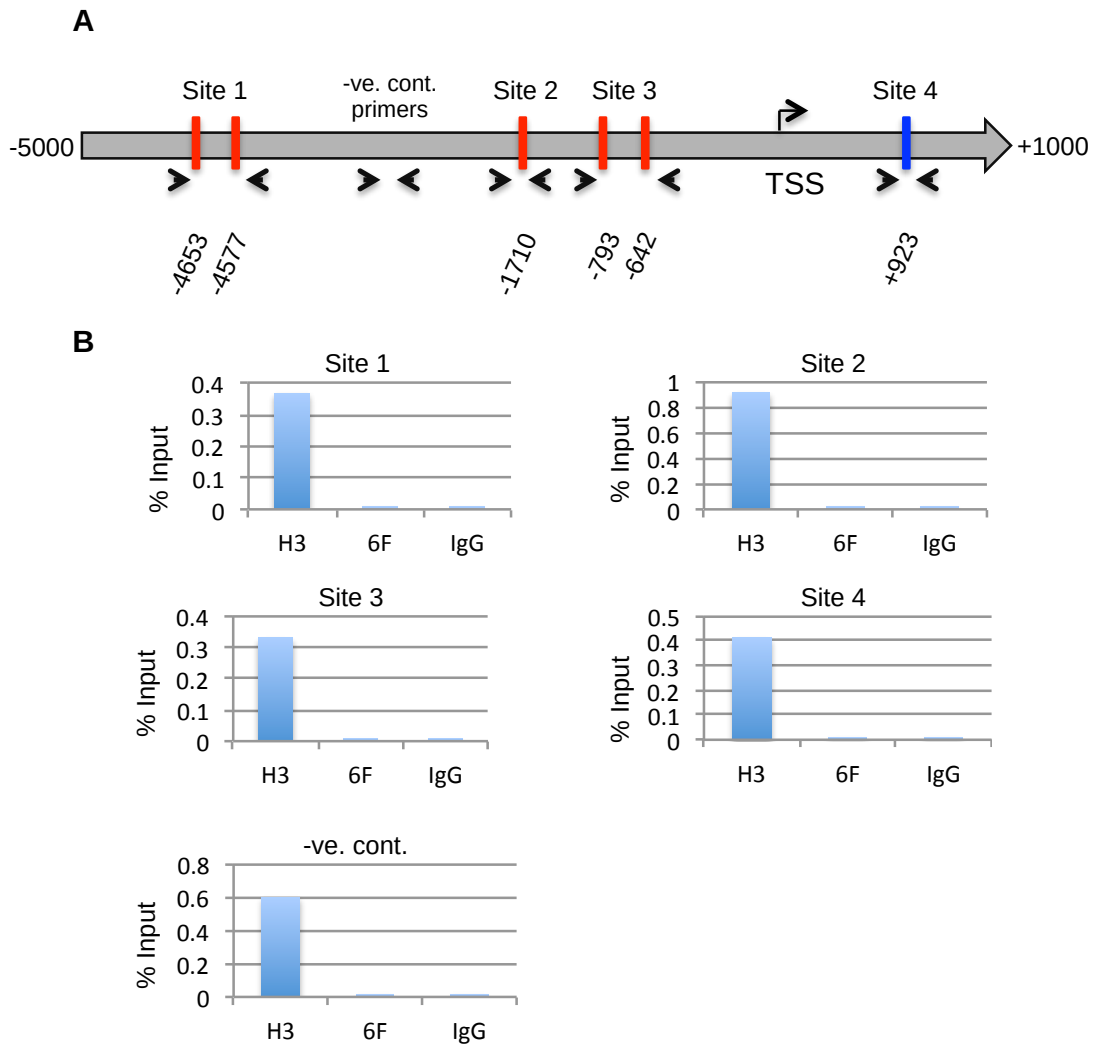


Figure A2.2. Kaiso does not interact with the *PPARG* promoter region. A) Schematic representation of the *PPARG* gene promoter analyzed by chromatin IP**.** Arrowheads denote location of primers used. Red bars – putative core KBS; Blue bar – putative full KBS. **B)** Chromatin from HCT116 cells was subject to ChIP-qPCR analysis of the *PPARG* promoter. Histone H3 and non-specific IgG antibodies were included as positive and negative control IPs, respectively.

A2.3.4 Kaiso-depletion attenuates PPARγ-mediated transactivation of the PPRE.

Since we found that Kaiso indirectly promotes PPARγ expression, we next wanted to investigate the consequences of Kaiso misexpression on PPARγ function. To this end, an artificial promoter-reporter construct containing 3 tandem copies of the murine PPRE (Appendix Fig. A2.3 A) was co-transfected with increasing amounts of pRS-Kaiso into HCT116 cells. As expected, transfection of pcDNA3-FLAG-PPARγ alone activated the PPRE (Appendix Fig. A2.3 B) and consistent with our hypothesis, Kaiso depletion (i.e. co-transfection with pRS-Kaiso) resulted in a dose-dependent decrease in PPRE-activity.

A2.3.5 Kaiso and PPARγ interact in a protein complex.

Since we found that Kaiso promotes PPARγ expression and function, but does not bind to the *PPARG* proximal promoter region, we sought to elucidate other possible mechanisms by which this might occur. To this end, IF and co-immunoprecipitation (coIP) were performed to determine whether both proteins co-localize and interact in a protein complex, respectively. IF revealed that Kaiso and PPARγ colocalize in the nucleus of HCT116 cells (Appendix Fig. A2.4 A). To determine whether Kaiso and PPARγ co-exist in a protein complex, stable shKaiso scrambled control and shKaiso HCT116 cells were co-immunoprecipitated using Kaiso- and PPARγ-specific antibodies; immunoprecipiation with anti-GFP antibody served as a negative control. A notable interaction between Kaiso and PPARγ was observed in control HCT116 cells expressing Kaiso, which was reduced in Kaiso-depleted cells (Appendix Fig. A2.4 B). While the significance of this interaction

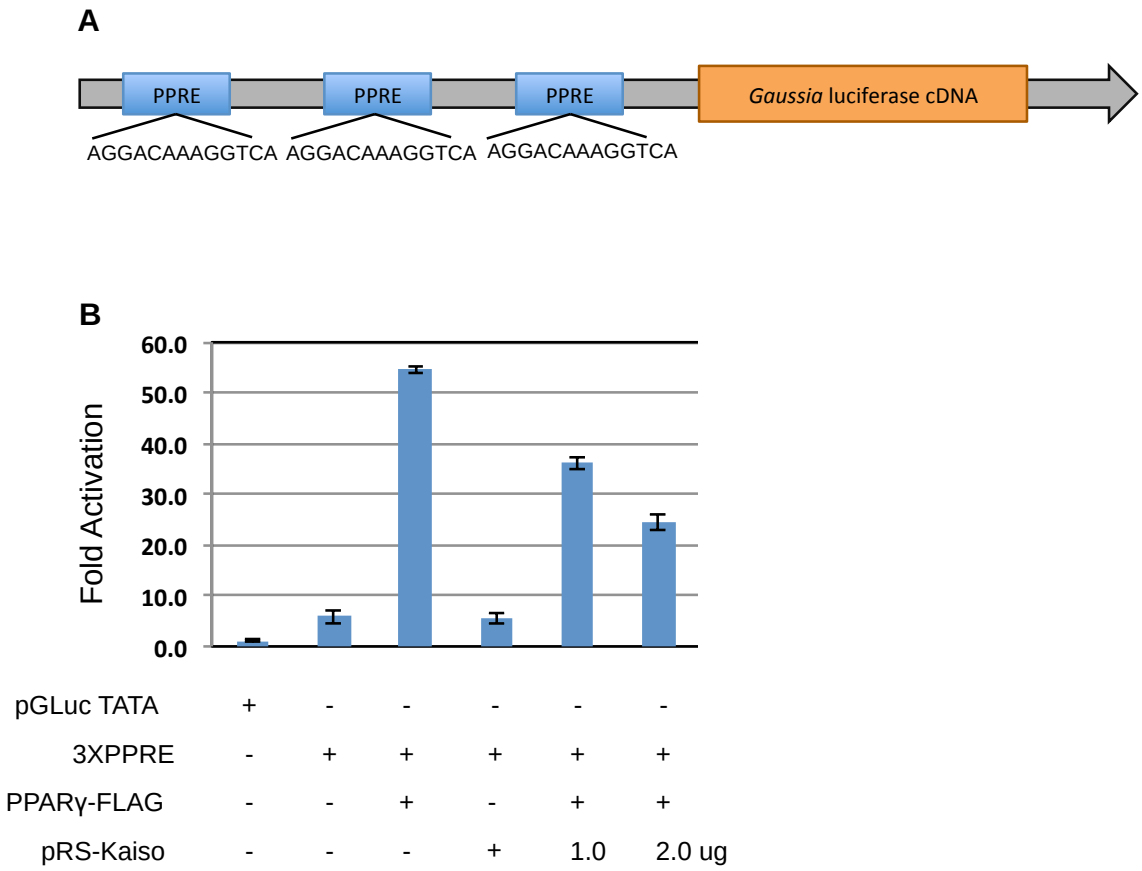


Figure A2.3. Kaiso-depletion attenuates PPARγ-mediated trans-activation. A) Schematic representation of the 3XPPRE-luciferase construct used in this study. **B)** HCT116 cells were co-transfected with pGLuc-3XPPRE, pcDNA3-FLAG-PPARγ and pRS-Kaiso. PPARγ-mediated transcriptional activation is reduced upon Kaiso-depletion.

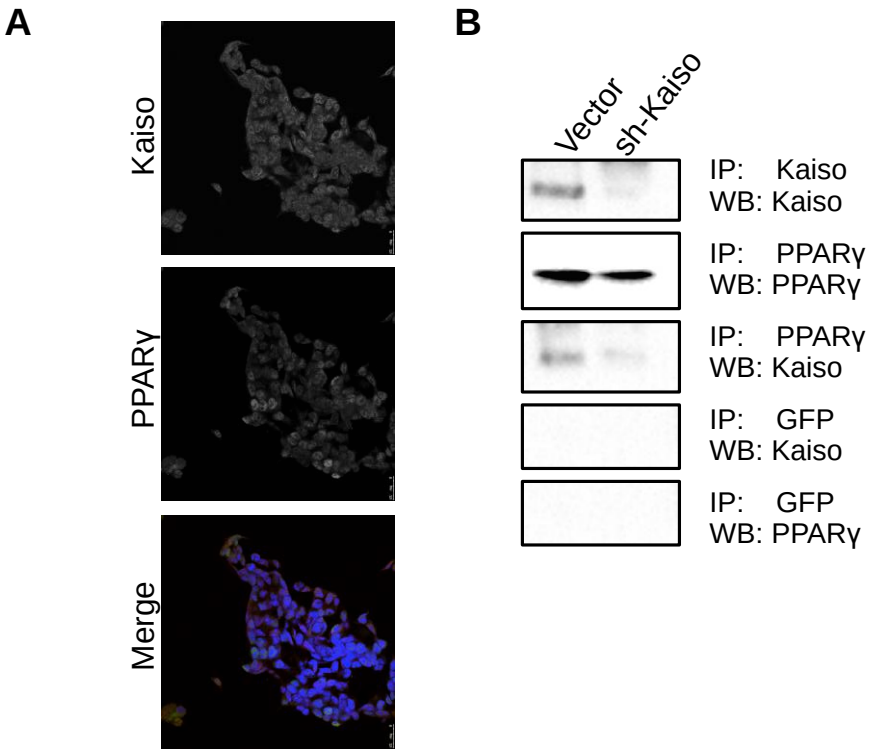
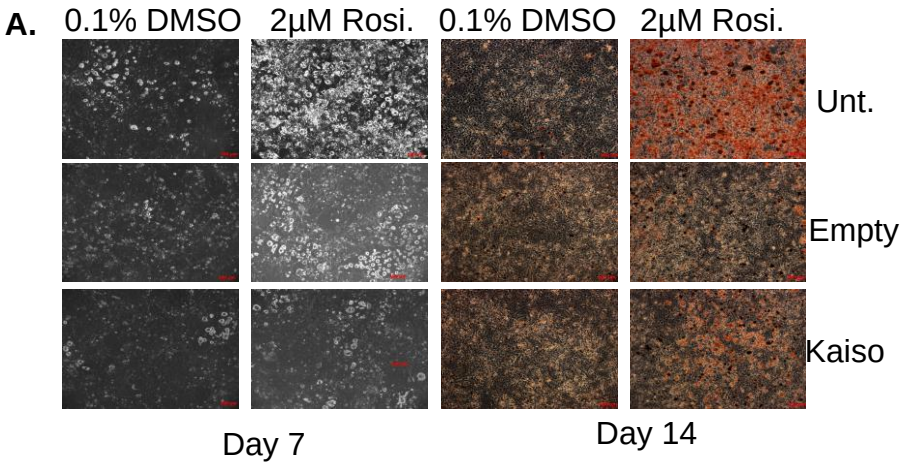


Figure A2.4. Kaiso and PPARγ co-localize and interact in a protein complex in HCT116 cells. (A) Kaiso colocalizes with PPARγ in the nucleus of HCT116 cells. **(B)** HCT116 pRS-empty and pRS-Kaiso cells were immunoprecipitated with Kaiso and PPARγ-specific antibodies; GFP was included as a negative control. Kaiso and PPARγ were found to interact in a protein complex. In agreement with previous findings, less PPARγ was precipitated in Kaiso-depleted cells.

remains to be elucidated, studies of other POZ-ZF transcription factors have shown altered transcriptional activities depending on their heterodimerization partner (Bowen *et al.*, 2002, Brenner *et al.*, 2005, Donaldson *et al.*, 2010, Herold *et al.*, 2002).

A2.3.6 The effects of Kaiso overexpression on in vitro differentiation of 3T3-L1 cells.

In culture, confluent 3T3-L1 preadipocytes exhibit contact inhibition. When induced to differentiate, growth-arrested 3T3-L1 cells re-enter the cell cycle and undergo mitotic clonal expansion (Tang *et al.*, 2003), followed by subsequent cell cycle arrest and terminal differentiation as PPARγ levels increase (Ntambi & Young-Cheul 2000). To complement studies by Raghav and colleagues, which showed accelerated 3T3-L1 differentiation upon Kaiso depletion (Raghav *et al.*, 2012), pilot experiments were conducted to overexpress Kaiso in 3T3-L1 cells. Confluent 3T3-L1 preadipocytes were transiently transfected with pcDNA3.1-Kaiso or pcDNA3-empty and induced to differentiate as outlined in Table A2.1. Untransfected 3T3-L1 cells (positive control) treated with differentiation medium containing 2 µM rosiglitazone (DMI+Rosi) exhibited greatly enhanced differentiation compared to those treated with DMI+DMSO at Day 7 and Day 14 (Appendix Fig. A2.5). Differentiation was significantly hindered in 3T3-L1 cells by transient transfection of the pcDNA3.1 empty vector. Nevertheless, fewer differentiated adipocytes were observed when transfected with Kaiso compared the empty vector control at Day 7 (Appendix Fig. A2.5). However, no marked differences in differentiation were observed between Kaiso-transfected and vector control cells at Day14 (Appendix Fig. A2.5).



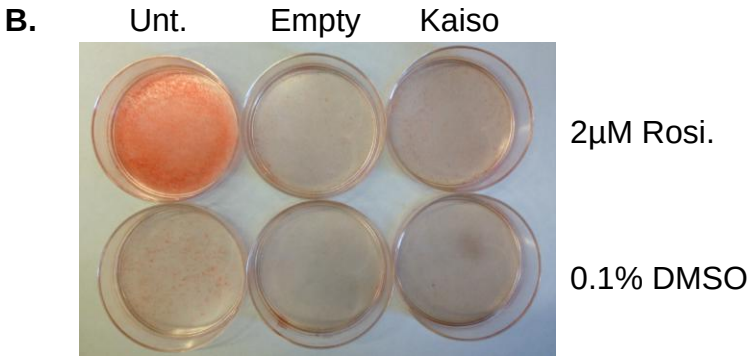


Figure A2.5. The effects of Kaiso-overexpression on PPARγ-mediated 3T3-L1 cell differentiation. (A, B) Cells were induced to differentiate in the presence or absence of rosiglitazone (Rosi). Fewer adipocytes are observed in cells transiently transfected with pcDNA3.1-Kaiso when compared to the empty control after 7 days of differentiation. Cells were stained with Oil Red O at the end of differentiation and enhanced differentiation was observed 14 days post-induction in Kaiso-transfected cells.

A2.4 Conclusions

Contrary to our hypothesis, preliminary findings from the Kaiso-PPARγ study suggest that Kaiso functions to promote PPARγ expression and function in intestinal cells, although the precise mechanism by which this occurs warrants further investigation. Our findings are unexpected since 1) published reports demonstrate that Kaiso inhibits adipogenesis, where PPARy is a key mediator; and 2) $Kaiso^{Tg}$ mice exhibit increased intestinal inflammation, and PPARγ has been shown to play a protective role in IBD. Thus, the data presented here suggests that intestinal inflammation in $Kaiso^{Tg}$ mice is unlikely mediated through negative regulation of PPARγ. Since Kaiso and PPARγ were found to interact in a protein complex, it is possible that this interaction precludes PPARγ's ability to bind and inhibit pro-inflammatory proteins, such as NFkB. However, additional studies are required to ascertain the precise biological role of the Kaiso/ PPARγ interaction.

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