THE BELLY DANCE OF KAISO IN MURINE INTESTINES

OVEREXPRESSION OF THE TRANSCRIPTION FACTOR KAISO IN MURINE INTESTINES INDUCES INFLAMMATION

By ROOPALI CHAUDHARY, B.Sc., M.Sc.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree of Doctor of Philosophy

DOCTOR OF PHILOSOPHY (2015) **McMaster University**

(Biology) **Hamilton, Ontario**

TITLE: Overexpression of the transcription factor Kaiso in murine intestines induces inflammation.

AUTHOR: Roopali Chaudhary, B.Sc. (University of Toronto), M.Sc. (University of Waterloo)

SUPERVISOR: Dr. Juliet M. Daniel

NUMBER OF PAGES: xv; 171

ABSTRACT:

Since the discovery of the $p120^{ctn}$ binding partner, Kaiso, a BTB/POZ transcription factor, several studies have implicated the protein in both development and tumourigenesis. Most information about Kaiso's function in vertebrate development has been gleaned from studies in *Xenopus laevis* embryos where Kaiso negatively regulates the Wnt signalling pathway. Since the Wnt signalling pathway is crucial in intestinal development, intestinal-specific Kaiso overexpressing mice were generated and characterized to elucidate Kaiso's role in a mammalian context. Kaiso transgenic ($Kaiso^{Tg/+}$) mice were viable and fertile but developed gross histopathological changes in the small intestine. The $Kaiso^{Tg/+}$ mice exhibited enlarged crypts accompanied by increased secretory cell differentiation reminiscent of inhibition of the Notch pathway. Indeed, the Notch effector protein, HES1, is decreased in $Kaiso^{Tg/+}$ mice. Additionally, $Kaiso^{Tg/+}$ mice display a neutrophil-specific intestinal inflammation reminiscent of the knockdown of $p120^{ctn}$. Interestingly, the $Kaiso^{Tg/+}$ mice display decreased $p120^{ctn}$ localization at the membranes and an increase in the neutrophil adhesion molecule, ICAM-1, both of which induce neutrophilia. Notably, the $Kaiso^{Tg/+}$ mice developed multiple crypt abscesses over time due to massive neutrophil infiltration of the epithelial cell layers. This is the first study to examine the *in vivo* roles of Kaiso in a mammalian context and our findings suggest a regulatory role for Kaiso in the inflammatory and Notch signalling pathways.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my committee: Dr. Roger Jacobs and Dr. Bradley Doble for their consistent guidance throughout my Ph.D., and a big THANK YOU goes to Dr. Juliet M. Daniel who took in a wide-eyed *Drosophila* geneticist and gave her the opportunity to explore a different field of research. You have mentored, coached, and guided me in more than just scientific research. Thank you for all the wonderful talks we have had, for being a source of guidance beyond the walls of the lab...and *thank you* for your vote of confidence in me. In addition to my committee, I would also like to thank Dr. Elena Verdu for her guidance in my project and for being a member of my comprehensive exam.

There are many people beyond my committee who helped me attain this milestone while helping me balance work and life. To my Main B's: Sonali Weerawardane, Shari Yasin, Christina Pierre, Shaiya Robinson, and, honorary member, Blessing Bassey, you guys have been a huge source of confidence, inspiration, and encouragement. Sonali, my brown counterpart in the lab, we have spent many a conversation pondering life. You have entertained my many ridiculous monologues about "scientific" explanations for zombies and vampires. Shari, our line dances in the lab were always a lot of fun. Christina, thank you for being the voice of reason on so many occasions. You can deny it as much as you like, but you are a true nerd at heart, too! I am glad to have shared my writing experience alongside you. Shaiya, my nerdy counterpart, you definitely appreciated my "jokes", and participated in quite a few "scientific" conversations about pop culture. Your random lyrics were always entertaining, and your reviews of my baking have always been on point. Blessing, we had a lot of conversations about life. I have loved talking about how similar our cultures are despite evolving independently in different continents. My Main B's, we have all been through a lot together! I am glad you were and are a continual presence in my life! Here's to many more milestones together ☺!!

There a lot of Daniel lab members, past and present, without whom completion of this thesis would not have been possible. Dr. Nickett Donaldson, your wise words have always been well-timed. You always knew what to say and how to express it. Dr. Snezana Milosavljevic, your research hands have been a huge help for me to complete this thesis. I have learned so much from you about patience and persistence! A special thank you goes to Daria Wojtal—you were my right hand person for almost 3 years!!!! We struggled together through so many IHC stains and microscopy sessions, leading to a quick run to the Phoenix for a shot…all good memories! I have seen you grow from a curious little second year student to a successful Master's student who now trains other students. You have made me proud little one! There were a multitude of other undergrads that helped out either directly or indirectly by keeping me sane in the lab. Shivanie Skandarajah, Joseph Longo, Lloyd He, Erin Walters, Kirandeep Kirpal, Meaghan Mavor, Catherine Crawford-Brown—all of you were an integral part of my Ph.D. career. I thank you all for being part of the Daniel Lab.

Outside the walls of the lab, there were many other people who directly helped me succeed in completion of my doctoral studies. A big THANK YOU to the Central Animal Facility staff, especially, Lillian Robson, Wendy Whittaker, Jackie Ferreira, April Scott, and Trish VanHoek—you were all vital for my success with mice! I learned a lot about mouse husbandry from you all! You were kind and patient with me while I learned how to handle and manage my mouse colonies. Your advice was always appreciated. Thank you very much! Additional thanks to the Jordana Lab—thank you for being patient with me while I finished my doctoral studies, and for all your encouragement that there is light at the end of the tunnel.

I cannot ignore all the people in my personal life that provided me with continual support, encouragement, and even a much needed ear for all my moans throughout the course of my studies. To Arshdeep, Sonia, Nina, Jumana, Joseph, and Emma: I have seen you all go through a lot in life, and you have been there to support me through all my lows and encouraged me to soar through all the difficulties. Your continual support has

been a huge boost to my self-esteem. Your words of encouragement kept me going, and you have all been inspirational in more ways than you know it. I cannot think of a life without any of you!

Saving the best for last: my source of identity and the people without whom I know I would have never succeeded: to my family, I love you and thank God that I have had you as my pillars throughout life. To Mom and Dad, you provided me with best of everything, and with this degree, I hope that I have been able to make you two the proudest and most boastful parents around☺. To Bhaiya and Bhabhi, though you may be far away, your words of encouragement and push to succeed were always by my side. And last but definitely not least, to Shefali, my little baby. You may be younger, but there were times when you were the older sister. You listened to all my practice presentations; you know about my research, you stayed by my side regardless of how ridiculous (or embarrassing) I was acting… you are my biggest fan! Our bickering and fights helped relieve stress, and I am so proud and lucky to have you as my sister. I love you all very much, and I dedicate this degree to you all! It is as much yours as it is mine.

Finally, I would like to thank God for all the wonderful people in my life. This career path would have been a lot more difficult without you all. I cannot believe I got here….everyone is coming to the party afterward! Yay!

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS, GRAPHS AND FIGURES

LIST OF TABLES

N/A

LIST OF ABBREVIATIONS AND SYMBOLS

AJ	**A**dherens **j**unction
Apc	**A**denomatous **P**olyposis **C**oli
AREB	**A**nimal **R**esearch **E**thics **B**oard
Atoh1	**Ato**nal **h**omolog **1**
AUP	**A**nimal **U**tilization **P**rotocol
Axin	**Ax**is **in**hibitor
β-TrCP	**β-t**ransducin **r**epeats-**c**ontaining **p**rotein
BMP	**B**one **M**orphogenic **P**rotein
BSA	**B**ovine **s**erum **a**lbumin
BTB/POZ	**B**ric a brac, **T**ramtrak, **B**road complex/**Po**x virus and **z**inc finger
C/EBP	**C**CAAT/**e**nhancer **b**inding **p**rotein
CAF	**C**entral **A**nimal **F**acility
CD	**C**rohn's **D**isease
ChIP	**Ch**romatin **i**mmuno**p**recipitation
CK1α	**C**asein **k**inase **1-α**
CRC	**C**olo**r**ectal **c**ancer
CSL	**C**BFI/**S**uppressor of Hairless/**L**ag2
Dll	**D**e**l**ta-**l**ike
Dsh	**D**i**sh**evelled
DSL	**D**elta/**S**errate/**L**ag-2
DSS	**D**extran **s**odium **s**ulfate
DTT	**D**i**t**hio**t**hreitol
E-cadherin	**E**pithelial **cadherin**
EDTA	**E**thylene**d**iamine**t**etra**a**cetic acid
EEC	**E**ntero**e**ndo**c**rine
FAP	**F**amilial **A**denomatous **P**olyposis
FBS	**F**etal **b**ovine **s**erum
FITC	Fluorescein **i**so**t**hio**c**yanate
FMO	**F**luorescence **m**inus **o**ne
Fz	**F**ri**z**zled
GI	**G**astro**i**ntestinal
GSK3	**G**lycogen **s**ynthase **k**inase **3**
H&E	**H**aematoxylin and **e**osin
HDAC	**H**istone **de**a**c**etylase
Hes	**H**airy/**E**nhancer of **S**plit
Hh	**H**edge**h**og

HTAB	**H**exadecyl**t**rimethyl**a**mmonium **b**romide
IBD	**I**nflammatory **B**owel **D**isease
ICAM-1	**I**ntercellular **a**dhesion **m**olecule-**1**
IEC	**I**ntestinal **e**pithelial **c**ell
IHC	**I**mmuno**h**isto**c**hemistry
Jag	**Jag**ged
JNK	**J**un **N**-terminal **k**inase
Kaiso$^{Tg/+}$	**Kaiso t**rans**g**enic
KBS	**K**aiso **B**inding **S**equence
LEF	**L**ymphoid **e**nhancer **f**actor
LI	**L**arge **i**ntestine
LPS	**L**ipo**p**oly**s**accharide
LRP	**L**ipoprotein **r**eceptor-related **p**rotein
Mam	**Ma**ster**m**ind
MATH1	**M**ouse **at**onal **h**omolog **1**
Min	**M**ultiple **i**ntestinal **n**eoplasms
mKaiso-MT	**M**yc-**t**agged **m**urine ***Kaiso***
MMP7	**M**atrix **m**etallo**p**roteinase 7
MPO	**M**yelo**p**er**o**xidase
mSin3A	**M**ammalian **S**wi-**in**dependent **3A**
NCoR	**N**uclear **co**r**r**epressor
NDS	**N**ormal **d**onkey **s**erum
NECD	**N**otch **e**xtra**c**ellular **d**omain
NEXT	**N**otch **ex**tracellular **t**ransmembrane
NFκB	**N**uclear **f**actor **k**appa-light-chain-enhancer- of activated **B** cells
NGS	**N**ormal **g**oat **s**erum
NICD	**N**otch **i**ntra**c**ellular **d**omain
NK	**N**atural **k**iller
non-Tg	**N**o**n**-**t**rans**g**enic
p120^{ctn}	**p120**-**c**a**te**n**i**n
PAS	**P**eriodic **A**cid-**S**chiff
PBS	**P**hosphate-**b**uffered **s**aline
PCR	**P**olymerase **c**hain **r**eaction
PDGF	**P**latelet-**d**erived **g**rowth **f**actor
PI	**P**ropidium **i**odide
PMN	**P**oly**m**orpho**n**uclear
PPARγ	**P**eroxisome **p**roliferator-**a**ctivated **r**eceptor-γ
RT-PCR	**R**everse **t**ranscriptase **PCR**

SAM	**S**enescence **a**ccelerated **m**ouse
SDS	**S**odium **d**odecyl **s**ulfate
SI	**S**mall **i**ntestine
SMRT	**S**ilencing **m**ediator of **r**etinoid and **t**hyroid receptor
SOP	**S**tandard **O**perating **P**rocedure
SPF	**S**pecific **p**athogen **f**ree
TBS-T	**T**ris-**b**uffered **s**aline with 0.05% **T**ween-20
TCF	**T**-**c**ell **f**actor
TFIID	**T**ranscription **f**actor **II D**
TJ	**T**ight **j**unction
TLE	**T**ransducin-**l**ike **e**nhancer of Split
TLR	**T**oll-**l**ike **r**eceptor
TNF	**T**umour **n**ecrosis **f**actor
Ub	**Ub**iquitinated
UC	**U**lcerative **C**olitis
Wnt	**W**ingless-type MMTV i**nt**egration site family member
ZO	**Z**onula **o**ccludens

CHAPTER 1: INTRODUCTION

The **g**astro**i**ntestinal (GI) tract is an important and fascinating vital organ system. In addition to digesting food and facilitating the uptake of nutrients, it also provides an internal line of defense against pathogens and other harmful agents, while allowing beneficial microbiota to flourish. Accomplishing these diverse tasks requires a healthy GI tract be maintained through the delicate balance of proper homeostasis. Disruptions in pathways involved in regulating intestinal homeostasis, such as the canonical Wnt (hereafter Wnt) and Notch signalling pathways, greatly increases the risk of developing diseases such as **I**nflammatory **B**owel **D**isease (IBD) and **C**olo**r**ectal **C**ancer (CRC).

Canada has one of the highest incidence rates of IBD in the world; ~1/150 Canadian live with IBD (~233,000 Canadians), and ~10,000 new cases are diagnosed every year (Canadian Colitis Association Annual Review, 2013). Due to the persistent inflammation of the colon, young patients diagnosed with IBD are at increased risk of developing CRC which accounts for ~13% of all newly diagnosed cancers in Canada, making it the second most diagnosed cancer in males and third in females (Canadian Cancer Society, 2014; Lutgens *et al.*, 2013). It is hypothesized that sporadic CRC and IBD both result from the same genetic mutations, though the exact mechanism is yet to be elucidated (reviewed in Ahmadi *et al.*, 2009). In this study, we investigated the role of the novel transcription factor, Kaiso, in intestinal homeostasis and its contribution to chronic intestinal inflammation.

1.1 INTESTINAL ANATOMY

Functionally, the intestines are segmented into the small and large intestines, which are responsible for nutrient and water absorption respectively (De Mey and Freund, 2013). The mucosa of the **s**mall **i**ntestine (SI) is organized into finger-like projections called villi, which serve to maximize the surface area for nutrient absorption by extending into the lumen of the gut (Vanuytsel *et al.*, 2013). Neighbouring villi are separated by deep invaginations, called crypts, where the stem cells and the highly proliferative progenitor cells reside (Vanuytsel *et al.*, 2013).

The SI epithelium is comprised of several short-lived cell types that must be continually renewed through differentiation of the progenitor cells located near the base of the crypts (Solanas and Batlle, 2011). The undifferentiated progenitor cells give rise to five known epithelial cell types that populate the crypt-villus axis; they are the absorptive enterocytes, mucous-secreting goblet cells, hormone-releasing **e**ntero**e**ndo**c**rine (EEC) cells, antimicrobial peptide-secreting Paneth cells, and the newly discovered tuft cells whose function is unclear (Solanas and Batlle, 2011). All but one (Paneth cells) of these intestinal epithelial cell types migrate up from the base of the crypts and are eventually shed into the lumen of the intestine at the villi tips after 3-5 days (Solanas and Batlle, 2011). The absorptive enterocytes are the most abundant cell type in the intestine and their primary function is nutrient absorption (Noah *et al.*, 2011). As the most abundant cells in the intestines, enterocytes come into contact with the external environment and act as the host's first line of defense against potentially harmful agents (microbes and/or chemicals) (Pastorelli *et al.*, 2013). Goblet cells are the second-most abundant cell type

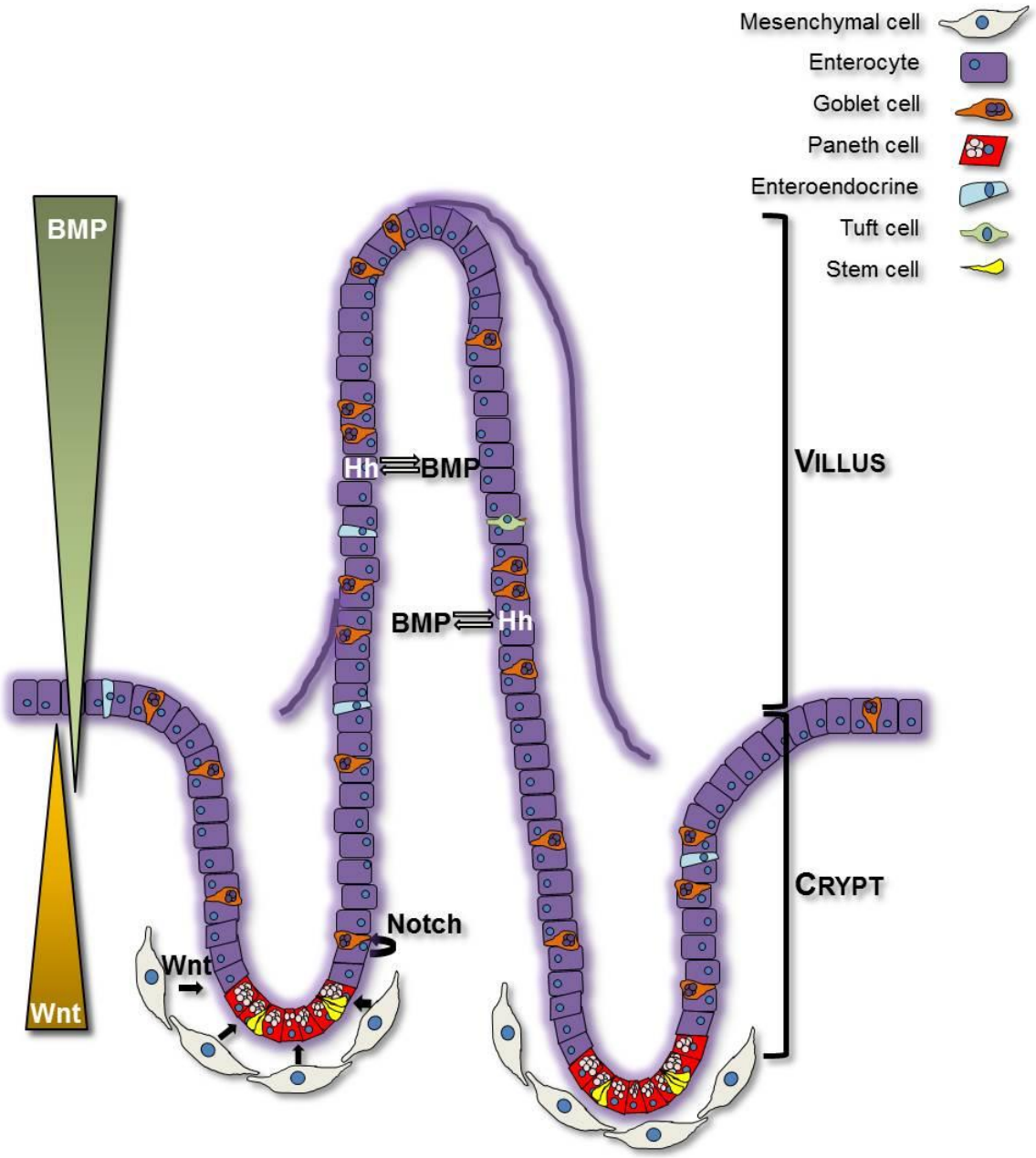
and they produce mucus that further protects the mucosal surface and reinforces the intestinal barrier (Pastorelli *et al.,* 2013). Unlike the other intestinal cell types, Paneth cells migrate downwards towards the crypt base where they localize and reside alongside intestinal stem cells (Solanas and Batlle, 2011) (Figure 1.1). Paneth cells secrete antimicrobial peptides that are involved in excluding bacteria from the epithelial surface to further establish a biochemical barrier in the SI (Peterson and Artis, 2014). Finally, the EEC and tuft cells represent <1% of all intestinal epithelial cells and are scattered throughout the crypts and the villi (Solanas and Batlle, 2011) (Figure 1.1). EEC cells are further differentiated into 16 subtypes, which secrete various gut hormones, and are associated with glucose homeostasis and thus energy intake (Noah *et al.*, 2011).

Unlike the small intestines, the mucosa of the **l**arge **i**ntestine (LI) is comprised of a flattened surface that lacks villi but is separated into crypts (Noah *et al.*, 2011). The epithelial precursor cells give rise to absorptive colonocytes, EEC, and goblet cells. These differentiated cells are present on the surface epithelium and the top third of the crypts of the LI (Kapoor *et al.*, 2007). The LI is responsible for most of the water and vitamin absorption (De Mey and Freund, 2013).

A healthy intestinal epithelium requires interactions between the epithelial cells and the surrounding mesenchyme, along with proper differentiation, proliferation, and communication between cells and beneficial microbes, all of which helps to maintain overall intestinal homeostasis (Maloy and Powrie, 2011).

Figure 1.1: Small intestine anatomy and homeostasis.

A depiction of a villus with connected crypts where progenitor cells contribute to the renewal of differentiated epithelial cells. Stem cells lie near the crypt base, intercalated between the Paneth cells. Differentiated epithelial cells that migrate up the crypt-villus axis include the enterocytes, goblet cells, enteroendocrine cells and the tuft cells. Various signalling pathways are involved in maintaining intestinal homeostasis and there is a gradient of signalling activity along the crypt-villus axis. The Hedgehog (Hh), Bone morphogenic protein (BMP), Wnt and Notch pathways function in different regions along the crypt-villus axis. Hh and BMP signalling occurs primarily in the apical region of the villus whereas the Wnt and Notch signalling pathways are active within the crypts of the intestines. Wnt signalling decreases from the crypts to the villi while BMP signalling increases. Wnt and Notch signalling are concentrated in the crypts where they influence differentiation and proliferation of the progenitor cells. Original illustration inspired from (Gregorieff and Clevers, 2005).


Mesenchymal cell
Enterocyte
Goblet cell
Paneth cell
Enteroendocrine
Tuft cell
Stem cell
BMP
Hh
BMP
BMP
Hh
VILLUS
CRYPT
Notch
Wnt
Wnt

1.2 INTESTINAL HOMEOSTASIS

Intestinal homeostasis refers to the proper maintenance of the intestine's internal environment, i.e. regulation of the microbiota, host innate immune system and the intestinal epithelial cells (Maloy and Powrie, 2011). Intestinal homeostasis requires a continual renewal of the single cell layered epithelium every 3-5 days. This epithelial layer maintains the physical barrier separating the intestinal microbiota from the underlying tissues, thus preventing bacterial infiltration (Vereecke *et al.*, 2011). A complex network of signalling pathways work together to ensure the proper regeneration of the intestinal epithelium. This network includes pathways such as the **H**edge**h**og (Hh), the **p**latelet-**d**erived **g**rowth **f**actor (PDGF), the **b**one **m**orphogenic **p**rotein (BMP), the Wnt, and the Notch signalling pathways, all of which function in different regions along the crypt-villus axis (reviewed in Crosnier *et al.*, 2006; Vanuytsel *et al.*, 2013). The Hh and PDGF signals are produced within the epithelium and their respective receptors are found in the mesenchymal cells (reviewed in Crosnier *et al.*, 2006). On the other hand, Wnt ligands are produced by the mesenchyme while the Wnt receptors are found on the epithelial cells (Crosnier *et al.*, 2006). Both BMP signals and receptors are found in the mesenchymal and epithelial cells, while Notch signals and receptors are found only on the epithelial cells (reviewed in Crosnier *et al.*, 2006; Vanuytsel *et al.*, 2013). The Hh, PDGF and BMP pathways are all involved in establishing the villar and crypt compartments of the intestines and are concentrated within the villar regions (reviewed in Vanuytsel *et al.*, 2013). In contrast, Wnt and Notch signalling are involved in maintaining the proliferation and differentiation within the stem cell compartment of the crypts, and

are thus concentrated within the crypt regions (reviewed in Vanuytsel *et al.*, 2013). This study focuses on the latter two pathways, which are discussed in greater detail below.

1.2.1 The Wnt signalling pathway

The Wnt signalling pathway has been extensively studied in the mammalian intestines where it plays critical roles in stem cell maintenance and proliferation (Solanas and Batlle, 2011; Vereecke *et al.*, 2011). The Wnt signalling pathways are categorized into the β-catenin dependent canonical pathway and the β-catenin independent non-canonical pathway (Rao and Kühl, 2010). The relative concentration of the Wnt ligands and receptors in the tissue microenvironment determines the downstream activation of either the canonical (detailed below) or the non-canonical Wnt pathways (Rao and Kühl, 2010). The non-canonical Wnt pathway involves different intracellular mediators (**j**un **N**-terminal **k**inase (JNK) or calcium) which determine planar cell polarity or tissue development respectively (Rao and Kühl, 2010).

The key effector protein of the canonical Wnt signalling pathway is the Armadillo catenin, β-catenin, which exists in two pools; one membrane-bound and the other cytoplasmic (Clevers and Nusse, 2012). The membrane-bound β-catenin pool interacts with other membrane-bound proteins to anchor the transmembrane cell adhesion protein **e**pithelial (E)-cadherin, and strengthen cell-cell adhesions (Hartsock and Nelson, 2008). In contrast, the cytoplasmic pool of β-catenin translocates to the nucleus in response to a Wnt signal and activates Wnt target genes (Clevers and Nusse, 2012).

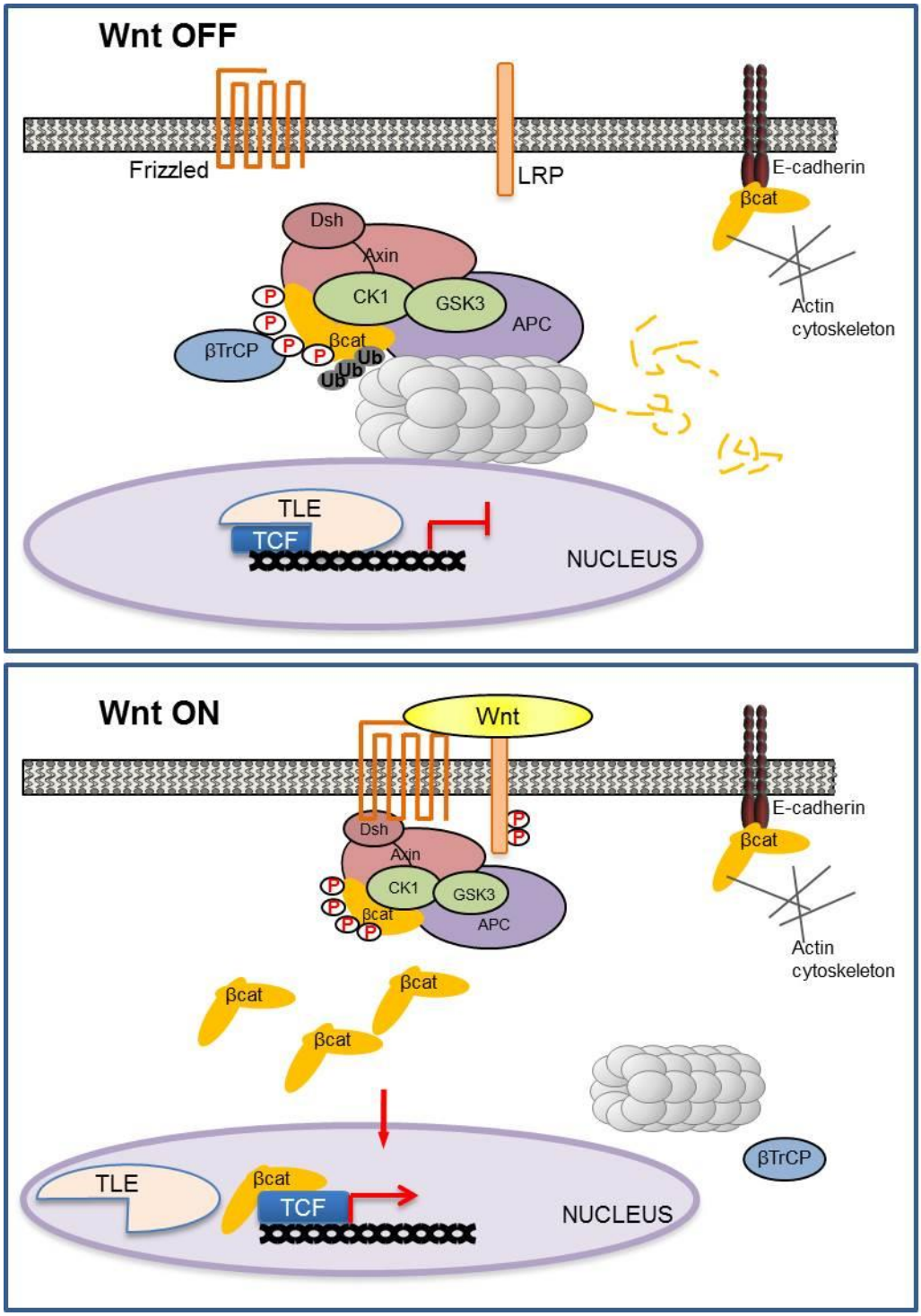
In the absence of a Wnt ligand, cytoplasmic β-catenin levels are kept low via a destruction complex that is comprised of the scaffolding proteins, **ax**is **in**hibitor (Axin),

adenomatous **p**olyposis **c**oli (APC), and **D**i**sh**evelled (Dsh), along with the kinases: **g**lycogen **s**ynthase **k**inase **3** (GSK3) and **c**asein **k**inase **1-α** (CK1α) (Clevers and Nusse, 2012). Cytoplasmic β-catenin is bound by Axin and phosphorylated first by CK1α, which primes β-catenin for subsequent phosphorylation by GSK3. Phosphorylated β-catenin is targeted for ubiquitination by β-TrCP (**β-t**ransducin **r**epeats-**c**ontaining **p**rotein), a component of the E3 ubiquitin ligase complex. Ubiquitinated β-catenin is then rapidly degraded by the proteasome, thus preventing its translocation to the nucleus (Clevers and Nusse, 2012) (Figure 1.2).

The Wnt signalling pathway is activated upon binding of a Wnt ligand to the heterodimeric receptor complex consisting of **F**ri**z**zled (Fz) and the **l**ipoprotein **r**eceptor-related **p**rotein (LRP)-5/6 co-receptor in target cells (Clevers and Nusse, 2012) (Figure 1.2). The binding of a Wnt ligand results in LRP receptor phosphorylation by GSK3, and the phosphorylated LRP then serves as a docking site for Axin (Clevers and Nusse, 2012). Dsh interacts with the cytoplasmic region of Fz, which further facilitates the Axin-LRP interaction (Clevers and Nusse, 2012). This Axin-LRP interaction prevents ubiquitination of phosphorylated β-catenin by β-TrCP, thus leading to the saturation of the destruction complex with phosphorylated β-catenin (Clevers and Nusse, 2012). Consequently, newly synthesized β-catenin translocates to the nucleus where it interacts with one of several members of the **T-c**ell **f**actor (TCF)/**l**ymphoid **e**nhancer **f**actor (LEF) transcription factors (Clevers and Nusse, 2012).

Figure 1.2: The canonical Wnt signalling pathway

In the absence of a Wnt ligand, β-catenin (β-cat) is phosphorylated (P) and ubiquitinated (Ub) by the destruction complex within the cytoplasm and is targeted for degradation. Consequently, **T**ransducin-**l**ike **e**nhancer of Split (TLE) binds TCF and Wnt target genes are repressed. However, in the presence of a Wnt ligand, the Frizzled and LRP co-receptors are activated and LRP is phosphorylated. The destruction complex then interacts with the phosphorylated LRP and is recruited to the membrane. Ubiquitination of β-catenin is blocked, and β-catenin accumulates in the cytoplasm and translocates into the nucleus where it binds TCF and co-activates Wnt target genes. Original illustration inspired from (Clevers and Nusse, 2012).


Wnt OFF
Frizzled
LRP
E-cadherin
βcat
Actin
cytoskeleton
Dsh
Axin
CK1
GSK3
APC
βTrCP
βcat
P
Ub
TLE
TCF
NUCLEUS
Wnt ON
Wnt
E-cadherin
βcat
Actin
cytoskeleton
Dsh
Axin
CK1
GSK3
APC
βcat
βTrCP
TLE
TCF
NUCLEUS

Nuclear β-catenin replaces **T**ransducin-**l**ike **e**nhancer of Split (TLE) bound to TCF and serves as a cofactor for TCF/LEF while recruiting other co-activators; this results in increased transcription and expression of Wnt target genes that promote cell proliferation (e.g. *cyclin D1, c-myc, matrilysin* and *Id2*) (Brabletz *et al.*, 1999; Crawford *et al.*, 1999; He *et al.*, 1998; Rockman *et al.*, 2001; Shtutman *et al.*, 1999; Tetsu and McCormick, 1999).

1.2.1.1 The Wnt pathway in intestinal homeostasis

The Wnt signalling pathway plays critical roles in embryonic development and regulates the development of various organs like the intestines, bone, heart, and muscle (Clevers and Nusse, 2012). Within the intestines, Wnt signalling is most active in the crypts where it is required to specify and maintain the proliferative, undifferentiated stem cell compartment, and for the maturation of Paneth cells as they migrate to the bottom of the crypts (Solanas and Batlle, 2011; Vereecke *et al.*, 2011). Thus aberrant Wnt signalling has detrimental results on the development and the maintenance of the intestines. Disruption of Wnt signalling in Tcf4 lacking mice leads to neonatal intestines devoid of proliferative cells due to undeveloped precursor crypt cells, and ultimately results in death shortly after birth (Korinek *et al.*, 1998). The tissue-specific ablation of the *β-catenin* gene results in the rapid loss of crypt structures and blocks proliferation of cells, revealing a role for Wnt signalling in maintaining intestinal homeostasis (Fevr *et al.*, 2007).

However constitutive activation of the Wnt signalling pathway leads to the development of **c**olo**r**ectal **c**ancer (CRC) (Bienz and Clevers, 2000; Clevers and Nusse, 2012; Gregorieff and Clevers, 2005). In humans, it is estimated that 80% of sporadic and hereditary CRC contain mutations in the *Apc* gene, which encodes a key component of the destruction complex; consequently there is constitutive Wnt signalling (Clevers and Nusse, 2012). In fact, germ line mutations in *Apc* are associated with **f**amilial **a**denomatous **p**olyposis (FAP) in humans, which is characterized by the spontaneous development of polyps in the colon, many of which progress to form carcinomas (Gregorieff and Clevers, 2005). The $Apc^{Min/+}$ mice, which develop **m**ultiple **i**ntestinal **n**eoplasms (Min), share similar mutations to human FAP patients, and are commonly used as models for CRC (Gregorieff and Clevers, 2005). In addition, constitutive activation of Wnt signalling via loss-of-function mutation in the *Apc* leads to hyperplasia in the intestinal crypts and impairs the differentiation of epithelial cells in murine intestines (Sansom *et al.,* 2004). The proper maintenance and functioning of the Wnt signalling pathway is thus crucial for intestinal homeostasis.

To date, several pathways have been identified that regulate Wnt signalling. In the intestines, Wnt signalling in villi is inhibited by BMP and Hh signalling, and thus Wnt signalling is most concentrated in the crypts (Kosinski *et al*., 2010; van Dop *et al*., 2009; reviewed inVanuytsel *et al*., 2013). However, within the crypts, Notch signalling also plays an important role in intestinal maintenance and cell differentiation. Wnt and Notch signalling have a significant amount of crosstalk, which is integral for the proper maintenance and differentiation of the intestinal progenitor cells (Crosnier *et al.,* 2006).

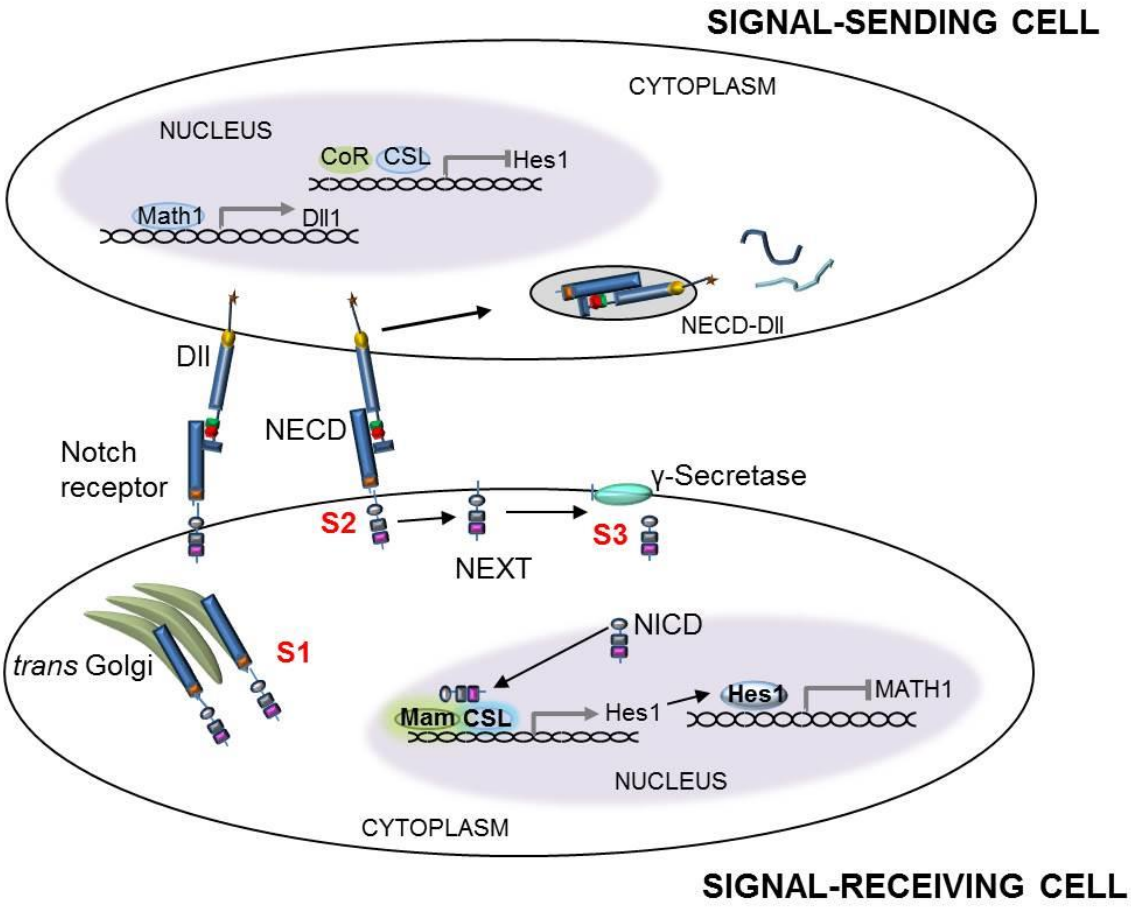
1.2.2 Notch signalling pathway

Unlike many signalling pathways, the Notch pathway works via lateral inhibition; a membrane-bound ligand on a signal-sending cell transmits its signal directly to an adjacent signal-receiving cell, while preventing Notch signalling within the signal-sending cell (Guruharsha *et al.*, 2012) (Figure 1.3). In mammals, there are five transmembrane Notch ligands belonging to the **D**e**l**ta-**l**ike (Dll1, Dll 3, and Dll4) and the **Jag**ged (Jag1 and Jag2) families, which collectively are called the **D**elta/**S**errate/**L**ag-2 (DSL) family (Guruharsha *et al.*, 2012). These ligands interact with Notch receptors (Notch 1-4) on neighbouring cells to activate the Notch signalling within the signal-receiving cells (Guruharsha *et al.*, 2012; Noah and Shroyer, 2013).

Three proteolytic cleavage steps are involved in the transmission of a Notch signal. The first proteolytic cleavage occurs in the *trans*-Golgi apparatus, and produces the mature Notch heterodimeric receptor that travels to the cell membrane (Guruharsha *et al.*, 2012). Upon interaction with the DSL ligand on a neighbouring cell, the Notch receptor undergoes a second proteolytic cleavage to produce: (1) the **N**otch **e**xtra**c**ellular **d**omain (NECD), which remains bound to the DSL ligand, but is endocytosed into the signal-sending cell, where it undergoes lysosomal degradation (Guruharsha *et al.*, 2012) (Figure 1.3); and (2) the **N**otch **ex**tracellular **t**ransmembrane (NEXT) fragment which remains anchored at the plasma membrane (Guruharsha *et al.*, 2012).

Figure 1.3: The Notch signalling pathway

Three proteolytic cleavage steps are required for the activation of canonical Notch signalling within the signal receiving cell. The first cleavage (S1) occurs in the *trans* Golgi and produces the heterodimeric Notch receptor. At the membrane, the Notch extracellular domain interacts with a ligand (Dll) on an adjacent cell (signal sending cell) and this triggers a second cleavage (S2) which removes the Notch extracellular domain (NECD). The ligand-NECD complex is endocytosed by the signal sending cell, where it undergoes lysosomal degradation. Subsequently, a third cleavage (S3) by γ-secretase releases the Notch intracellular domain (NICD) from the membrane of the signal receiving cell and the NICD then translocates to the nucleus to activate transcription of Notch target genes. Original illustration inspired from (Noah and Shroyer, 2013).


SIGNAL-SENDING CELL
CYTOPLASM
NUCLEUS
CoR
CSL
Hes1
Math1
Dll1
NECD-Dll
Dll
NECD
Notch
receptor
γ-Secretase
S2
S3
NEXT
NICD
trans Golgi
S1
Mam
CSL
Hes1
Hes1
MATH1
NUCLEUS
CYTOPLASM
SIGNAL-RECEIVING CELL

The NEXT fragment on the signal-receiving cell undergoes a third proteolytic cleavage by γ-secretase to produce the transcriptionally active **N**otch **i**ntra**c**ellular **d**omain (NICD) (Figure 1.3). NICD translocates to the nucleus of the signal-receiving cell and forms a complex with the transcription factor **C**BFI/**S**uppressor of Hairless/**L**ag2 (CSL, also known as RBPjκ), and the coactivator **Ma**ster**m**ind (Mam), which then activates target gene transcription (Guruharsha *et al.*, 2012; Noah and Shroyer, 2013). Many Notch target genes encode transcriptional regulators (e.g. **H**airy/**E**nhancer of **S**plit (Hes) and Hes-related families) that influence cell fate decisions (Guruharsha *et al.*, 2012) (Figure 1.3).

1.2.2.1 The Notch pathway in intestinal homeostasis

Maintenance and differentiation of intestinal progenitor and stem cells is regulated by the Notch signalling pathway (Noah and Shroyer, 2013). Like Wnt signalling, the Notch signalling pathway is most active within the intestinal crypts, and is critical for the differentiation of the progenitor cells into either absorptive enterocytes or one of the secretory cell types (Vereecke *et al.*, 2011). Inactivated Notch signalling in mice treated with γ-secretase inhibitors, conditional RBPjκ deletion, or HES1 null mice resulted in an abnormal intestinal architecture due to conversion of progenitor cells into goblet cells or EEC at the expense of enterocytes (Milano et al., 2004; Obata et al., 2012; Ueo et al., 2012; van Es et al., 2005). HES1, the Notch effector protein in the intestines, represses the production of Dll ligands at the cell surface, thus limiting the activation of the Notch pathway in adjacent cells (Noah and Shroyer, 2013). HES1 also represses its target gene, *Atoh1* (***Ato****nal* ***h****omolog 1*), also known as **m**ouse **at**onal **h**omolog **1** (*MATH1*), which

opposes Notch/Hes activation and promotes secretory cell lineage differentiation (Yang *et al.*, 2001). The HES1 target, *MATH1*, is essential for intestinal secretory cell commitment; overexpression of *MATH1* in embryonic intestines was sufficient to force secretory cell differentiation (VanDussen and Samuelson, 2010). Additionally, ectopic Notch signalling using constitutively active NICD led to a decrease in secretory cells in the intestinal epithelium of transgenic mice (Fre *et al*., 2005; Stanger *et al*., 2005).

1.2.3 Wnt/Notch signalling crosstalk in intestinal homeostasis

During normal intestinal development, both Wnt and Notch signalling pathways work cooperatively for continuous proliferation of intestinal stem and progenitor cells. Mouse intestinal tissues lacking Notch signalling by conditional removal of RBPjκ or treatment with γ-secretase display not only an overproduction of secretory cell types, but also a decrease in proliferation within the crypts suggesting that a Notch-mediated regulation of proliferation also occurs within the intestines (van Es *et al.*, 2005). Indeed, enhanced Notch signalling is observed in the adenomatous tissue of $Apc^{Min/+}$ mice (which have constitutive Wnt signalling), and abolishing Notch signalling in $Apc^{Min/+}$ mice blocks proliferation within tumour tissues (van Es *et al.*, 2005). Conversely, overactive Notch signalling by constitutive NICD production has been shown to promote tumour formation in $Apc^{Min/+}$ mice supporting a role for Notch signalling in mediating proliferation (Fre *et al.*, 2005). In addition to Notch-mediated proliferation in a constitutive Wnt background, inhibition of Notch signalling with γ-secretase inhibitor in human colon cancer cell lines decreased both Cyclin D1 (Wnt target) and HES1 (Notch target) levels, and reduced cell proliferation (Gopalakrishnan *et al*., 2014). Interestingly

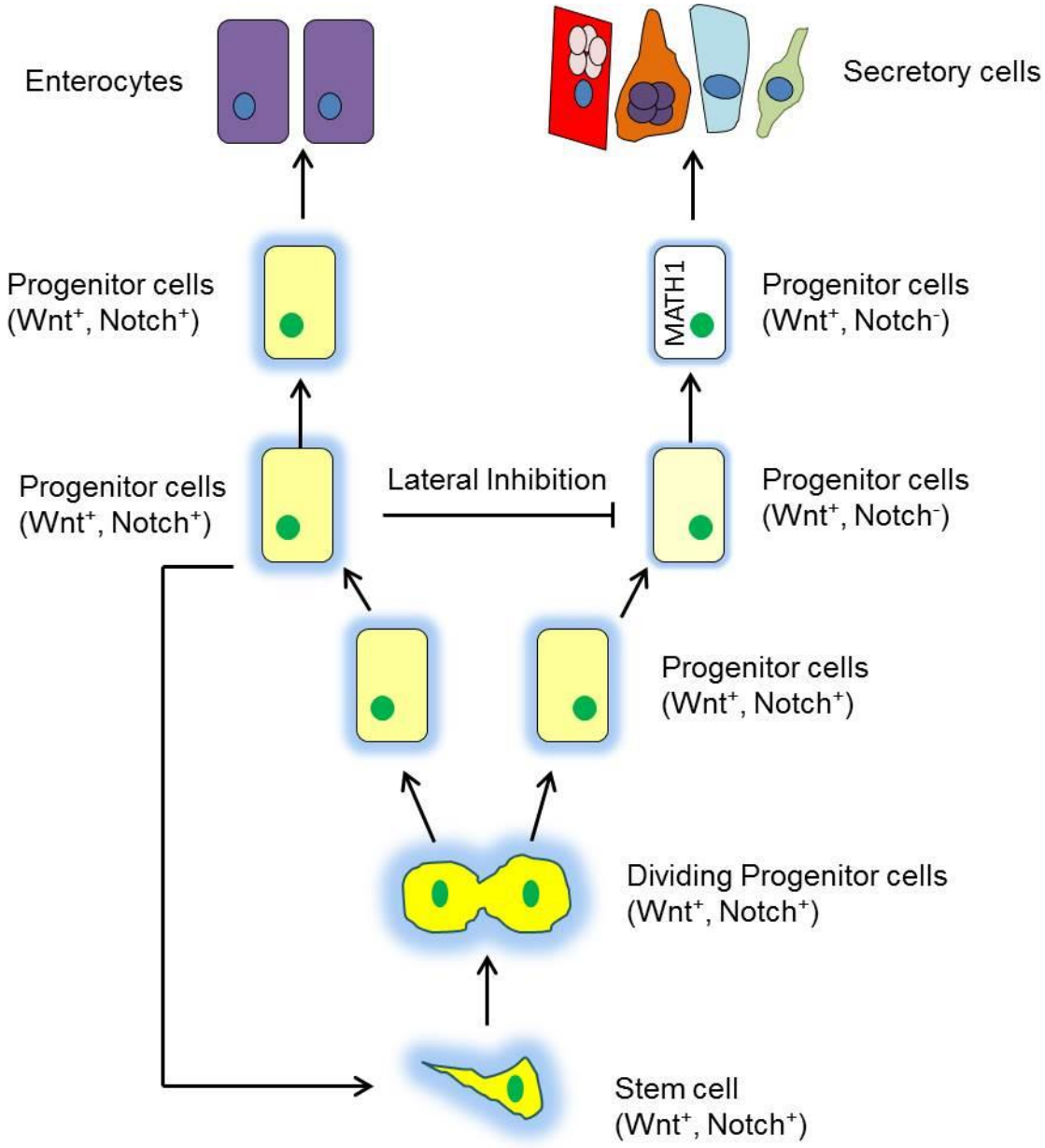
however, no obvious effect on β-catenin nuclear localization is observed when the Notch pathway is misregulated (Fre *et al.*, 2005; van Es *et al.*, 2005). Although Wnt signalling was reported to regulate proliferation within the intestines (Fevr *et al*., 2007; Korinek *et al*., 1998), the above studies demonstrate that Wnt and Notch signalling are both involved in regulating proliferation within the intestines.

One proposed mechanism for progenitor cell differentiation is the escape of cells from the Wnt/Notch-activated progenitor cell population (reviewed in Crosnier *et al.*, 2006; Nakamura *et al.*, 2007). Wnt signalling has been shown to drive the expression of Notch pathway components, including HES1 and Jag1 (Fre *et al.*, 2005; Rodilla *et al.*, 2009; van Es *et al.*, 2005). Notch components mediate lateral inhibition within the Wnt-activated cells (Wnt^+); thus some cells expressing Notch ligands escape Notch activation ($Notch^-$), while others expressing Notch receptors are Notch activated ($Notch^+$). Cells characterized as Wnt^+-$Notch^+$ continue to divide and produce more stem and progenitor cells (reviewed in Crosnier *et al.*, 2006; Nakamura *et al.*, 2007). However, cells characterized as Wnt^+-$Notch^-$ differentiate into one of the secretory cell types (reviewed in Crosnier *et al.*, 2006; Nakamura *et al.*, 2007) (Figure 1.4).

The differentiation of the Wnt^+-$Notch^-$ cells into secretory cell fate is coupled with the acquisition of MATH1 function (Yang *et al.*, 2001). MATH1 expression is not only regulated by Notch, but also by the Wnt pathway (Tsuchiya *et al.*, 2007). MATH1 is targeted for ubiquitination and degradation upon GSK3-mediated phosphorylation (Tsuchiya *et al.*, 2007). Upon Wnt activation, GSK3 is redirected from β-catenin to

Figure 1.4: Wnt and Notch pathways in intestinal epithelial proliferation and differentiation.

Intestinal stem cells and progenitor cells near the base of the crypts have active Wnt and Notch signalling (Wnt^+, $Notch^+$). Some Wnt^+ cells express Notch pathway components leading to some cells being Wnt^+; $Notch^+$, and others as Wnt^+; $Notch^-$ progenitor cells. The Wnt^+; $Notch^+$ progenitors either differentiate into enterocytes or produce more stem cells. In contrast, Wnt^+; $Notch^-$ cells differentiate into one of the secretory cell types. Original illustration inspired from (Crosnier *et al*., 2006).


Enterocytes
Secretory cells
Progenitor cells
(Wnt+, Notch+)
MATH1
Progenitor cells
(Wnt+, Notch-)
Progenitor cells
(Wnt+, Notch+)
Lateral Inhibition
Progenitor cells
(Wnt+, Notch-)
Progenitor cells
(Wnt+, Notch+)
Dividing Progenitor cells
(Wnt+, Notch+)
Stem cell
(Wnt+, Notch+)

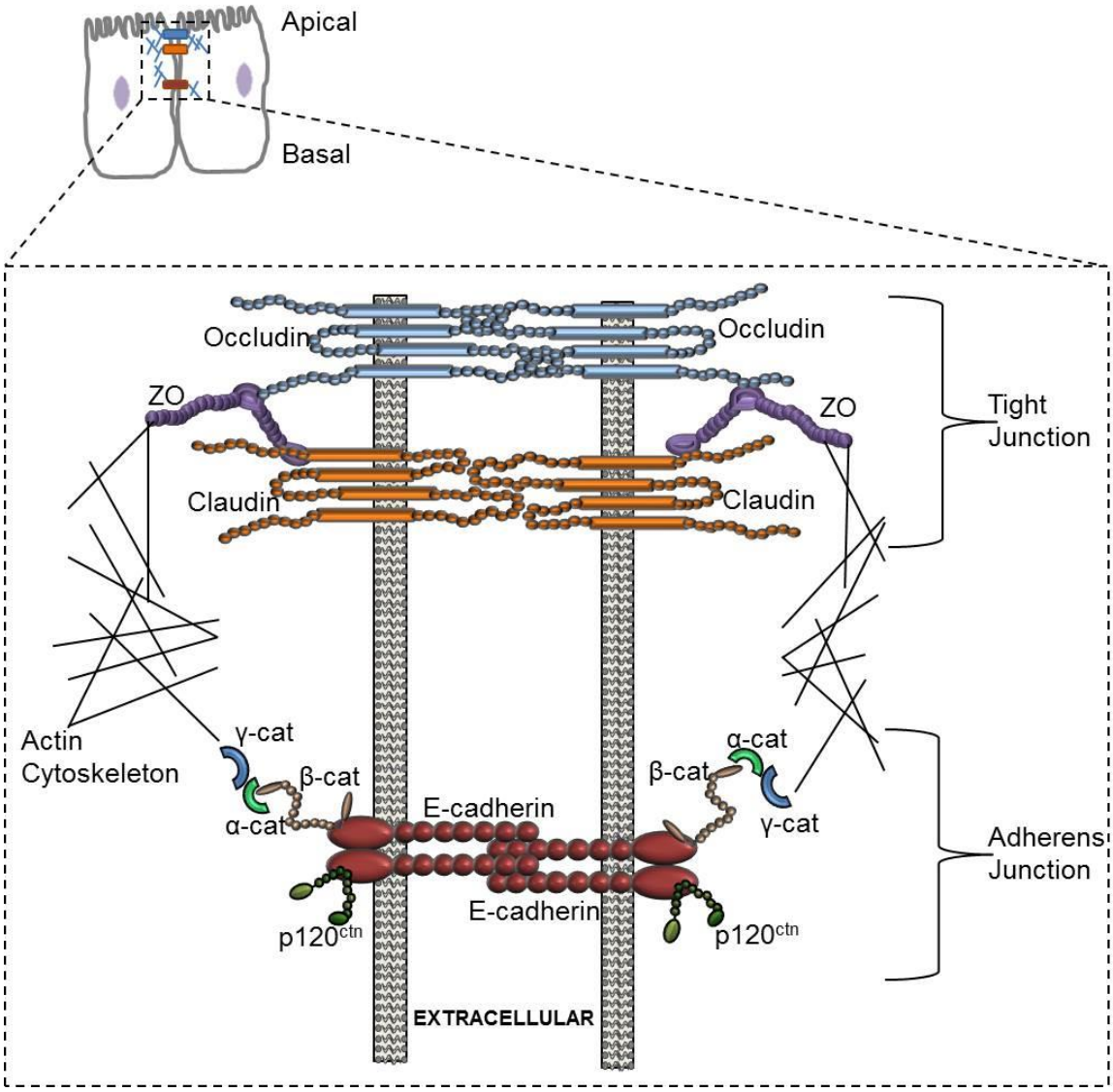
MATH1, and upon loss of the Wnt signal, MATH1 becomes stable as β-catenin is destabilized by GSK3 (Tsuchiya *et al.*, 2007). As the cells migrate up the crypt/villus axis, the Wnt signal is weakened, and the MATH1 expression is stabilized in the Wnt^{+}-$Notch^{-}$ cells allowing for differentiation of secretory cell types (Nakamura *et al.*, 2007). The Wnt^{+}-$Notch^{+}$ cells also migrate up the crypt/villus axis away from the Wnt signal, and these differentiate into enterocytes (Crosnier *et al.*, 2006) (Figure 1.4). As cells continually proliferate and differentiate within the intestinal crypts, differentiated epithelial cells migrate along the crypt/villus axis to create the mature intestinal epithelium. The intestinal epithelium also acts as a robust barrier separating the intestinal lumen from the underlying tissues. Thus, the formation of dynamic and flexible cell adhesion between epithelial cells must be intimately coupled with the migratory process.

1.2.4 Cell adhesion

The intestinal epithelium constitutes the largest exposed surface area of the human body, and its integrity is finely regulated by two key cell adhesion complexes. **T**ight **j**unctions (TJ) are large multi-protein complexes which, together with ***a****dherens* **j**unctions (AJ), link intestinal epithelial cells to each other (Figure 1.5). TJs and AJs on epithelial cells seal the intercellular spaces on the luminal surface of the intestine, and regulate paracellular passage of molecules between the cells (Pastorelli *et al.*, 2013). TJs and AJs are also involved in the proper migration of epithelial cells along the crypt/villus axis (Pastorelli *et al.*, 2013).

Figure 1.5: A schematic of *adherens* and tight junctions.

A simplified model depicting *adherens* and tight junctions, both of which play critical roles in the formation of an epithelial barrier within the intestines. The tight junctions (TJ) are comprised of proteins called occludins and claudins that are internally stabilized by Zonula occludens (ZO) proteins which link the tight junctions to the actin cytoskeleton below. Adherens junctions (AJ), on the other hand, are comprised of cadherins (e.g. E-cadherin) that interact with cytosolic cofactors called catenins (α, β, $p120^{ctn}$). Whereas α and β-catenins anchor E-cadherin to the actin cytoskeleton, $p120^{ctn}$ regulates E-cadherin stability and turnover. Original illustration.


Apical
Basal
Occludin
Occludin
ZO
ZO
Tight
Junction
Claudin
Claudin
Actin
Cytoskeleton
γ-cat
β-cat
α-cat
α-cat
β-cat
γ-cat
E-cadherin
Adherens
Junction
E-cadherin
p120ctn
p120ctn
EXTRACELLULAR

1.2.4.1 Adherens junctions

AJs are comprised of cadherin-catenin complexes that are essential for mediating epithelial cell-cell adhesion (Hartsock and Nelson, 2008). The most important protein in AJs is the transmembrane protein, **e**pithelial **cadherin** (E-cadherin) that forms calcium-dependent homophilic interactions with E-cadherin molecules on adjacent cells (Solanas and Batlle, 2011).

Within the intestinal epithelium, E-cadherin plays an important role in cell adhesion (reviewed in Solanas and Batlle, 2011). E-cadherin is anchored at the membrane via intracellular interactions with cofactors called catenins. The membrane-bound pool of β-catenin binds E-cadherin via the catenin-binding domain (Hartsock and Nelson, 2008; Yamada *et al.*, 2005). Interestingly, loss of β-catenin in the intestine does not lead to cell-cell adhesion defects, rather intestines display crypt loss and alterations in differentiation due the loss of β-catenin's signalling role as the downstream effector of the Wnt signalling pathway (Fevr *et al.*, 2007). E-cadherin-null intestines, on the other hand, display improper positioning and maturation of goblet and Paneth cells, as well as defects in cell polarity, cell-cell and cell-matrix adhesions (Schneider *et al.*, 2010). Conversely, ectopic expression of E-cadherin in intestinal cells slowed the cell migration rate along the crypt/villus axis without affecting cell differentiation, thus supporting a role for E-cadherin in intestinal epithelial cell migration (Hermiston *et al.*, 1996).

Unlike the classical β-catenin that functions to anchor E-cadherin to the actin cytoskeleton, **p120 ca**t**eni**n (p120^{ctn}) binds E-cadherin at a distinct region called the

juxtamembrane domain (Hartsock and Nelson, 2008; Reynolds et al., 1994; Thoreson et al., 2000) and regulates E-cadherin stability and turnover (Davis and Reynolds, 2006; Ireton *et al.*, 2002; reviewed in Xiao *et al.*, 2007). Intestinal-specific $p120^{ctn}$ knockout mice exhibited cell-cell adhesion defects that resulted in mucosal erosion and terminal intestinal bleeding into the lumen (Smalley-Freed *et al.*, 2010) similar to E-cadherin null intestines (Schneider *et al.*, 2010). Moreover, $p120^{ctn}$ ablation in the intestines reduced E-cadherin and β-catenin levels, and caused an epithelial barrier defect (Smalley-Freed *et al.*, 2010).

The epithelial barrier constitutes the physical and functional barrier between the host and the external environment, and is mediated by the TJs. AJs help facilitate the assembly of TJs by recruiting the TJ scaffolding protein, **Z**onula **o**ccluden (ZO), to the early cadherin-containing intercellular contacts (Tunggal *et al.*, 2005). Though the absence of ZO has no effect on AJ formation, it does provide cytoskeletal connections between the two junctional complexes (reviewed in Hartsock and Nelson, 2008).

1.2.4.2 Tight junctions

Like AJs, TJs are large multiprotein complexes, but unlike AJs that mediate cell-cell adhesion, TJs regulate the flow of ions and molecules in and out of the intercellular space (i.e. paracellular permeability) (Solanas and Batlle, 2011). TJs are comprised of the transmembrane proteins occludins and claudins which interact with the actin cytoskeleton via the ZO scaffolding proteins (Hartsock and Nelson, 2008).

ZO proteins localize to the AJs at the earliest occurrence of cell-cell contacts, prior to interacting with occludins and claudins of TJs (Tunggal *et al.*, 2005). Consequently, they mediate interactions between AJs and TJs which allows for dynamic cell-cell contacts. Local clustering of ZO proteins mediates the incorporation of occludins and claudins into TJs, thus directing junctional assembly (reviewed in Fanning and Anderson, 2009; Lu et al., 2013).

Claudins are tetraspan transmembrane proteins that are regarded as the backbone of TJ, and the key to paracellular permeability (reviewed in Lu *et al*., 2013). To date, 24 members of the claudin family have been identified, each with a specific organ and tissue distribution (reviewed in Lu *et al*., 2013). Some claudins directly regulate the ion selectivity of TJs, and others regulate the competition between permeable molecules (reviewed in Hartsock and Nelson, 2008). In the intestines, claudin-1, -3, -4, -5 and -8 regulate tightening of the TJs, thus decreasing paracellular permeability, while claudin-2 and -15 form charge-selective paracellular pores within the membrane (reviewed in Lu *et al*., 2013). The expression of different members of the claudin family vary over the length of the GI tract, and this alters the strength, size and ion specificity of the TJ barrier (reviewed in Lu *et al*., 2013).

In addition to their role in paracellular permeability, claudins also function to recruit occludin to the TJ (Furuse *et al.*, 1998). Like claudin, occludin is also a tetraspan transmembrane protein and was the first protein to be identified within the TJ complex (Furuse *et al.*, 1993). Occludin is involved in regulating the permeability of

macromolecules via homophilic interactions with occludin molecules on adjacent cells (Suzuki, 2013). Though the function of occludin is not completely understood, it is known to have crucial roles in TJs structure and permeability (Suzuki, 2013). Accumulating evidence shows that defects in the integrity of the intestinal epithelial cell barrier is one contributing factor in the development of IBD (Solanas and Batlle, 2011).

1.3 INFLAMMATORY BOWEL DISEASE

Inflammatory Bowel Disease (IBD) represents a group of syndromes, the most common of which are **C**rohn's **D**isease (CD), which affects any part of the GI tract, and **U**lcerative **C**olitis (UC), whose effects are restricted to the colonic mucosa (reviewed in Maloy and Powrie, 2011). Though both diseases have complex etiologies, their main features are a robust inflammatory response with mucosal injury, increased epithelial permeability, invasion of commensal bacteria into the lamina propria and massive recruitment of leukocytes (reviewed in Maloy and Powrie, 2011). Currently, there is no cure for IBD and the treatment of IBD involves alleviation of symptoms using anti-inflammatory agents.

Despite extensive research, the exact pathogenesis of IBD remains elusive. However IBD etiology is known to involve complex interactions between the microbiome, host genetics, and immune responses (Zhang and Li, 2014). Disruptions in the interaction of the intestinal epithelium and its surrounding microbiome, i.e. the environment, can provoke IBD (reviewed in Actis *et al.*, 2014). Recent studies have also uncovered over a hundred polymorphisms in the immune response pathways, including

innate immune receptors, epithelial barrier function, and immune- and cytokine-related genes, increasing the genetic complexity of IBD (reviewed in Ek *et al.*, 2014). Studies further suggest that the abnormal immune responses in IBD patients are directed against the intestinal microbiome, thus exacerbating the inflammatory phenotype (Zanello *et al.*, 2014). Some of the factors contributing to IBD are discussed further below.

1.3.1 Cell adhesion defects and IBD

AJ and TJ proteins are misregulated in IBD patients (reviewed in Lu et al., 2013; Solanas and Batlle, 2011). In fact, loss of the AJ proteins, E-cadherin and $p120^{ctn}$, have been detected at the mucosal edges around epithelial ulcerations in all cases of active UC and 50% of active CD (reviewed in Solanas and Batlle, 2011). Additionally, conditional knockdown of $p120^{ctn}$ in murine intestines results in cell-cell adhesion and permeability defects, and inflammation characterized by massive neutrophil infiltration, a common finding in some IBD patients (Smalley-Freed *et al.*, 2010; Smalley-Freed *et al.*, 2011).

The epithelium of inflamed intestinal segments of IBD patients is also characterized by alterations of TJ proteins (reviewed in Lu *et al*., 2013; Tang *et al*., 2010). Mounting evidence shows that alterations in TJs lead to a compromised intestinal barrier allowing for greater interactions between commensal microorganisms and the host immune system (Peterson and Artis, 2014). *In vitro* experiments suggest that claudin-1 is internalized in epithelial cells contributing to the disrupted barrier (Ivanov *et al.*, 2004), although claudin-1 expression is significantly increased in IBD biopsies and is correlated with active inflammation (Weber *et al.*, 2008). In addition to claudin-1, the reduction of occludin and ZO-1 in colonic mucosa results in thickening of the colonic wall and heavy

infiltration of leukocytes in the intestinal lamina propria (Vaziri *et al.*, 2012) suggesting that multiple TJ proteins may regulate intestinal barrier. Surprisingly, deficiency of occludin alone does not induce intestinal barrier defects or defects in intestinal TJ morphology in murine tissues (Saitou *et al.*, 2000), however, occludin expression is significantly decreased in intestinal tissues of IBD patients (Gassler *et al.*, 2001).

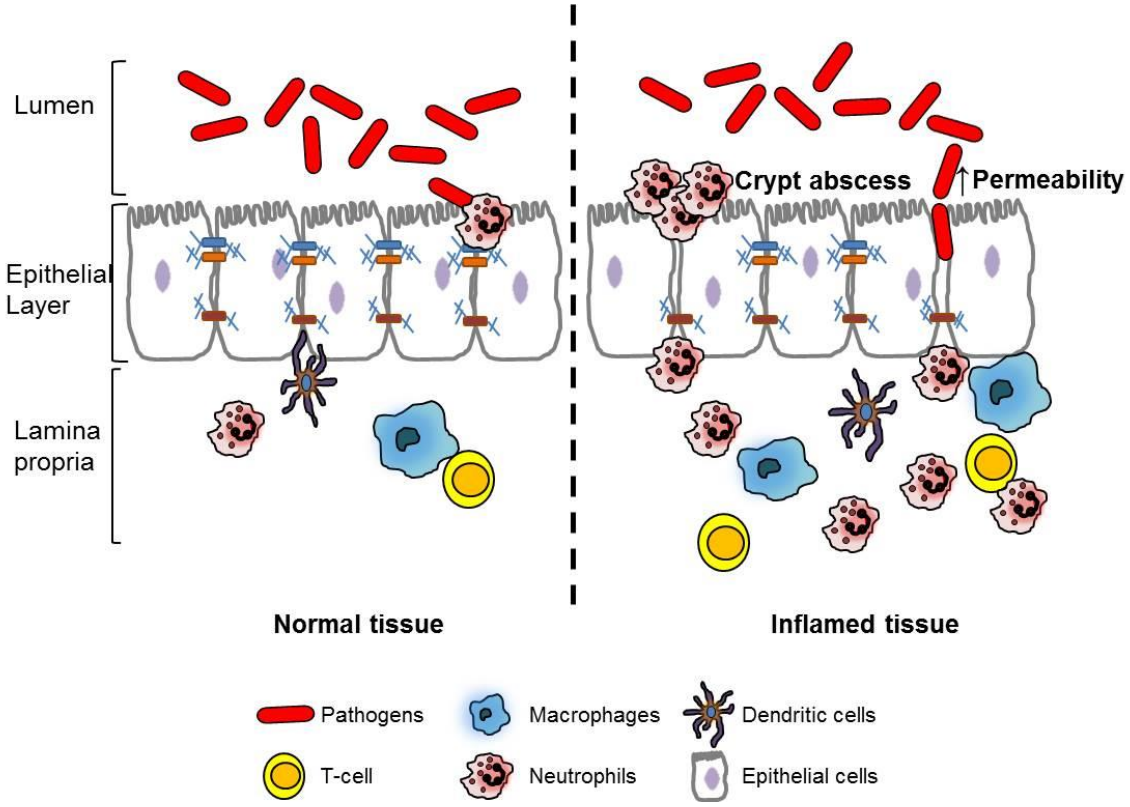
Although TJ- and AJ-mediated permeability increase correlates with IBD, several lines of evidence suggest that barrier function defects are insufficient to cause the disease (reviewed in Turner, 2009). While increased TJ-mediated barrier permeability *in vivo* caused bacterial translocation and mucosal immune cell activation, it was insufficient to induce colitis in the murine intestines (Khounlotham *et al.*, 2012; Laukoetter *et al.*, 2007; Su *et al.*, 2009). These data suggest that the compensatory immune mechanism can act to protect against the colitis (Figure 1.6).

1.3.2 Innate immune system and IBD

Host-microbial interactions are crucial for developing and modulating the immune system. The first line of defense against harmful pathogens is the gut epithelium, which is comprised of mucin-secreting goblet cells, enterocytes, and phagocytic cells (neutrophils, dendritic cells, and tissue-resident macrophages) within the lamina propria (reviewed in Wallace *et al.*, 2014) (Figure 1.6). Together these phagocytic cells make up the intestinal innate immune system which recognizes and kills infected cells and microorganisms, and, subsequently, activates the adaptive immune system (T and B cells) (Wallace *et al.*, 2014). In fact, mice lacking an adaptive immune system but intact innate immune system

Figure 1.6: Inflamed intestinal tissue has increased permeability and neutrophil influx

The intestinal barrier is comprised of the epithelial cells and innate immune system including the macrophages, neutrophils and dendritic cells. Upon inflammation, the TJ proteins are decreased allowing for greater permeability of pathogens into the underlying lamina propria of the intestine where they contact phagocytic innate immune cells (e.g. neutrophils and macrophages). Excess neutrophil influx and transepithelial migration into the tissue results in crypt abscess formation in IBD tissues. Original illustration.


Lumen
Epithelial Layer
Lamina propria
Crypt abscess
↑Permeability
Normal tissue
Inflamed tissue
Pathogens
Macrophages
Dendritic cells
T-cell
Neutrophils
Epithelial cells

do not develop spontaneous inflammation, but are susceptible to **d**extran **s**odium **s**ulfate (DSS)-induced colitis (Dieleman *et al.*, 1994). These data suggest that the intestinal innate immune system is sufficient for the development of acute colitis upon DSS administration independent of the adaptive immune system (reviewed in Wallace *et al.*, 2014).

The microbiota within the lumen of the intestine directly interacts with the epithelial cells and some innate immune cells. The epithelial and innate immune cells subsequently communicate with the immune cells in the underlying lamina propria. The microbiota signals enterocytes and immune cells via pattern recognition receptors such as the **T**oll-**l**ike **r**eceptors (TLRs) (Wallace *et al.*, 2014). Expression of TLRs is highly regulated to maintain tissue homeostasis and prevent an inappropriate immune response (Wallace *et al.*, 2014). Activation of TLRs triggers expression of pro-inflammatory mediators like **t**umour **n**ecrosis **f**actor (TNF) that make mice more susceptible to DSS-induced colitis (Saleh and Trinchieri, 2011).

Microbial antigens within the lamina propria activate the innate immune system via pattern recognition receptors and subsequently activate the adaptive immune system (Däbritz, 2014). Tissue-resident macrophages play important roles in presenting antigens to, and activating, other innate immune cells in the lamina propria (Däbritz, 2014). Macrophages are also responsible for clearing out short-lived immune cells to maintain intestinal homeostasis (Fournier and Parkos, 2012). One of the innate immune cell types activated and cleared by macrophages are neutrophils (Brazil *et al.*, 2013).

Neutrophils, also known as **p**oly**m**orpho**n**uclear (PMN) leukocytes, are part of the normal gut inflammatory response and function primarily to kill invading microbes (Fournier and Parkos, 2012). Although neutrophils are mostly viewed as playing a beneficial role to the host, their improper activation may also lead to tissue damage during an autoimmune or exaggerated inflammatory reaction (Németh and Mócsai, 2012). Neutrophils produce and release various agents to digest microbes, some of which are harmful to the host; these include reactive oxygen species, antimicrobial peptides, myeloperoxidase, hydrolytic enzymes, and proteases (reviewed in Fournier and Parkos, 2012).

Depletion of neutrophils within the intestine in a murine colitis model yielded an increase in bacterial translocation and exacerbated the inflammation (Kühl *et al.*, 2007). Interestingly, TJ disruption induces bacterial translocation and increases PMN levels in the lamina propria, but these mice do not develop spontaneous colitis (Laukoetter *et al.*, 2007). These data suggest that recruitment of neutrophils to the lamina propria may serve a protective role against development of inflammation (Fournier and Parkos, 2012).

Although neutrophils are well suited to kill bacteria and serve a protective role against inflammation, their antimicrobial toxic molecules can also damage the host tissue and initiate an inflammatory response (Brazil *et al.*, 2013). Massive transmigration of neutrophils into the intestinal lumen decreases expression levels of TJ, thereby creating "holes" within the epithelium, increasing the barrier permeability (Kucharzik *et al.*, 2001) (Figure 1.6). Neutrophils may also partially disrupt AJ, which enhances the loss of barrier

function (Kucharzik *et al.*, 2001). This loss of barrier function further augments the inflammation by facilitating transmigration of the bacteria from the lumen to mucosa, thus further activating neutrophils (Fournier and Parkos, 2012).

In the process of transepithelial migration, neutrophils can form crypt abscesses in the intestines (Brazil *et al.*, 2013). Crypt abscesses are a classic feature of active IBD and represent collections of massive numbers of active PMN that have migrated across the epithelium (Brazil *et al.*, 2013; Fournier and Parkos, 2012). This retention of active neutrophils in the crypts results in deformation of the crypt architecture and correlates with IBD disease (Xavier and Podolsky, 2007). Hence neutrophil accumulation is beginning to be recognized as a hallmark of the active inflammation in IBD (reviewed in Brazil *et al.*, 2013). Multiple factors mediate neutrophil infiltration within the intestine and surprisingly one of these is the AJ protein, $p120^{ctn}$.

1.3.3 $p120^{ctn}$ and inflammation

Though $p120^{ctn}$ plays roles in cell adhesion and signal transduction, increasing evidence shows $p120^{ctn}$ also functions as an anti-inflammatory mediator in many tissues. Depletion of $p120^{ctn}$ in conditional knockout mice results in pro-inflammatory cytokine release and immune cell infiltration into intestinal tissues (Smalley-Freed *et al.*, 2010; Smalley-Freed *et al.*, 2011). Conditional knockdown of $p120^{ctn}$ in murine intestines resulted in cell-cell adhesion defects, increased epithelial barrier permeability, and inflammation characterized by massive $COX2^{+}$ neutrophil infiltration, a common finding in IBD patients (Smalley-Freed *et al.*, 2010; Smalley-Freed *et al.*, 2011). Not surprisingly, $p120^{ctn}$ depletion resulted in cell autonomous loss of AJs and accumulation

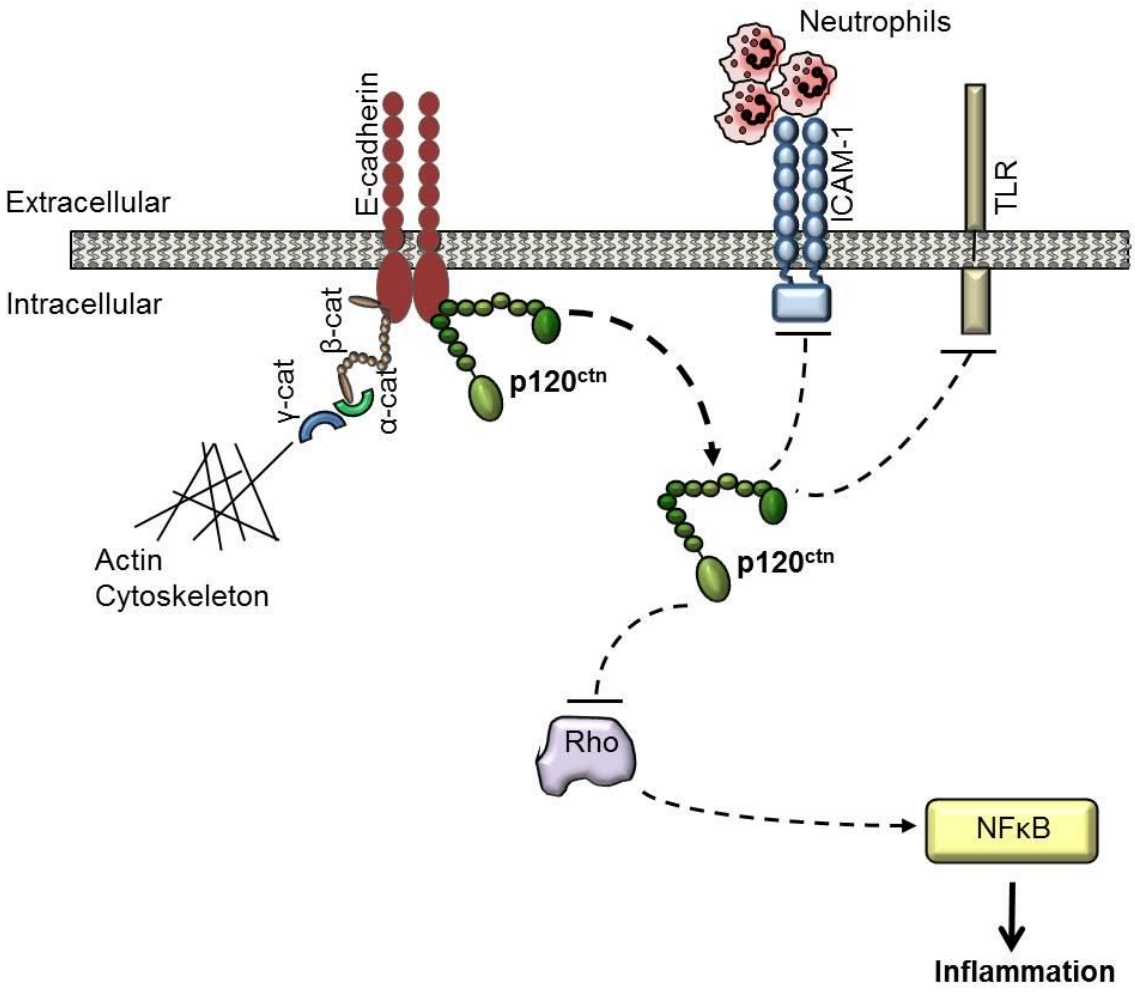
of neutrophils in the p120^{ctn}-null areas (Smalley-Freed *et al.*, 2010). Interestingly, neutrophil binding was increased in p120^{ctn} depleted cells (Smalley-Freed *et al.*, 2010). p120^{ctn} depletion in murine intestines also resulted in intestinal adenoma formation as the mice age, which may be a consequence of the inflammation (Smalley-Freed *et al.*, 2011).

p120^{ctn} depletion also leads to cytokine and immune cell infiltration in the epidermis (Perez-Moreno *et al.*, 2006; Perez-Moreno *et al.*, 2008). p120^{ctn}-null epidermal cells have elevated levels of activity of the pro-inflammatory mediator, NFκB (Perez-Moreno *et al.*, 2006). p120^{ctn} links to inflammation and the NFκB pathway have also been observed in airway inflammation following **l**ipo**p**oly**s**accharide (LPS) treatment (Qin *et al.*, 2014; Wang *et al.*, 2011). In both models, p120^{ctn} appears to exert its pro-inflammatory effects through the NFκB signalling pathway in a Rho-dependent (Perez-Moreno *et al.*, 2006; Qin *et al.*, 2014) or a TLR-dependent manner (Wang *et al.*, 2011) (Figure 1.7).

Although p120^{ctn} has been implicated in regulating the inflammatory response, p120^{ctn} has also been inversely linked to the transcriptional regulation of the pro-inflammatory adhesion molecule, ICAM-1 (**i**ntercellular **a**dhesion **m**olecule-**1**) in human endothelial cells (Alcaide *et al.*, 2012; O'Donnell *et al.*, 2011; Wang *et al.*, 2011). ICAM-1 is an integrin receptor that is involved in leukocyte-endothelial cell interaction, and is important for neutrophil adhesion and transepithelial migration (Thomas and Baumgart, 2012), suggesting a role for p120^{ctn} in regulating the adhesion of neutrophils within tissues.

Figure 1.7: Anti-inflammatory role of p120^{ctn}

p120^{ctn}, a structurally related protein, is also involved in regulating multiple intracellular pathways inhibiting inflammation. p120^{ctn} inhibits Rho activities thus preventing the downstream activation of NFκB. In different tissues, p120^{ctn} can also inhibit the TLR pathway thus preventing the activation of the NFκB pathway. The neutrophil adhesion protein is also negatively regulated by p120^{ctn}. Thus, the overall inhibition of the NFκB pathway by p120^{ctn} inhibits the activation of inflammation. Original illustration.


Neutrophils
E-cadherin
ICAM-1
TLR
Extracellular
Intracellular
β-cat
γ-cat
α-cat
p120ctn
Actin
Cytoskeleton
p120ctn
Rho
NFκB
Inflammation

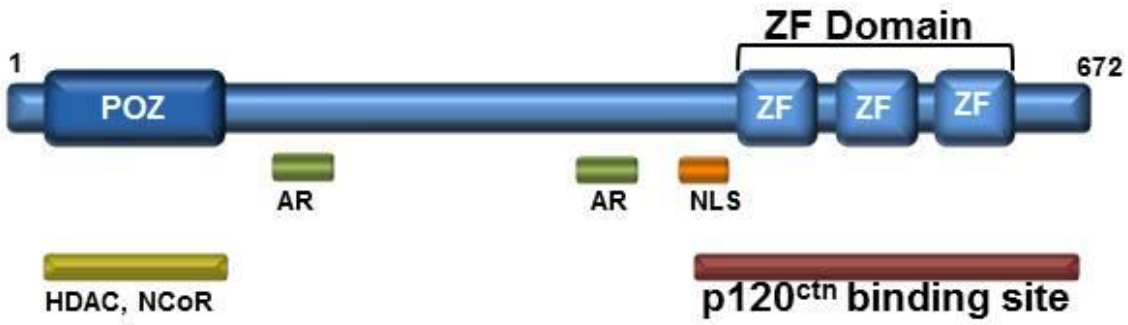
All of these studies show a role for p120^{ctn} as an anti-inflammatory regulator (Figure 1.7) that may contribute to the phenotype seen in Kaiso (p120^{ctn} binding partner) overexpressing mice (Chapter 3, Chapter 4).

1.4 THE P120^{CTN} BINDING PARTNER, KAISO

Kaiso is a unique transcription factor that was first identified in a yeast two-hybrid screen to identify p120^{ctn} binding partners (Daniel and Reynolds, 1999). Kaiso is a member of the BTB/POZ (**B**ric à brac, **T**ramtrak, **B**road complex/**Po**x virus and **z**inc finger) zinc finger family of proteins that are characterized by an amino-terminal POZ protein-protein interaction domain, and carboxy-terminal zinc finger (ZF) motifs that mediate DNA binding (Daniel and Reynolds, 1999) (Figure 1.8). Kaiso functions primarily as a transcriptional repressor that recruits corepressor complexes comprised of **h**istone **d**e**ac**etylases (HDAC), **s**ilencing **m**ediator of **r**etinoid and **t**hyroid receptor (SMRT), **n**uclear **cor**epressor (NCoR), and **m**ammalian **S**wi-**in**dependent **3A** (mSin3A) (Daniel and Reynolds, 1999; reviewed in Kelly and Daniel, 2006; Kelly *et al*., 2004a; Park *et al*., 2005; Prokhortchouk *et al*., 2001; Spring *et al*., 2005; Yoon *et al*., 2003). However, Kaiso is also one of the few POZ-ZF transcription factors that also activate gene expression (Kelly and Daniel, 2006); Kaiso activates transcription of the neuromuscular gene *rapsyn* (Rodova *et al.*, 2004), but its mechanism of transcriptional activation in currently unknown.

Figure 1.8: Structural and functional domains of Kaiso.

Like most POZ-ZF transcription factors, Kaiso possesses an amino-terminal POZ protein-protein interaction domain, which interacts with co-repressors (HDAC and NCoR), and a carboxy-terminal **z**inc **f**inger (ZF) domain that binds to DNA. Kaiso has a **n**uclear **l**ocalization **s**ignal (NLS), and two **a**cidic **r**ich regions (AR) that may play a role in transcriptional activation. Kaiso interacts with $p120^{ctn}$ via a carboxy-terminal region that overlaps with its DNA-binding domain, and consequently $p120^{ctn}$ binding regulates Kaiso's transcriptional activity. Original illustration.


ZF Domain
1
672
POZ
ZF
ZF
ZF
AR
AR
NLS
HDAC, NCoR
p120ctn binding site

Unlike most POZ-ZF transcription factors, Kaiso is a unique dual-specificity transcription factor that recognizes and binds methylated CpG dinucleotides and the non-methylated DNA sequence TCCTGCnA, known as the **K**aiso **B**inding **S**equence (KBS) (Daniel *et al*., 2002; Prokhortchouk *et al*., 2001). $p120^{ctn}$ interacts with Kaiso through Kaiso's zinc finger domain and thus inhibits Kaiso's DNA-binding abilities and regulates its transcriptional activity (Daniel and Reynolds, 1999; Prokhortchouk *et al*., 2001). Indeed, a $p120^{ctn}$ mutant incapable of nuclear translocation failed to inhibit Kaiso-mediated transcriptional repression (Kelly *et al.*, 2004b), while nuclear $p120^{ctn}$ relieves Kaiso-mediated transcriptional repression of a Kaiso target gene, *matrilysin* (Ogden *et al.*, 2008). Surprisingly, most Kaiso target genes identified to date are Wnt target genes, suggesting that Kaiso may play a role in regulating Wnt signalling.

1.4.1 Kaiso and Wnt signalling

Most insight into Kaiso's role in Wnt signalling and development comes from studies in *Xenopus* where Kaiso depletion resulted in severe gastrulation defects (Kim *et al*., 2004; Park *et al*., 2005). Kaiso-depleted *Xenopus* embryos displayed increased expression of genes involved in cell growth control and apoptosis, suggesting a role for Kaiso in regulating these processes (Ruzov *et al.*, 2004). Interestingly, Kaiso overexpression suppressed the duplicate-axis phenotype induced by constitutive Wnt signalling (β-catenin overexpression)—a finding that implicated Kaiso as a negative regulator of Wnt signalling in *Xenopus* (Park *et al.*, 2005). However, recent independent studies in *Xenopus* implicated Kaiso as both a positive and negative regulator of Wnt signalling . Kaiso depletion decreased Wnt-mediated reporter activity, while low ectopic

Kaiso expression enhanced Wnt signalling in *Xenopus* embryos . Additionally, Kaiso expression can alleviate negative HDAC effects on the β-catenin/TCF complex in the nucleus, suggesting a positive role for Kaiso in the Wnt signalling pathway . In mammalian cells, Kaiso binds and represses several tumourigenesis-related Wnt target genes, including c*yclin D1* and *matrilysin* (Donaldson *et al*., 2012; Spring *et al*., 2005).

1.4.2 Kaiso in tumourigenesis

Although increasing evidence implicates Kaiso in promoting tumourigenesis, a clear role of Kaiso as an oncogene or tumour suppressor remains unresolved. Cytoplasmic Kaiso correlates with poor prognosis of non-small cell lung cancer and chronic myeloid leukemia which suggests a potential tumour suppressor role for Kaiso (Cofre *et al.*, 2012; Dai *et al.*, 2009; Dai *et al.*, 2011). However, nuclear Kaiso has been positively correlated with primary and metastatic prostate and breast cancers (Jones *et al*., 2014; Jones *et al*., 2012), suggesting an oncogenic role for Kaiso. In murine models, when Kaiso-null mice are mated with the $Apc^{Min/+}$ mice model of colon cancer, the $Kaiso^{null}$;$Apc^{Min/+}$ progeny have delayed tumour onset and smaller intestinal polyps (Prokhortchouk *et al*., 2006), further supporting an oncogenic role for Kaiso.

The delayed tumour onset observed in $Kaiso^{null}$ mice in an $Apc^{Min/+}$ background was an unexpected result, as previous studies supported a negative regulatory role for Kaiso in Wnt signalling (Donaldson et al., 2012; Kim et al., 2004; Park et al., 2005; Spring et al., 2005). However, the lower tumour burden in $Kaiso^{null}$:$Apc^{Min/+}$ double mutant mice suggested a positive regulatory role for Kaiso in Wnt signalling within mammalian intestines (Prokhortchouk *et al.*, 2006). Kaiso protein levels were increased in intestinal

tumours compared to matched normal tissue in murine intestinal tumours (Prokhortchouk *et al.*, 2006). Kaiso also binds and represses methylated tumour suppressor and DNA repair genes in colon cancer cells, suggesting Kaiso may be an oncogene whose DNA-binding ability is dependent on the methylation status of genes in colon cancer (Lopes *et al.*, 2008).

In addition to the above findings, gene expression profiling revealed increased Kaiso expression in human colorectal cancers compared to normal biopsies, which was inversely related to the expression levels of its binding partner, $p120^{ctn}$ (Pierre *et al*., 2015; manuscript in revision). Mice with intestine-specific Kaiso overexpression in an $Apc^{Min/+}$ background display an increase in tumour burden, decrease in tumour size and shorter life spans further supporting an oncogenic role for Kaiso (Pierre *et al*., 2015; manuscript in revision).

1.5 Rationale and Summary

Most evidence collected to date with regards to Kaiso in colon cancer suggests an oncogenic role for Kaiso. Interestingly, though Kaiso depletion has developmental defects in *Xenopus* (Kim *et al.*, 2002; Park *et al.*, 2005; Ruzov *et al.*, 2004), Kaiso-null mice were viable and fertile and it was postulated that the Kaiso-like proteins, ZBTB4 and ZBTB38, were functionally redundant to Kaiso, and masked the Kaiso depletion effects during murine development (Prokhortchouk *et al.*, 2006). The lower tumour burden observed in the $Kaiso^{null}$ mice when bred to $Apc^{Min/+}$ mice suggests a positive regulation of Wnt signalling in the murine intestines, contrary to previous literature. To

address this discrepancy with regards to Kaiso's role in regulating Wnt signalling, intestinal-specific Kaiso overexpressing transgenic mice (*Kaiso*$^{Tg/+}$) were created for subsequent mating to the *Apc*$^{Min/+}$ mouse model of colon cancer. The hypothesis of this study was that Kaiso would positively regulate the Wnt signalling pathway in the context of the intestine. This study focuses on the unbiased characterization of the intestinal-specific *Kaiso*$^{Tg/+}$ mice to determine whether intestinal-specific Kaiso overexpression has any major effects on murine development and function. Characterization of the *Kaiso*$^{Tg/+}$ mice revealed increased numbers of secretory cells, enlarged crypts, villar blunting and fusion, and chronic inflammation. Further characterization of the intestinal inflammation phenotype revealed that the phenotype involved epithelial barrier defects and neutrophilia.

CHAPTER 2: MATERIALS AND METHODS

2.1 ETHICS STATEMENT

All mouse work was conducted according to the guidelines of the McMaster University **A**nimal **R**esearch **E**thics **B**oard (AREB). Protocols for mouse husbandry, breeding, genotyping and euthanasia were approved by AREB under **A**nimal **U**tilization **P**rotocol (AUP) 10-05-32. Euthanasia was achieved via CO_2 asphyxiation followed by cervical dislocation.

2.2 GENERATION OF *VILLIN-KAISO* TRANSGENIC MICE

Kaiso transgenic mice were created at the London Regional Transgenic Facility, University of Western Ontario. **M**yc-**t**agged **m**urine ***Kaiso*** (*mKaiso-MT*) was cloned downstream of the murine 9 Kb intestinal-specific *villin* promoter fragment in the pBluescript II vector provided by Dr. Sylvie Robine (Institut Curie, Paris, France) (Pinto *et al.*, 1999). The *villin-mKaiso-MT* fragment was excised from the plasmid by restriction enzyme digest with *SalI*. The isolated fragment was microinjected into 1-cell C57BL6/CBA hybrid mouse embryos *in vitro*, which were then implanted into pseudo-pregnant foster mothers to produce transgenic founders. Transgenic pups were identified by **p**olymerase **c**hain **r**eaction (PCR) analysis of DNA from tail biopsies using primer pairs corresponding to sequences in the *Myc* tag and *mKaiso* sequences (forward *5'-ATC ATC AAA GCC GGG TGG GCA-3'* and reverse *5'-TTT TCT ACT CTC CAT TTC ATT CAA GTC CTC-3'*). The transgenic lines were backcrossed to C57BL/6N mice (Taconic) for a minimum of 8 generations to obtain stable transgenic offspring, and thus three

transgenic founder lines were obtained. All transgenic offspring were genotyped by PCR using DNA obtained from ear snips upon weaning. Mice were fed a standard mouse chow diet and breeders were housed in the pathogen-free barrier facility, while post-genotyping pups were housed in a **s**pecific **p**athogen **f**ree (SPF) room with 12h/12h light/dark cycle in accordance with McMaster **C**entral **A**nimal **F**acility's (CAF) **S**tandard **O**perating **P**rocedures (SOPs).

2.3 TRANSGENE COPY NUMBER

Copy number standards were prepared by spiking wild-type tail DNA with specified amounts of purified transgenic DNA. PCR was performed using standard DNA and transgenic DNA from each founder line using the primers described above. The intensity of the band amplified in each of the transgenic animals was compared to that of the standards to estimate transgene copy number.

2.4 MOUSE TISSUE HARVEST

Mice were sacrificed via CO_2 asphyxiation according to the McMaster CAF SOPs. Small and large intestines were immediately removed from the sacrificed animals and flushed with cold **p**hosphate-**b**uffered **s**aline (PBS) on ice. Tissues were either flash frozen in liquid nitrogen for long-term storage or rolled into "Swiss rolls" for fixation in 10% neutral-buffered formalin for 48 hours, followed by 70% ethanol dehydration at room temperature. The small intestine was divided into four equal sections for formalin fixation. Fixed tissues were sent to McMaster Core Histology Research Services for

paraffin-embedding and sectioning at 5 μm within one week of tissue harvest, and placed onto glass slides for **i**mmuno**h**isto**c**hemistry (IHC) analysis as outlined below.

2.5 MORPHOLOGICAL ANALYSIS

Crypt depth and villi length were evaluated using **h**aematoxylin and **e**osin (H&E) stained slides from both transgenic lines (n=3 mice per genotype/founder line). Paneth cells were counted as eosin-filled cells at the base of the crypts. **P**eriodic **A**cid-**S**chiff (PAS) stain for Goblet cells was performed by the McMaster Core Histology Research Services according to standard protocols. All images were collected using the Aperio ScanScope system, and ImageScope software was used for all measurements. For each small intestine, 800 open crypts and 80 complete villi were assessed per mouse by two independent blind observers.

2.6 IMMUNOHISTOCHEMISTRY

Tissue slides were incubated in xylenes at room temperature for 10 min (2 washes) to remove paraffin, followed by rehydration in an ethanol gradient. Tissue was permeabilized with **T**ris-**b**uffered **s**aline with 0.05% **T**ween-20 (TBS-T), and antigen retrieval was accomplished by boiling samples in 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in TBS. Slides were incubated in 5% **n**ormal **g**oat **s**erum (NGS), 10% **b**ovine **s**erum **a**lbumin (BSA) in TBS-T with avidin blocking solution (Vector Labs) for 1 hour at room temperature. For Lysozyme staining (Pierce), antigen retrieval was performed by treating tissues with 200 μg/mL of Proteinase K (Roche) solution in 50 mM Tris, pH 7.4 for 5

minutes, and blocked in 10% **n**ormal **d**onkey **s**erum (NDS) in PBS with avidin blocking solution for 1 hour at room temperature. The slides were then incubated with biotin blocking solution (Vector Labs) and primary antibodies: rabbit anti-Kaiso polyclonal (gift from Dr. Albert Reynolds) at 1:1000 dilution, mouse monoclonal anti-c-Myc (Santa Cruz) at 1:60, rabbit anti-Lysozyme (Peirce) 1:50, rat monoclonal anti-MMP7 (Vanderbilt) at 1:50, and rabbit polyclonal anti-PPARγ (Millipore) 1:75 at 4°C overnight. For rat anti-Ki67 (DAKO clone TEC-3 at a 1:20 dilution), mouse anti-Synaptophysin (DAKO at 1:20 dilution), rabbit anti-HES1 (Santa Cruz at 1:75 dilution) and rabbit anti-Cyclin D1 (US Biological at a 1:100 dilution) staining, antigen retrieval was accomplished by boiling samples at 95°C in Target Retrieval Solution Citrate pH 6.0 (DAKO). Slides were blocked in 5% NDS in TBS-T for Ki67 and Cyclin D1; in 5% NDS, 10% BSA in PBS for HES1; and in 10% NGS, 10% BSA in PBS for Synaptophysin. Primary antibody incubation was performed for 2 hours at room temperature. After three 2 min washes in TBS-T, and one in TBS, slides were incubated in secondary antibodies (biotinylated donkey anti-rabbit at a 1:1000 dilution, biotinylated goat anti-mouse at a 1:1000 dilution, or biotinylated rabbit anti-rat at a 1:200 dilution) for 2 hours at room temperature. Slides were washed as before, and incubated for 30 min in an avidin-biotin horseradish peroxidase complex, Elite ABC (Vector Labs). After a brief wash in TBS, Vectastain DAB substrate (Vector Labs) was applied for 3 minutes for satisfactory colour development. Ki67 and Cyclin D1 staining required a DAB time of 7 minutes. Tissues were counterstained with Harris hematoxylin (Sigma), differentiated in acid ethanol (0.3% HCl in 70% ethanol), blued in Scott's tap water substitute, and

dehydrated in a gradient of ethanol. Slides were then dried in xylenes and mounted using PolyMount (Polysciences Inc). Images were acquired using the Aperio ScanScope, and processed using ImageScope.

2.7 IMMUNOFLUORESCENCE

Tissue slides were incubated in xylenes at room temperature for 10 min (2 washes) to remove paraffin, followed by rehydration in an ethanol gradient as described above. Tissue was permeabilized with 0.05% TBS-T, and antigen retrieval was accomplished by boiling samples in 10 mM sodium citrate buffer (pH 6.0). Tissues were incubated in 5% NGS and 10% BSA in TBS-T for 1 hour at room temperature. The slides were next incubated with primary antibodies (rabbit polyclonal anti-Kaiso at a dilution of 1:500, BD Biosciences mouse monoclonal anti-p120 at a dilution of 1:500; BD Biosciences mouse monoclonal anti-E-cadherin at a dilution of 1:500; Santa Cruz mouse monoclonal anti-Claudin-1 at a dilution of 1:100) at 4°C overnight. After three 10 min washes in TBS-T, and one in TBS, slides were incubated in secondary antibodies (Alexa-488 goat anti-mouse and Alexa-546 goat anti-rabbit, at a dilution of 1:500 each) for 2 hours in the dark at room temperature. Slides were washed as before, and incubated for 30 min in the dark with TOTO-3 dye (Invitrogen; 1:1000) to stain the nuclei. Slides were mounted in ProLong Gold (Invitrogen) overnight in the dark and stored at -20°C until imaging. Images were captured and processed using a Leica Confocal Microscope.

2.8 INTESTINAL EPITHELIAL CELL ISOLATION

Fresh intestinal tissues were isolated from sacrificed mice as stated above. Tissues from two mice were processed at a time. The intestines were washed and flushed with saline solution (5 M NaCl, 1 M imidazole, pH 7.2, 10% sodium azide). The intestinal tissues were cut open longitudinally and two inch sections were cut and placed into a sucrose buffer (200 mM sucrose, 20 mM KH_2PO_4 monobasic, 80 mM Na_2HPO_4 dibasic, 0.5 M EDTA, 10% sodium azide). The intestinal sections were incubated in sucrose buffer with vigorous continuous stirring at 4°C for 2 hours. The sucrose buffer with isolated epithelial cells was sifted using a cheese cloth to remove large tissue sections, and centrifuged at 200g for 10 min at 4°C. The pellet containing isolated epithelial cells was resuspended and washed in fresh sucrose buffer and centrifuged at 200 g for 10 min at 4°C. The pellet was finally washed in 10 mL cold 1X PBS, and centrifuged at 5000 rpm for 5 min at 4°C. The PBS was aspirated off the pelleted cells, and cells were flash frozen in liquid nitrogen. The epithelial cells were stored at -80°C for future protein isolation, as described in Section 2.9 below.

2.9 PROTEIN ISOLATION AND IMMUNOBLOT

50 mg of flash frozen mouse tissue was minced with a sterile blade and homogenized in 1 mL cold RIPA buffer (1% NP-40, 50 mM Tris, 150 mM NaCl, 0.5% sodium deoxycholate, 1% SDS, 0.5% Na_3VO_4 and cOmplete ULTRA Tablet (1 tablet/5 mL buffer, Roche) in a chilled tissue grinder (Kontes). Harvested lysates were poured into chilled microfuge tubes followed by further homogenization using a 21-Gauge syringe. Lysates were incubated on ice for 30 minutes, followed by centrifugation at 13,000g for

10 min at 4°C. **I**ntestinal **e**pithelial **c**ell (IEC) pellets were lysed in 100 μL of 1% SDS and boiled for 20 min until the pellet was completely dissolved. Lysates were spun at 14,000 rpm for 30 min at 4°C.

The supernatants were transferred to new pre-chilled microfuge tubes. Total protein content was quantified by Bradford assay (tissue lysate) or Lowry assay (IEC lysate), and 25 mg of protein was resuspended in Laemmli sample buffer, boiled for 5 min and subjected to electrophoresis in an SDS polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane using a Hoeffer semi-dry transfer apparatus (Amersham Biosciences). To prevent non-specific antibody binding, the membranes were blocked with 3% milk/TBS (pH 7.4) and incubated at 4°C overnight with antibody diluted in 3% milk/TBS. Antibodies used were as follows: rabbit polyclonal anti-Kaiso antibody (gift from Dr. Albert Reynolds) at a 1:30,000 dilution, rabbit polyclonal anti-Cyclin D1 antibody (US Biological) at a 1:5,000 dilution, rabbit polyclonal anti-NFκB p65 (Santa Cruz) at 1:1000, mouse monoclonal anti-ICAM-1 (Santa Cruz) at 1:150, mouse monoclonal anti-E-cadherin (BD Biosciences) at 1:240,000, rat monoclonal anti-MMP7 (Vanderbilt) at 1:200, and mouse monoclonal anti-β-actin antibody (Sigma Aldrich) at a 1:30,000 dilution. The membranes were washed 5 x 5 minutes each with TBS and incubated at room temperature with HRP-conjugated donkey anti-mouse or goat anti-rabbit secondary antibody both at a dilution of 1:40,000 in 3% milk/TBS. Membranes were washed as previously described and processed with Enhanced Chemiluminescence (Amersham Biosciences) according to the manufacturer's protocol.

2.10 RNA ISOLATION

Mouse tissues were homogenized and total RNA purified using the RNeasy Kit (Qiagen). Briefly, ~20 mg frozen tissues were chopped finely with a clean blade, resuspended in 600 µl Qiagen digestion buffer, and homogenized on ice in a glass tissue grinder. Total RNA was then purified from the homogenized lysate using the RNeasy kit according to manufacturer's instructions.

2.11 RT-PCR

Reverse **t**ranscriptase **PCR** (RT-PCR) analysis was performed using SuperScriptII One-Step RT-PCR with Platinum Taq (Invitrogen). Briefly, 1 µg of RNA was DNaseI treated (Invitrogen) to remove any genomic DNA contamination. 100 ng total RNA was used for each reaction with primers specific to the *villin-mKaiso* transcript and **t**ranscription **f**actor **II D** (TFIID) as a loading control. The primer pairs used were as follows*: villin-mKaiso*: forward *5'-CAA CTT CCT AAG ATC TCC CAG GT-3'* and reverse *5'-CAA GGA GTT CAG CAG ACT GG -3'*; *TFIID*: forward *5'-CCA CGG ACA ACT GCG TTG AT-3'* and reverse *5'-GGC TCA TAG CTA CTG AAC TG-3'*. The RT-PCR program included one round of cDNA synthesis at 50°C for 30 minutes, followed by denaturation at 95°C for 2 minutes. 25 cycles of DNA amplification was performed as follows: denaturation at 95° for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec. Final extension occurred at 72°C for 10 mins.

2.12 QUANTITATIVE RT-PCR

Total RNA was purified from ~ 20 mg of small intestinal tissue as described above. 1 µg of RNA was DNaseI treated (Invitrogen) to remove any genomic DNA contamination, and cDNA synthesis was accomplished using the SuperScript III First-Strand Synthesis System (Invitrogen). RNA abundance was compared using PerfeCTa SYBR Green SuperMix Reaction Mixes (Quanta Biosciences). The standard curve method was used to calculate relative expression of HES1 and Kaiso following normalization to the housekeeping gene, GAPDH, and then normalizing to the **non-t**rans**g**enic (non-Tg) tissue level. Primer sequences used were as follows: *villin-mKaiso* as stated above; *mHES1*: forward *5'-AAA ATT CCT CCT CCC CGG TG-3'* and reverse *5'-TTT GGT TTG TCC GGT GTC G-3'*; *mPPARγ*: forward *5'- GTC ACA CTC TGA CAG GAG CC-3'* and reverse *5'- TCA CCG CTT CTT TCA AAT CTT GT-3'*; and *mGAPDH:* forward *5'–ATG ACC ACA GTC CAT GCC ATC–3'* and reverse *5'-CCT GCT TCA CCA CCT TCT TG-3'*.

2.13 MYELOPEROXIDASE (MPO) ASSAY

Approximately 50 mg of flash frozen ileum and colon were homogenized in 50 mg/mL of 0.5% HTAB buffer (0.5% **h**exadecyl**t**rimethyl**a**mmonium **b**romide in 50 mM phosphate buffer, pH 6.0) via sonication at 30Hz for 4 minutes. Homogenates were cleared by centrifugation at 12,000 rpm for 15 minutes at 4°C. MPO Assay was performed by adding 200 µL of o-dianisidine dihydrochloride solution (16.8 mg/mL o-dianisidine dihydrochloride in 5 mM phosphate buffer, pH 6.0 with 50 µL of 1.2% H_2O_2) to 96-well plates. Samples (7 µL) were added to each well of the 96-well plate in triplicate, and absorbance measured at 450 nm every 30 sec (3 readings). The MPO activity was

measured in units (U), where 1 U represents the amount MPO needed to degrade 1 μmoL of H_2O_2/minute at 25°C, which gives an absorbance of 1.13 X 10^{-2} nm/min. MPO activity in each sample was determined as the change in absorbance $[\Delta A(t_2\text{-}t_1)]/\Delta min]/(1.13 \text{ X } 10^{-2})$. MPO activity/mg of tissue was calculated by dividing MPO U by 0.35 mg of tissue (7 μL homogenate X 50 mg/mL buffer).

2.14 FITC-DEXTRAN ASSAY

FITC-Dextran (4 kDa; Sigma) was diluted to 50 mg/mL in distilled water. Mice were weighed and fasted (food and water) for 7 hours. The mice were then given 0.6 mg/g of body weight of the FITC-Dextran solution by oral gavage. Two (2) hours after the oral gavage, blood was collected via retro-orbital bleeding into whole blood collection tubes. Blood was centrifuged at 12,000 rpm for 3 min at 4°C to separate the plasma (supernatant) and serum (pellet). Plasma was analyzed for FITC-Dextran levels using a fluorescent spectrophotometer with excitation at 485 nm and emission at 535 nm (Perkin Elmer Instruments LS Reader Plate Fluorometer). Standard curves for calculating the FITC-Dextran concentration in all samples were generated using known dilutions of FITC-Dextran in distilled water.

2.15 ISOLATION OF INTESTINAL LAMINA PROPRIA CELLS

Freshly isolated intestines were washed and cleaned in cold PBS. The intestines were opened longitudinally and cut into 2-3 mm pieces and incubated in 1 mM DL-**dit**hio**t**hreitol (DTT) (Sigma-Aldrich) in PBS for 15 min in a 37°C shaker to remove mucus. The intestinal sections were vortexed briefly (2-3 sec) and poured through a metal

strainer to separate mucus from tissue pieces. The tissue pieces were then washed three (3) times in 10% **f**etal **b**ovine **s**erum (FBS) in PBS and 2 mM EDTA (**e**thylene**d**iamine**t**etra**a**cetic acid) for 15 min each in a 37°C shaker to remove the epithelial cell fraction, repeating the collection process through the metal strainer as above. After the final wash, the remaining tissue was incubated for 50-60 min in a 37°C shaker and digested with 0.239 mg/mL Collagenase A (Roche) with DNase (Roche) in RPMI media. The samples were then filtered through a 40 μm nylon mesh filter in RPMI to release the lamina propria cells. The cells were collected and centrifuged at 1250 rpm at 4°C for 10 mins and resuspended in 6 mL of 40% Percoll (GE Healthcare) in RPMI. 2 mL of 70% Percoll in RPMI was carefully added to the bottom of the 40% Percoll cell suspension using a long glass micropipette. The samples were centrifuged at 2400 rpm for 30 min at 23°C. The cells collected at the 40-70% Percoll interface were transferred to fresh RPMI media and centrifuged at 1300 rpm for 10 min at 4°C. The pelleted cells were re-suspended in FACS buffer and used for Flow Cytometry as described below.

2.16 Flow Cytometry

Isolated intestinal lamina propria cells were pre-incubated with anti-FcγRII/IIIb antibodies for 15 min in the dark on ice to prevent non-specific binding of the flurochrome-conjugated antibodies. The cells were then incubated with the following fluorochrome-conjugated antibodies for 30 min on ice in the dark: F7/4-FITC (1:200), CD63-PE (1:200), Ly-6G- (1:100), CD11b-eFluor605 (1:100), F4/80- (1:100), CD45-APC-eFluor780 (1:100). Dead cells were excluded by **p**ropidium **i**odide (PI) uptake and

gated on singlets. **F**luorescence **m**inus **o**ne (FMO) controls were used for gating. Data were acquired on an LSR II (BD) and analyzed using FlowJo (Tree Star).

2.17 CELL CULTURE

HCT-116 cells were purchased from ATCC (Manassas, USA) and cultured as per the ATCC protocols. In addition to the parental cell lines, three HCT-116 stable *Kaiso* misexpressing and control cell lines were generated by Dr. Kevin Kelly (Daniel Lab): pRS-Kaiso where Kaiso is depleted via siRNA and a negative control pRS–Empty (pRetroSuper) vector, lacking the Kaiso-specific siRNA sequence. Cells were cultured in DMEM (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Thermo Scientific), 0.1 mg/mL penicillin/streptomycin (Invitrogen) and 0.25 μg/mL fungizone (Invitrogen). The stable cell lines were grown in media as listed above with addition of 2 μg/mL of puromycin for continual selection. Cells were lysed and protein isolated as listed in Section 2.9.

2.18 STATISTICAL ANALYSIS

When necessary, the Student's T-test was used to determine whether any observed differences were statistically significant ($p < 0.05$) using GraphPad Prism 6.0, unless stated otherwise.

CHAPTER 3: THE POZ-ZF TRANSCRIPTION FACTOR KAISO (ZBTB33) INDUCES INFLAMMATION AND PROGENITOR CELL DIFFERENTIATION IN THE MURINE INTESTINE

PREFACE:

This chapter outlines the studies performed during the initial characterization of the **Kaiso t**rans**g**enic (*Kaiso*$^{Tg/+}$) mice in the following publication:

***Chaudhary R**, Pierre CC, Nanan K, Wojtal D, Morone S, Pinelli C, Wood GA, Robine S, Daniel JM. (2013) The POZ-ZF Transcription Factor Kaiso (ZBTB33) Induces Inflammation and Progenitor Cell Differentiation in the Murine Intestine. PLoS ONE 8(9): e74160. doi:10.1371/journal.pone.0074160*

Revisions were made to maintain formatting consistency with other chapters. Significant revisions include elimination of methods section (details in Chapter 2), removal of reference list (included in combined thesis bibliography), and re-organization of figures in order of appearance in the text.

I performed ~85% of the experiments in this article, assembled the figures, and co-wrote the manuscript. Christina C. Pierre co-wrote the manuscript and performed the western blots shown in Figure 3.1D and Figure 3.8C. The Line A and B mice were originally created by Kyster Nanan, who showed the difference in copy number between the varying lines along with the intestinal specific expression of the transgene depicted in Figure 3.1B and C. I created *Kaiso*$^{Tg/+}$ Lines E and F upon the loss of Line B to ensure phenotypic changes were independent of transgene insertion sites. Daria Wojtal

performed the c-myc IHC to show the cellular specificity of the transgene seen in Figure 3.2. Drs. Pinelli and Wood were integral in understanding the pathology and histology of the intestines. Dr. Simona Morone was very helpful in the initial dissections and tissue collection. Dr. Robine provided the *villin* promoter construct used to create the transgene. Dr. Daniel, Christina C. Pierre and I all contributed to the design and intellectual input of the manuscript, along with the numerous revisions to the manuscript before submission and acceptance.

This study summarized the overall phenotypic analysis of the *Kaiso*$^{Tg/+}$ mice. *Kaiso*$^{Tg/+}$ mice were created to have Kaiso overexpression specifically in the intestines using the *villin* promoter. Immunoblot and immunohistochemistry were used to show the tissue specific increase of Kaiso expression in the intestinal epithelial cells. The *Kaiso*$^{Tg/+}$ mice exhibited increased crypt depth, though there was a decrease in proliferation. The *Kaiso*$^{Tg/+}$ mice also display signs of inflammation, which may be attributed to the nuclear localization of p120^{ctn}. In this study, we also reported an increase in secretory cell types (Goblet, Paneth and Enteroendocrine (EEC) cells) that may be explained by the decrease expression of the differentiation factor and Notch pathway effector protein, HES1. This study was the first to link Kaiso to the Notch signalling pathway.

3.1 ABSTRACT

Since its discovery, several studies have implicated the POZ-ZF protein Kaiso in both developmental and tumorigenic processes. However most of the information regarding Kaiso's function to date has been gleaned from studies in *Xenopus laevis* embryos and cultured mammalian cells. To examine Kaiso's role in a relevant, mammalian organ-specific context, we generated and characterized a Kaiso transgenic mouse model expressing a murine Kaiso transgene under the control of the intestine-specific *villin* promoter. Kaiso transgenic mice were viable and fertile but pathological examination of the small intestine revealed distinct morphological changes. Kaiso transgenics ($Kaiso^{Tg/+}$) exhibited a crypt expansion phenotype that was accompanied by increased differentiation of epithelial progenitor cells into secretory cell lineages; this was evidenced by increased cell populations expressing goblet, Paneth and EEC markers. Paradoxically however enhanced differentiation in $Kaiso^{Tg/+}$ was accompanied by reduced proliferation, a phenotype reminiscent of Notch inhibition. Indeed, expression of the Notch signalling target HES1 was decreased in $Kaiso^{Tg/+}$ animals. Finally, our Kaiso transgenics exhibited several hallmarks of inflammation, including increased neutrophil infiltration and activation, villi fusion and crypt hyperplasia. Interestingly, the Kaiso binding partner and emerging anti-inflammatory mediator, $p120^{ctn}$, is recruited to the nucleus in $Kaiso^{Tg/+}$ mice suggesting that Kaiso may elicit inflammation by antagonizing $p120^{ctn}$ function.

3.2 INTRODUCTION

Since its discovery as a binding partner for the Src kinase substrate and cell adhesion protein $p120^{ctn}$, mounting evidence suggests that the POZ-ZF transcription factor Kaiso functions in vertebrate development and tumourigenesis (Cofre *et al*., 2012; Dai *et al*., 2010; Dai *et al*., 2009; Jiang *et al*., 2012; Lopes *et al*., 2008; Prokhortchouk *et al*., 2006; Vermeulen *et al*., 2012; Wang *et al*., 2012). To date however, Kaiso's role in these processes in mammalian systems remains unclear, and much controversy surrounds several aspects of Kaiso's function, including the mechanism by which it binds to DNA (Blattler *et al*., 2013; Daniel *et al*., 2002; Donaldson *et al*., 2012; Prokhortchouk *et al*., 2001; Ruzov *et al*., 2004; Ruzov *et al*., 2009a; Ruzov *et al*., 2009b; Sasai *et al*., 2010; Yoon *et al*., 2003) and its function in regulating the canonical Wnt signalling pathway that plays a key role in vertebrate development and tumourigenesis (Donaldson et al., 2012; Park et al., 2005; Prokhortchouk et al., 2006; Ruzov et al., 2009a).

One study investigated the effect of Kaiso depletion on murine development and found that Kaiso null mice exhibited no overt developmental phenotypes (Prokhortchouk *et al.*, 2006). This unexpected lack of a developmental phenotype may be attributed to the existence of two Kaiso-like proteins in mammals, ZBTB4 and ZBTB38, that may function redundantly with Kaiso (Filion *et al.*, 2006; Sasai *et al.*, 2010), and highlights what may be an important consideration in deciphering Kaiso's role in mammalian systems. Surprisingly however, Kaiso depletion extended the lifespan, and delayed tumour onset in the $Apc^{Min/+}$ model of intestinal tumourigenesis (Prokhortchouk *et al.*, 2006). This observation implicated Kaiso as an oncogene and is consistent with the report

that Kaiso binds and represses methylated tumour suppressor and DNA repair genes in colon cancer cells (Lopes *et al.*, 2008). Given that constitutive Wnt signalling resulting from mutation of *Apc* functions as the first "hit" in $Apc^{Min/+}$-mediated tumourigenesis, the *Kaiso*-null/$Apc^{Min/+}$ phenotype suggests that Kaiso is a positive regulator of Wnt signalling. This result is surprising, since Kaiso has been implicated as a negative regulator of canonical Wnt signalling in *Xenopus laevis* embryos and in cultured mammalian cells (Donaldson *et al*., 2012; Park *et al*., 2006; Park *et al*., 2005; Spring *et al*., 2005). However it remains possible that Kaiso may potentiate intestinal tumorigenesis in the $Apc^{Min/+}$ model via a non-Wnt related mechanism.

Consistent with this possibility, studies to elucidate the role of the Kaiso binding partner $p120^{ctn}$ in the intestine hinted at a non-cell autonomous mechanism for $p120^{ctn}$-mediated tumourigenesis (Smalley-Freed *et al.*, 2010; Smalley-Freed *et al.*, 2011). Smalley Freed *et al.* found that mice with limited ablation of $p120^{ctn}$ developed adenomas in addition to an intestinal barrier defect and chronic inflammation (Smalley-Freed *et al.*, 2011). Surprisingly, conditional depletion of $p120^{ctn}$ in the murine intestine resulted in severe **i**nflammatory **b**owel **d**isease (IBD) and lethality (Smalley-Freed *et al.*, 2010; Smalley-Freed *et al.*, 2011). Thus it was postulated that the adenomas arising in mice with limited $p120^{ctn}$ ablation was a result of chronic inflammation, which is considered a risk factor for colorectal cancer (Terzic *et al.*, 2010).

Since several studies have implicated Kaiso in intestinal cancer development and progression (Lopes *et al.*, 2008; Prokhortchouk *et al.*, 2006), we generated an intestinal-specific Kaiso overexpression mouse model to clarify Kaiso's role in the context of

murine intestinal epithelium development. We generated multiple *Kaiso*$^{Tg/+}$ founder lines, each with varying copy numbers of the transgene. *Kaiso*$^{Tg/+}$ mice were viable and fertile with no deleterious developmental phenotypes. However we noticed several phenotypes in the intestines of *Kaiso*$^{Tg/+}$ mice that were reminiscent of Notch inhibition. *Kaiso*$^{Tg/+}$ mice exhibited increased differentiation of intestinal epithelial progenitor cells into secretory cell lineages (Paneth, goblet, EEC) accompanied by reduced proliferation, a phenotype consistent with Notch inhibition (Ogaki *et al*., 2013; VanDussen *et al*., 2012; Zecchini *et al*., 2005). Indeed, expression of the Notch signalling target HES1 was also reduced in *Kaiso*$^{Tg/+}$ mice. Interestingly, p120^{ctn} localized mainly to the nucleus in the small intestine in *Kaiso*$^{Tg/+}$ mice, and this was accompanied by increased infiltration of inflammatory cells and increased myeloperoxidase activity (a surrogate marker for inflammation) suggesting that *Kaiso*$^{Tg/+}$ mice are more susceptible to inflammation. Together these data suggest that Kaiso functions in a pro-inflammatory role in the murine intestine by antagonizing the anti-inflammatory functions of p120^{ctn}.

3.3 RESULTS

3.3.1 Generation of *villin-Kaiso* transgenic mice

Kaiso transgenic (*Kaiso*$^{Tg/+}$) mice were generated by cloning the sequence encoding N-terminal myc-tagged murine *Kaiso* downstream of a 9 Kb regulatory region of the mouse *villin* gene (Figure 3.1A). The *villin-Kaiso* construct was injected into fertilized C57BL6/CBA embryos that were subsequently transferred to pseudo-pregnant foster mothers and resulted in four transgenic founder mice (Line A, B, C, D). Upon

backcrossing to C57BL/6N mice, only lines A, B and C transmitted the transgene to their progeny at rates of 15%, 32% and 57%, respectively. Since pronuclear injections result in random genome integration, transgene copy number was estimated by PCR (Figure 3.1B). The three founders possessed varying copy numbers of the Kaiso transgene, with Line A having the highest copy number and Line C having the lowest copy number. Unfortunately, Line C died prior to being established and thus Lines A and B were used for further analysis. Upon founder line establishment (8 generations of backcrossing), Lines A and B transmitted the transgene at rates of 33.8% and 35.9% respectively, which is lower than the expected Mendelian rate of 50%.

To confirm tissue-specific expression of the *Kaiso* transgene, RT-PCR was performed with transgene-specific primers. As expected, the transgene was detected in all 3 villin-positive tissues: kidneys, **s**mall **i**ntestine (SI) and **l**arge **i**ntestine (LI) (Figure 3.1C). Kaiso protein expression was confirmed by Western blot analysis of protein harvested from SI and LI (Figure 3.1D). Consistent with the transgene copy number observed via PCR, higher Kaiso protein expression was detected in Line A transgenics compared to Line B, with the lowest protein expression in Line C (data not shown).

To further evaluate and confirm Kaiso expression and localization in $Kaiso^{Tg/+}$ and Non-Tg tissues, IHC was performed on SI and LI tissues harvested from Line A and Line B mice using a Kaiso-specific antibody. Line A $Kaiso^{Tg/+}$ mice exhibited stronger nuclear Kaiso expression in the villi and increased nuclear expression in the crypts of the small intestine compared to their Non-Tg siblings (Figure 3.2A). However, Line B $Kaiso^{Tg/+}$, which expressed less Kaiso than Line A, exhibited predominantly cytoplasmic localized

Kaiso (Figure 3.2B). In the LI, both transgenic lines exhibited stronger Kaiso staining than their Non-Tg siblings (Figure 3.2C). Furthermore, strong nuclear Kaiso expression was observed in the epithelial cells near the top of the crypts, with lower expression at the bottom of the crypts (Figure 3.2). To confirm that increased Kaiso expression in *Kaiso*$^{Tg/+}$ mice was due to the transgene rather than an enhancement of endogenous *Kaiso* gene expression, we evaluated c-Myc expression in Line A small intestines. Indeed, *Kaiso*$^{Tg/+}$ mice exhibited stronger staining in comparison to Non-Tg mice, consistent with the expression of myc-tagged Kaiso (Figure 3.2). All subsequent analyses were performed on Line A *Kaiso*$^{Tg/+}$ (unless noted otherwise).

3.3.2 Kaiso transgenic mice exhibit inflammation in the intestinal mucosa

After establishing that Kaiso was robustly expressed in the intestine via our transgene we next sought to determine the effect of ectopic Kaiso on intestinal morphology and function. Examination of H&E stained sections from SI and LI tissues of 1-year old Line A mice revealed longer crypts with no difference in villi length in the small intestine (Figure 3.3A), although this phenotype was not observed in Line B mice. We also noticed that several villi were fused and blunted in our Line A *Kaiso*$^{Tg/+}$ mice in comparison to the characteristic elongated, finger-like appearance of villi in Non-Tg mice (Figure 3.3). To rule out the possibility that this phenotype was an artefact resulting from the transgene insertion site, we examined H&E sections from additional *Kaiso*$^{Tg/+}$ lines that had been backcrossed for only 3 generations. Two of these lines, Lines E and F, exhibited even more robust Kaiso staining than Line A mice, concomitant with extensive

villi fusion and blunting (Figure 3.4). These lines have continued to be backcrossed to 10 generations.

Crypt hyperplasia accompanied by fused, blunted villi has been previously reported in both humans and mice exhibiting chronic inflammation of the intestinal mucosa (Goldstein, 2006; Jacques and Elewaut, 2008; Kuhnert *et al.*, 2004; Ostanin *et al.*, 2006), suggesting that ectopic Kaiso expression may cause intestinal inflammation. Indeed, closer examination of *Kaiso*$^{Tg/+}$ intestines (Line A, E and F) revealed increased immune cell infiltration of the lamina propria compared to their Non-Tg siblings (Figure 3.3B and Figure 3.4); however no such phenotype was observed in Line B mice (Figure 3.3C). We also measured the levels of MPO, which is a surrogate marker for inflammation, in *Kaiso*$^{Tg/+}$ and Non-Tg intestinal tissues. MPO activity was increased in the distal SI (ileum) of Lines A, E and F *Kaiso*$^{Tg/+}$ mice compared to their age-matched Non-Tg siblings (Figure 3.3B and Figure 3.4) while no change in MPO activity was detected in Line B mice (Figure 3.3C). Furthermore, the proximal colon of Lines E and F also exhibited increased MPO activity while mice from Lines A and B exhibited no such change (data not shown). These data suggest that ectopic Kaiso expression may predispose the murine intestine to inflammation, but this effect may be dose-dependent.

3.3.3 Ectopic Kaiso overexpression results in nuclear accumulation of p120^{ctn}

Given that *Kaiso*$^{Tg/+}$ mice exhibited an inflammatory response similar to that elicited by limited p120^{ctn} depletion (Smalley-Freed *et al.*, 2010), albeit less severe, we examined p120^{ctn} expression in the small intestines of our *Kaiso*$^{Tg/+}$ mice. Interestingly,

in *Kaiso*$^{Tg/+}$ mice we observed nuclear localization of p120^{ctn} and reduced p120^{ctn} staining at the membrane in the distal small intestine (Figure 3.4 and 3.5). However in Non-Tg siblings, p120^{ctn} was largely membrane bound (Figure 3.5). Taken together this data suggests that Kaiso overexpression results in nuclear accumulation of p120^{ctn}, and decreased membrane-bound p120^{ctn}, which phenocopies the consequences of p120^{ctn} depletion (Smalley-Freed *et al.*, 2011).

3.3.4 Kaiso transgenic mice exhibit enhanced differentiation of progenitor cells into secretory cell fates

While characterizing the effect of ectopic Kaiso expression on intestinal morphology, we noted a significant expansion of goblet cells in both the SI and LI of Line A *Kaiso*$^{Tg/+}$ mice. Thus, we performed PAS staining for the goblet cell-specific marker, Mucin, and quantification of Mucin(+) cells confirmed a significant increase in the goblet cell population in both the SI and LI of Line A mice compared to Line B and Non-Tg mice (Figure 3.6A & Figure 3.7). Interestingly, staining for the other secretory cell types using the Paneth and EEC markers, lysozyme and synaptophysin respectively, revealed that these cell populations were also expanded in the SI and LI of Line A *Kaiso*$^{Tg/+}$ mice but not in Line B or non-Tg mice (Figure 3.6B, C & Figure 3.7).

The expansion of secretory cell lineages in the *Kaiso*$^{Tg/+}$ mice led us to hypothesize that Kaiso may be driving progenitor cell differentiation. However, since we also observed crypt expansion in *Kaiso*$^{Tg/+}$ mice, we questioned whether the increase in secretory cells was indicative of increased progenitor cell proliferation. Hence we examined the expression of the cell proliferation marker Ki67. Surprisingly, Ki67

expression was decreased in Line A *Kaiso*$^{Tg/+}$ mice and Ki67 positive cells were localized more apical to the normal crypt/villus boundary (Figure 3.8A). We next evaluated the expression of the Kaiso target gene *cyclin D1* (Donaldson *et al.*, 2012; Jiang *et al*., 2012) that has been shown to drive proliferation in the intestinal epithelium and is frequently overexpressed in colon cancer (Yang *et al.*, 2006). Similar to Ki67, Cyclin D1 expression was also decreased in Line A *Kaiso*$^{Tg/+}$ mice but surprisingly the apparent decreased numbers of Cyclin D1(+) cells in *Kaiso*$^{Tg/+}$ intestines was not statistically significant (Figure 3.8B, C).

Previous studies have reported an expansion of secretory cell lineages and a reduction in the number of proliferating columnar base cells upon inhibition of the Notch signalling pathway in the intestine (Jensen *et al*., 2000; Milano *et al*., 2004; VanDussen *et al*., 2012; Zecchini *et al*., 2005). Specifically, depletion of the Notch target gene *Hes1* resulted in increased expression of secretory cell markers in the intestine of *Hes1* null mice, suggesting that HES1 is necessary for specification of secretory cells in the intestine (Jensen *et al.*, 2000). This prompted us to examine the expression of HES1 in our *Kaiso*$^{Tg/+}$ mice. Line A *Kaiso*$^{Tg/+}$ mice exhibited decreased HES1 staining and reduced expression of *Hes1* mRNA compared to Non-Tg littermates (Figure 3.9). Together our data demonstrate that ectopic Kaiso elicits enhanced differentiation of progenitor cells into secretory lineages, perhaps through the down-regulation of the Notch target *Hes1*.

3.4 DISCUSSION

Since Kaiso's discovery over a decade ago, several studies have utilized *Xenopus laevis* and cultured cells as models to elucidate Kaiso's biological roles (Cofre *et al.*, 2012; Daniel, 2007; Jones *et al.*, 2012; Martin Caballero *et al.*, 2009; Prokhortchouk *et al.*, 2006; Ruzov *et al.*, 2009b; van Roy and McCrea, 2005; Vermeulen *et al.*, 2012). Here we describe the first study to examine the role of Kaiso in a relevant organ-specific context, the murine intestine. Using the murine *villin* promoter we were able to successfully drive intestinal-specific expression of the *Kaiso* transgene. In all founder lines, Kaiso was expressed along the entire crypt-villus axis with the most robust expression in the villi, which is consistent with the normal expression pattern of villin (Pinto *et al.*, 1999).

A previous report examining the effect of Kaiso depletion on $Apc^{Min/+}$-mediated tumorigenesis found that Kaiso depletion resulted in fewer tumours (Prokhortchouk *et al.*, 2006), suggesting that Kaiso functions as an oncogene. However ectopic Kaiso expression was not sufficient to drive spontaneous tumour formation in our mouse model. Nonetheless, our $Kaiso^{Tg/+}$ Line A mice exhibited enlarged crypts accompanied by fused, blunted villi, increased immune cell infiltration and increased MPO activity (indicative of neutrophil accumulation and inflammation) suggesting that $Kaiso^{Tg/+}$ mice have greater susceptibility to inflammation. Indeed, preliminary cytokine analysis of $Kaiso^{Tg/+}$ intestinal tissue revealed increased activity of the pro-inflammatory cytokine TNF-α compared to Non-Tg intestines (data not shown). Analysis of additional Kaiso transgenic lines (Lines E and F) revealed a similar intestinal phenotype to Line A, with concomitant

increased neutrophil activation as measured by MPO activity. Increased MPO activity is often correlated with **u**lcerative **c**olitis (UC), a form of IBD and patients with IBD are at a higher risk of colon cancer (Rubin *et al.*, 2012; Saleh and Trinchieri, 2011; Terzic *et al.*, 2010; Xavier and Podolsky, 2007). Thus in accordance with Knudson's multiple hit theory of tumorigenesis, it is possible that Kaiso's full oncogenic potential may only be unmasked in the presence of a second oncogenic insult such as *Apc* mutation or p53 loss of function. Intriguingly, preliminary analysis of intestinal tissues from a **d**extran **s**odium **s**ulfate (DSS)-induced model of colitis (kind gift of Dr. Elena Verdú), revealed increased expression of Kaiso compared to non-DSS treated mice (Figure 3.10), further supporting the notion that Kaiso overexpression plays a role in intestinal inflammation.

The enhanced inflammation observed in *Kaiso*$^{Tg/+}$ mice may be linked to altered p120^{ctn} function. Kaiso overexpression resulted in the nuclear localization of p120^{ctn}, suggesting that Kaiso may somehow recruit or sequester p120^{ctn} to the nucleus. Given that p120^{ctn} was mainly localized to the cytoplasm and the cell membrane in non-transgenic mice, this change in localization may be indicative of altered or lost p120^{ctn} function that may phenocopy the p120^{ctn} loss observed by Smalley Freed *et al.* (Smalley-Freed *et al.*, 2011). Further studies are needed to determine whether p120^{ctn} directly contributes to the Kaiso overexpression inflammatory phenotype.

Interestingly, the phenotypes observed in Lines A, E and F mice were not observed in Line B mice which express significantly lower levels of ectopic Kaiso; this suggests that a threshold level of Kaiso expression is necessary for the observed inflammatory phenotype. Additionally, no change in MPO activity was seen in Line B mice, further

supporting our hypothesis of threshold effects of Kaiso expression. This is not surprising since varying amounts of Kaiso were shown to have completely opposite effects in *Xenopus laevis* embryos . Hence in Line B mice, it is likely that Kaiso expression is below the threshold at which it elicits inflammation and leads to expanded crypts.

Finally, *Kaiso*$^{Tg/+}$ mice exhibited increased populations of goblet, Paneth and EEC cells. This expansion of secretory cell populations accompanied by decreased cell proliferation is consistent with the phenotype observed upon pharmacological inhibition of Notch signalling (VanDussen *et al*., 2012) and in HES1^{null} mice (Jensen *et al.*, 2000). One study found that Notch signalling is activated in intestinal epithelium in response to inflammation and is required for proper regeneration of the intestinal epithelium following colitis-induced damage (Okamoto *et al.*, 2009). It should be noted that 90-day old *Kaiso*$^{Tg/+}$ mice exhibit increased Goblet cells but do not exhibit any overt signs of inflammation or myeloperoxidase activity (data not shown). This suggests that inflammation in these mice develops over time although Notch inhibition is present at a very early age. Thus it is possible that the intestinal epithelium in our *Kaiso*$^{Tg/+}$ mice are incapable of regeneration following bacterial or physical insult and consequently develop chronic inflammation over time.

In summary, Kaiso overexpression promotes inflammation and inhibits Notch signalling in the murine intestine. These findings support a model in which *Kaiso*$^{Tg/+}$ mice develop inflammation, possibly by altering p120^{ctn} localization and consequently function (Figure 3.11). Kaiso's inhibition of the Notch pathway may hinder the ability of these mice to repair and regenerate the epithelium in response to inflammation, resulting

in chronic inflammation that increases in severity over time, thus making the mice more susceptible to inflammation-induced tumorigenesis.

Figure 3.1: Development of transgenic mouse lines ectopically expressing *Villin-Kaiso*.

(A) Myc-tagged murine *Kaiso* cDNA was cloned downstream of the 9 kb v*illin* promoter sequence. **(B)** The transgene copy number in each transgenic line was evaluated via PCR. Line A transgenic animals have the greatest copy number. **(C)** RT-PCR shows that the Kaiso transgene is being expressed in *villin* expressing tissues in transgenic mice, *i.e.* the small intestine, large intestine, and kidneys. **(D)** Immunoblot shows an increase in Kaiso expression in both small and large intestines in Kaiso transgenic ($Kaiso^{Tg/+}$) mice compared to non-transgenic (Non-Tg) siblings.

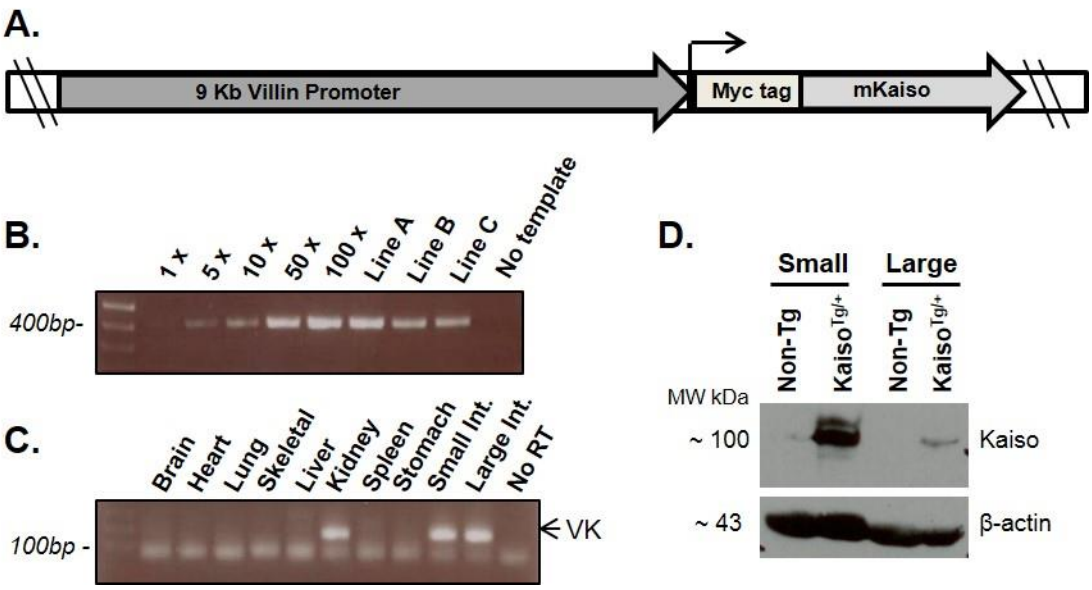

A.
9 Kb Villin Promoter
Myc tag
mKaiso
B.
1 x
5 x
10 x
50 x
100 x
Line A
Line B
Line C
No template
400bp-
C.
Brain
Heart
Lung
Skeletal
Liver
Kidney
Spleen
Stomach
Small Int.
Large Int.
No RT
100bp -
VK
D.
Small
Large
Non-Tg
Kaiso Tg/+
Non-Tg
Kaiso Tg/+
MW kDa
~ 100
Kaiso
~ 43
β-actin

Figure 3.2: Ectopic Kaiso expression in the intestine of Kaiso transgenic mice.

(A) Kaiso transgenic mice (Line A) display strong nuclear Kaiso in the villi and crypts, however non-transgenic mice (Non-Tg) display weak Kaiso staining with Kaiso mainly localizing to the cytoplasm. In addition, *Kaiso*$^{Tg/+}$ mice display strong nuclear c-Myc staining corresponding to ectopic myc-tagged Kaiso expression, while Non-Tg mice display cytoplasmic c-Myc expression. **(B)** Line B *Kaiso*$^{Tg/+}$ mice display sporadic nuclear stain and strong cytoplasmic Kaiso stain in the epithelial cells of the villi, and lack Kaiso staining in the crypts, compared to the Non-Tg mice. **(C)** Non-Tg mice display low nuclear Kaiso expression in the colon, while Line A and B *Kaiso*$^{Tg/+}$ show strong nuclear Kaiso expression in the colon, with the apical epithelial cells displaying the most Kaiso expression. Colon tissues from Line A show greater Kaiso expression than the colon tissue from Line B.

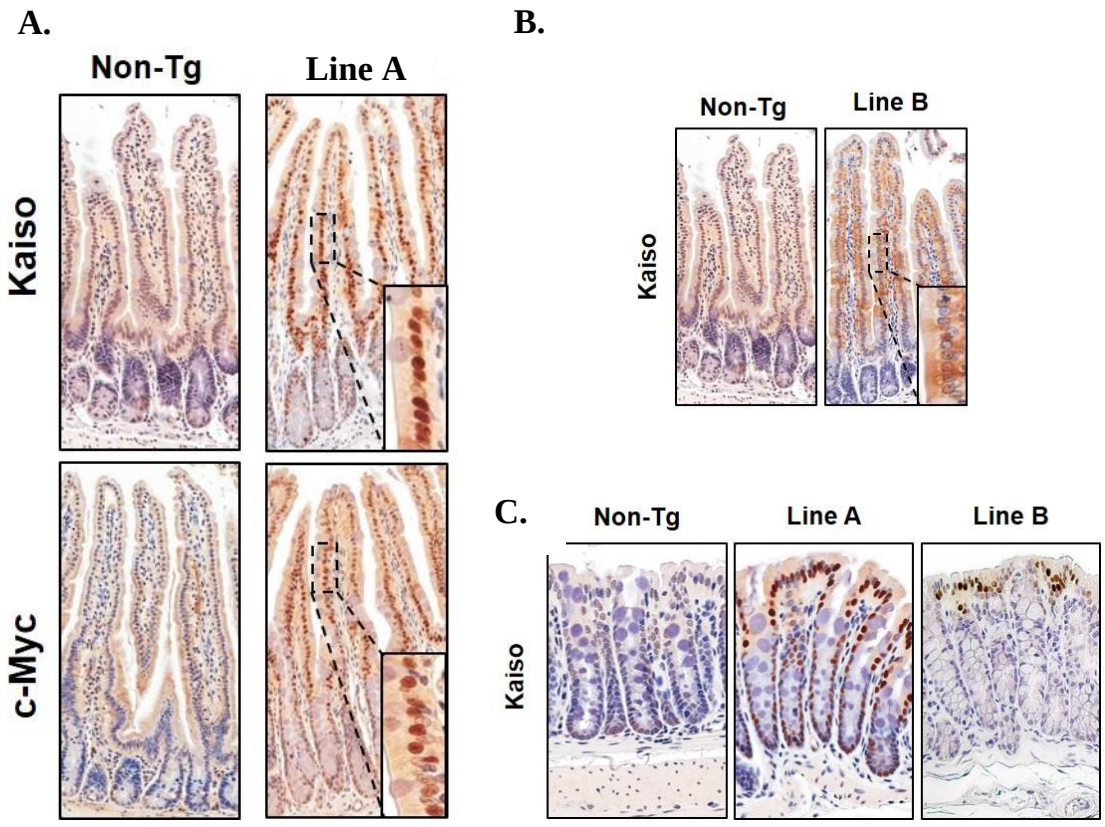

A.
Non-Tg
Line A
Kaiso
c-Myc
B.
Non-Tg
Line B
Kaiso
C.
Non-Tg
Line A
Line B
Kaiso

Figure 3.3: Kaiso transgenic mice exhibit inflammation of the intestinal mucosa.

(A) H&E stained sections were used to measure villi length (red bracket; ~80 villi/mouse) and crypt depth (black bracket; ~800 open crypt/mouse). *Kaiso*$^{Tg/+}$ display increased crypt depth compared to their non-transgenic (Non-Tg) siblings. **(B)** Line A *Kaiso*$^{Tg/+}$ mice exhibit increased immune cell infiltration of the lamina propria (yellow demarcated area) accompanied by increased MPO activity compared to their Non-Tg siblings. **(C)** Line B mice do not exhibit immune cell infiltration or enhanced MPO activity compared to Non-Tg. * represents p-value <0.05.

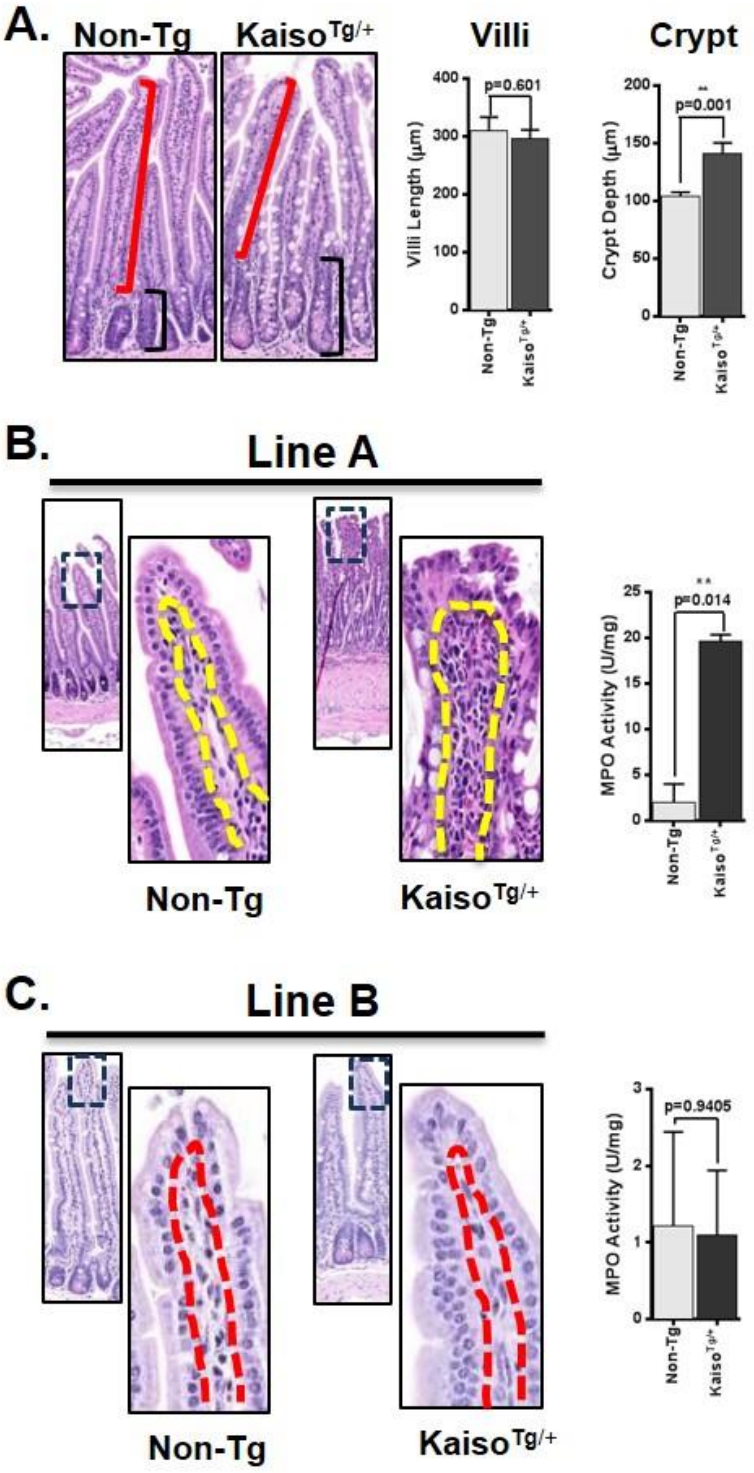

A.
Non-Tg
Kaiso Tg/+
Villi
Crypt
p=0.601
p=0.001
Villi Length (µm)
Crypt Depth (µm)
Non-Tg
Kaiso Tg/+
B.
Line A
Non-Tg
Kaiso Tg/+
p=0.014
MPO Activity (U/mg)
C.
Line B
Non-Tg
Kaiso Tg/+
p=0.9405
MPO Activity (U/mg)

Figure 3.4: Ectopic Kaiso expression in the small intestine of multiple Kaiso transgenic lines increases inflammatory cell infiltrates.

Kaiso$^{Tg/+}$ mice display strong nuclear Kaiso expression in the villi and crypt cells, however Non-Tg mice display weak Kaiso staining with most Kaiso localizing to the cytoplasm. Lines E and F show strong Kaiso staining from the base of the crypts to the top of the villi. Interestingly, in all three *Kaiso*$^{Tg/+}$ lines analysed, ectopic Kaiso expression also appears to induce villi fusion (black arrows). Histological analysis showed increased neutrophil infiltration into the villi of Lines A, E, F *Kaiso*$^{Tg/+}$ mice (yellow demarcated area). An MPO assay of Line A, E and F ileums show increased MPO activity when compared to age-matched Non-Tg. Immunofluorescence shows nuclear p120^{ctn} in both Line E and F in the villi.

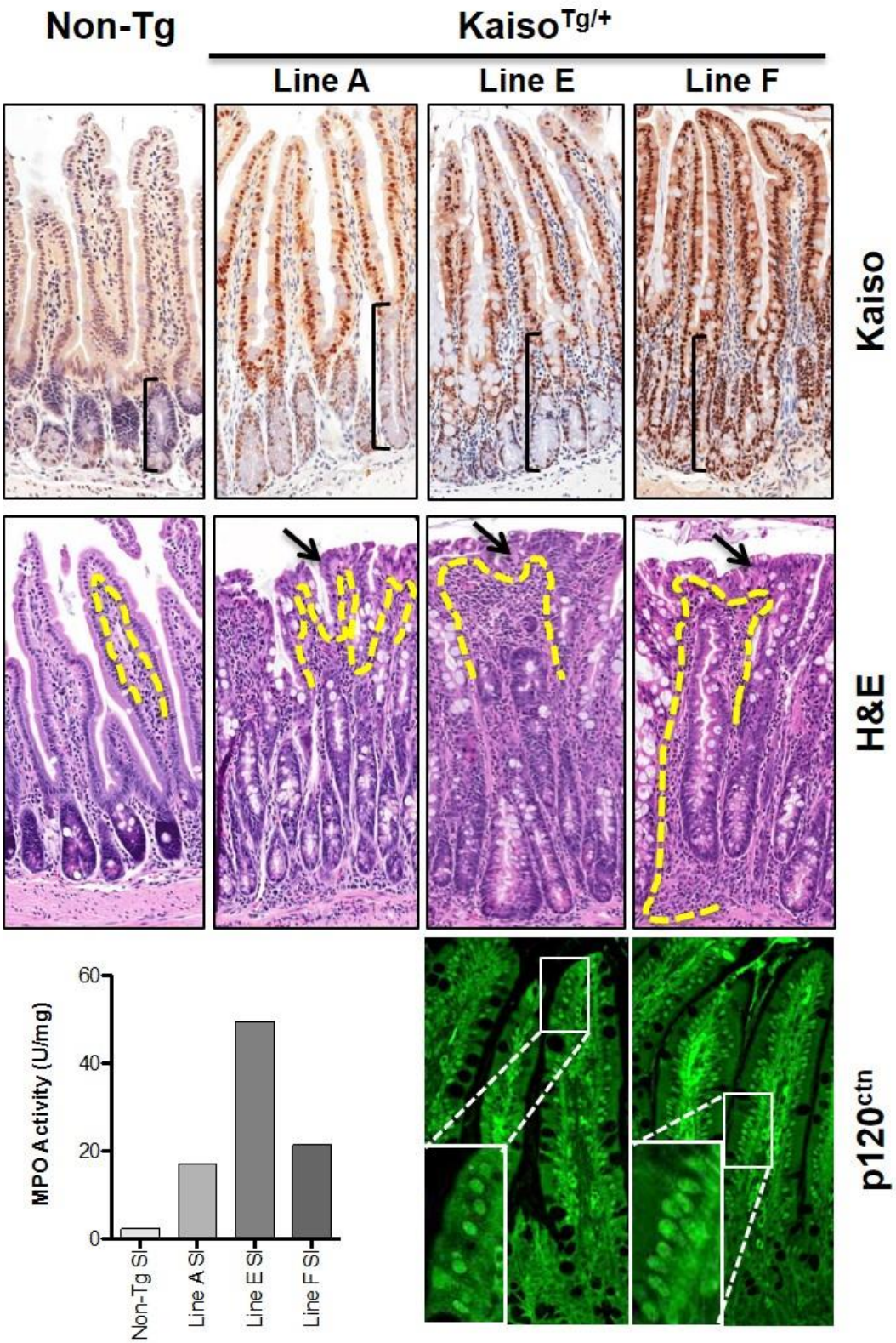

Non-Tg
Kaiso Tg/+
Line A
Line E
Line F
Kaiso
H&E
p120ctn
MPO Activity (U/mg)
60
40
20
0
Non-Tg SI
Line A SI
Line E SI
Line F SI

Figure 3.5: Kaiso transgenic mice display nuclear p120^{ctn} in villi of the small intestine.

Immunofluorescence staining for p120^{ctn} showed nuclear localization of p120^{ctn} in epithelial cells of villi overexpressing Kaiso (*Kaiso*$^{Tg/+}$), while Non-Tg mice displayed membrane localized p120^{ctn}.

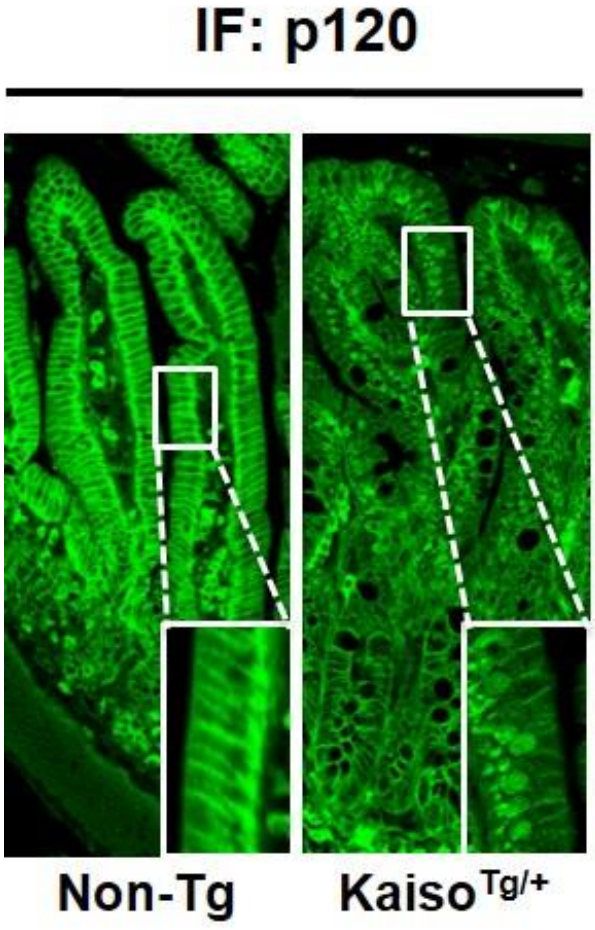

IF: p120
Non-Tg
Kaiso Tg/+

Figure 3.6: Secretory cell lineages are expanded in the intestines of *Kaiso*$^{Tg/+}$ mice.
(A) PAS stain for Goblet cells (black arrowheads) revealed increased numbers of Goblet cells in both the villi and crypts of *Kaiso*$^{Tg/+}$ intestines. **(B)** Lysozyme staining revealed increased Paneth cell numbers in *Kaiso*$^{Tg/+}$ mice. **(C)** Synaptophysin positive enteroendocrine cells (arrowheads) are increased in *Kaiso*$^{Tg/+}$ mice. n=3 mice/genotype; cell numbers determined by two independent blind observers; * represents p-value <0.05.

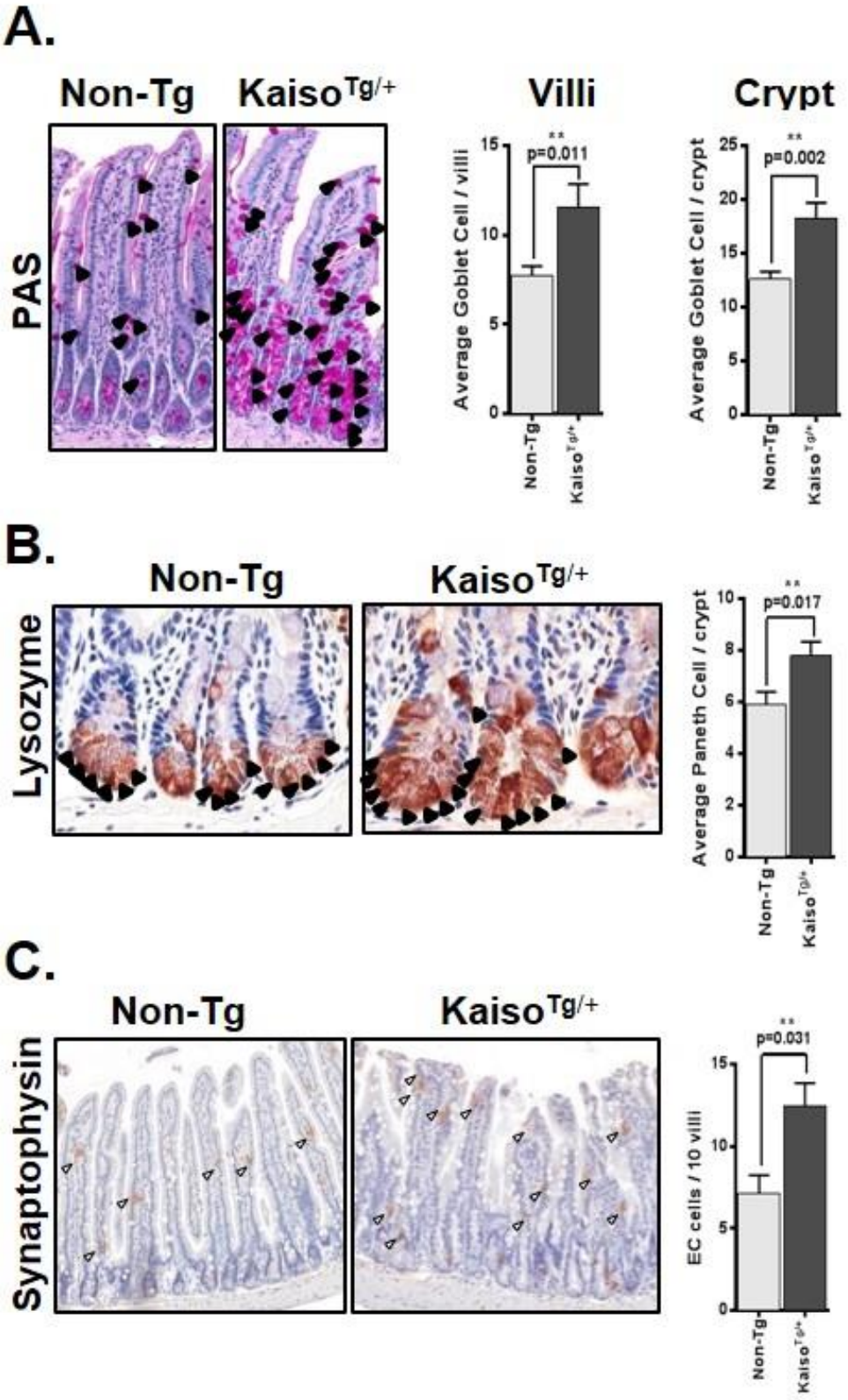

A.
Non-Tg
Kaiso Tg/+
PAS
Villi
Crypt
Average Goblet Cell / villi
Average Goblet Cell / crypt
p=0.011
p=0.002
Non-Tg
Kaiso Tg/+
B.
Non-Tg
Kaiso Tg/+
Lysozyme
Average Paneth Cell / crypt
p=0.017
C.
Non-Tg
Kaiso Tg/+
Synaptophysin
EC cells / 10 villi
p=0.031

Figure 3.7: Line A *Kaiso*$^{Tg/+}$ mice display increased numbers of differentiated secretory cells in the colon.

Kaiso$^{Tg/+}$ mice display a significant increase in Goblet (PAS stain) and enteroendocrine cells (synaptophysin) compared to their Non-Tg littermates in the colon. The colon does not have Paneth cells.

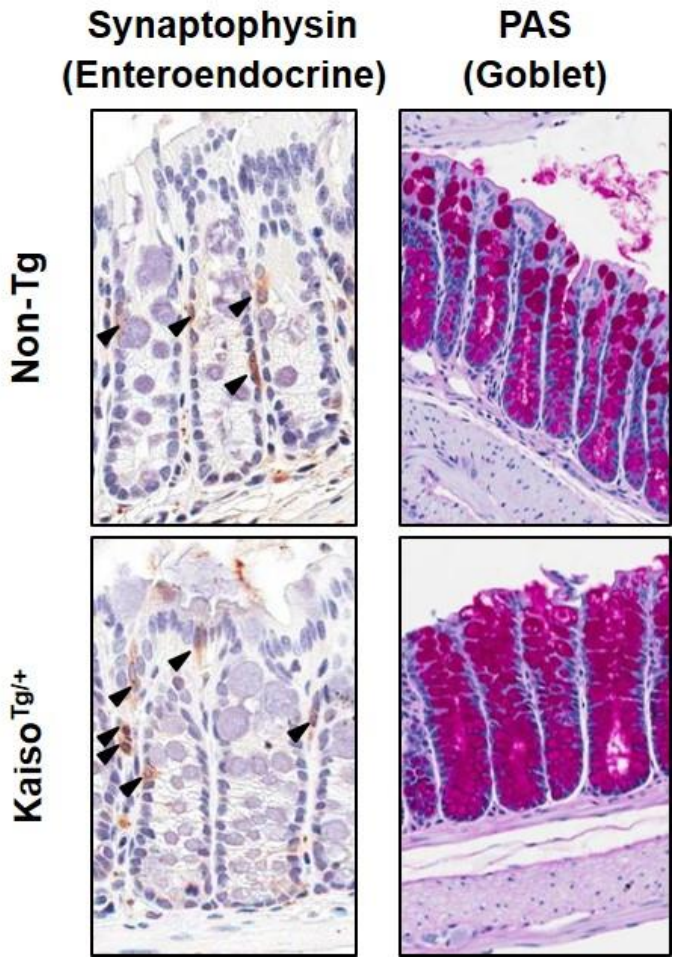

Synaptophysin
(Enteroendocrine)
PAS
(Goblet)
Non-Tg
Kaiso Tg/+

Figure 3.8: Cell proliferation is decreased in *Kaiso*$^{Tg/+}$ mice.

Cell proliferation was evaluated by Ki67 **(A)** and Cyclin D1 **(B)** staining, both of which show strong nuclear staining in the cells of the crypts. *Kaiso*$^{Tg/+}$ mice display decreased staining of both proteins compared to their Non-Tg siblings. **(C)** Immunoblot revealed that *Kaiso*$^{Tg/+}$ mice have decreased CyclinD1 expression compared to Non-Tg siblings. * represents p-value <0.05.

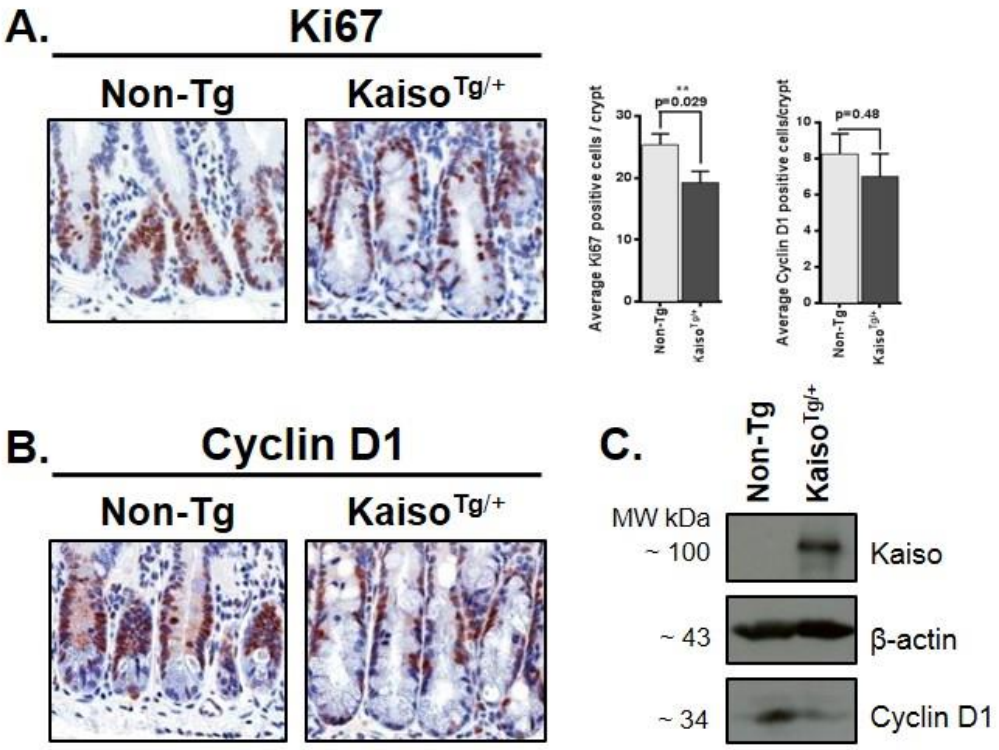

A.
Ki67
Non-Tg
Kaiso Tg/+
**
p=0.029
Average Ki67 positive cells / crypt
30
20
10
0
Non-Tg
Kaiso Tg/+
p=0.48
Average Cyclin D1 positive cells/crypt
10
8
6
4
2
0
Non-Tg
Kaiso Tg/+
B.
Cyclin D1
Non-Tg
Kaiso Tg/+
C.
Non-Tg
Kaiso Tg/+
MW kDa
~ 100
Kaiso
~ 43
β-actin
~ 34
Cyclin D1

Figure 3.9: *Kaiso*$^{Tg/+}$ mice display decreased HES1 expression in the small intestine. Both Non-Tg and *Kaiso*$^{Tg/+}$ tissues displayed nuclear HES1 expression in the crypts of the small intestine, however *Kaiso*$^{Tg/+}$ tissue displays significantly decreased HES1 expression in Line A mice. Quantitative RT-PCR showed a significant decrease in HES1 expression in *Kaiso*$^{Tg/+}$ mice. Values were first normalized to the GAPDH housekeeping gene, followed by normalizing to Non-Tg HES1 expression. * represents p-value <0.05.

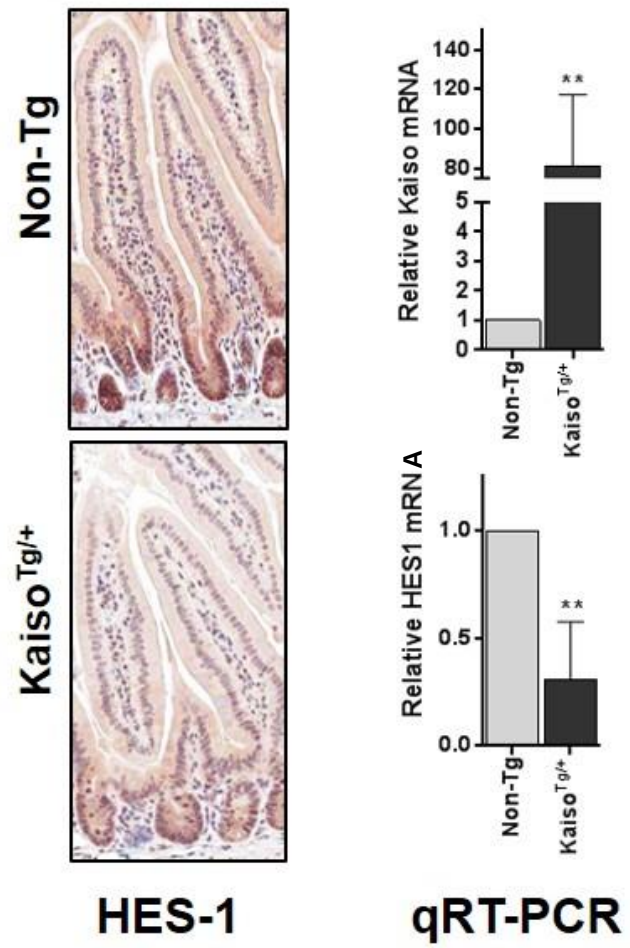

Non-Tg
Kaiso Tg/+
HES-1
Relative Kaiso mRNA
140
120
100
80
5
4
3
2
1
0
**
Non-Tg
Kaiso Tg/+
Relative HES1 mRNA
1.0
0.5
0.0
**
Non-Tg
Kaiso Tg/+
qRT-PCR

Figure 3.10: Kaiso expression is increased in DSS-treated murine colon tissues.

Preliminary analysis of DSS-induced murine colitis model intestinal tissues revealed increased nuclear Kaiso expression in DSS-treated colon tissues whereas non-treated mice show low cytoplasmic Kaiso expression.

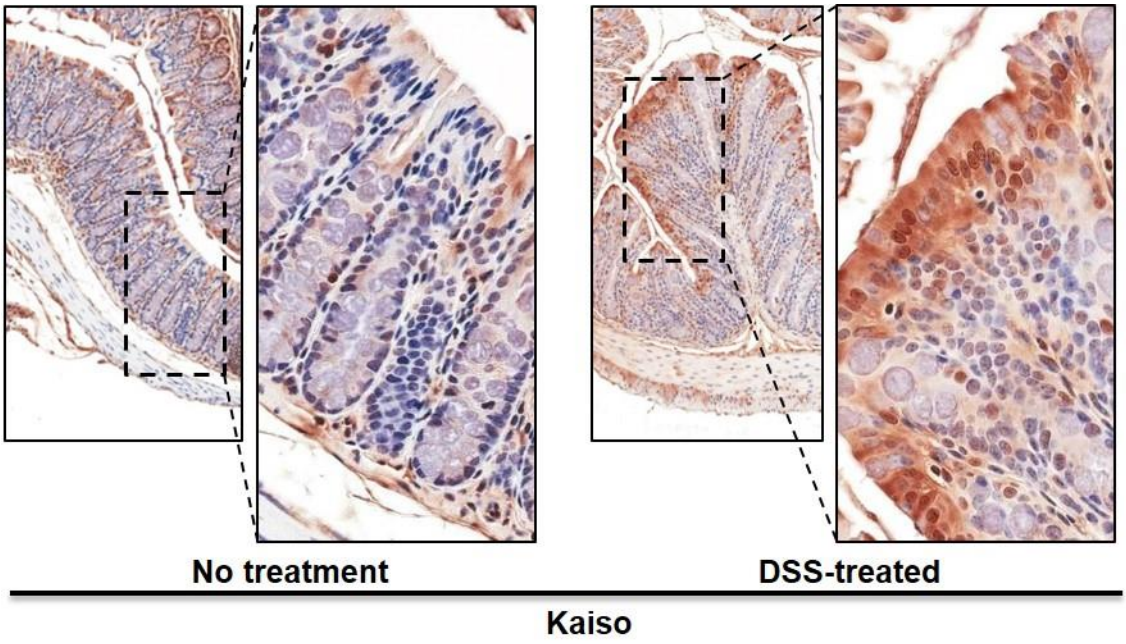
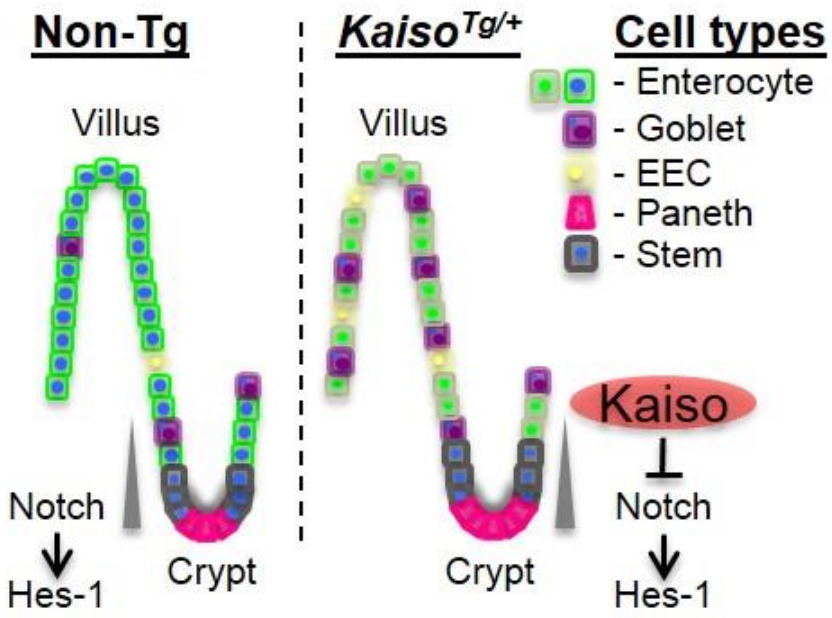

No treatment
DSS-treated
Kaiso

Figure 3.11: Schematic model depicting Kaiso's effects in the intestine.

Notch signalling in the crypts modulates differentiation of progenitor cells into the various epithelial cell lineages: enterocytes, Goblet, Paneth, and enteroendocrine (EEC) cells. HES1 is necessary for the proper specification of these cell types. $p120^{ctn}$ localizes to the membrane in enterocytes of Non-Tg mice (green boundary), but is recruited to the nucleus in $Kaiso^{Tg/+}$ mice (green nuclei), thus inducing inflammation.


Non-Tg
Villus
Notch
Hes-1
Crypt
Kaiso^Tg/+
Villus
Crypt
Kaiso
Notch
Hes-1
Cell types
- Enterocyte
- Goblet
- EEC
- Paneth
- Stem

CHAPTER 4: INTESTINAL KAISO OVEREXPRESSION LEADS TO A NEUTROPHILIC INFLAMMATORY RESPONSE.

PREFACE:

This chapter outlines the studies performed to understand Kaiso's role in intestinal inflammation and summarizes the findings in the following manuscript in preparation:

***Chaudhary R**, Jimenez-Saiz R, Longo J, Skandarajah S, Milosavljevic S and Daniel JM. Intestinal Kaiso overexpression leads to a neutrophilic inflammatory response. Manuscript in preparation.*

Revisions were made to the manuscript to maintain formatting consistency with other chapters. Significant revisions include elimination of methods section (details in Chapter 2), removal of reference list (included in combined thesis reference list), and re-organization of figures in order of appearance in the text.

I performed 85% of the experiments in this article, assembled the figures and wrote the text for the manuscript. Dr. Jimenez-Saiz performed the FACS analysis on the mice shown in Figure 4.2. Joseph Longo performed the HCT116 cell immunoblots shown in Figure 4.5, while Shivanie Skandarajah performed the MMP7 IHC experiments depicted in Figure 4.3. Dr. Daniel and I both contributed to the manuscript; we designed the experiments, and wrote and edited the manuscript.

Though this is an ongoing study, our findings show that the higher Kaiso overexpressing transgenic line (Line E) has an earlier onset of inflammation compared to

the lower Kaiso expressing Line A. Interestingly, the Kaiso transgenics show an increase in neutrophil population numbers and their concomitant activation in the lamina propria specifically. To determine if the inflammation is due to an intestinal epithelial barrier defect, FITC-dextran studies were performed on the mice. The Line E mice have a leaky barrier compared to the Line A mice at an age when only Line E mice show active inflammation. This suggests that the leaky barrier phenotype may be due to the inflammation. To further characterize the barrier defect, we examined the expression and subcellular localization of some epithelial and junctional markers and found they were mislocalized in the *Kaiso*$^{Tg/+}$ mice. We also found an increase in the matrix metalloproteinase, MMP7, which regulates the influx of neutrophils in the *Kaiso*$^{Tg/+}$ mice. This suggested a mechanism for neutrophil recruitment into the intestines. Preliminary results also show an increase in a neutrophil attachment protein, ICAM-1, in the intestinal epithelial cells of the *Kaiso*$^{Tg/+}$ mice. Collectively our data suggests that intestinal Kaiso overexpression induces neutrophil recruitment and activation possibly via pathways that involve MMP7 and ICAM-1 which are known to play roles in inflammation.

4.1 ABSTRACT:

Kaiso transgenic mice (*Kaiso*$^{Tg/+}$), which specifically overexpress the transcription factor Kaiso in the intestines, were previously reported to have a spontaneous intestinal inflammatory response. Since the underlying cause of this inflammation is unknown, we evaluated two independent Kaiso transgenic lines that express either low (Line A) or high (Line E) levels of Kaiso to further elucidate the mechanism of inflammation. The *Kaiso*$^{Tg/+}$ mice develop tissue damage with thickening of the intestinal mucosa and the underlying muscularis layer. Both lines exhibit "lesions" with fused blunted villi in a discontinuous pattern throughout the small intestine, and develop multiple crypt abscesses. Line E mice develop inflammation as early as 8 weeks of age, while Line A mice display inflammation at ~8 months of age. The *Kaiso*$^{Tg/+}$ mice develop a neutrophilic inflammation in the small intestine, accompanied by an increase in the expression of the matrix metalloproteinase MMP7 that regulates the influx of neutrophils. Although both lines experience an increase in Claudin-1 expression, only Line E mice show a leaky intestinal barrier. The intestinal epithelial cells also have an increase in the neutrophil adhesive protein, ICAM-1, which may further exacerbate neutrophil homing to the gut. Overall, the inflammatory response observed in the *Kaiso*$^{Tg/+}$ mice is mediated by neutrophils and likely facilitated by the higher intestinal expression of MMP7 and ICAM-1.

4.2 INTRODUCTION

Inflammatory **b**owel **d**isease (IBD) is a disorder of the intestinal tract that is categorized into two main types: **C**rohn's **d**isease (CD) or **u**lcerative **c**olitis (UC). Though the etiology of the diseases is complex, both diseases are characterized by an overactive immune response to commensal bacteria, and differ in the site and nature of the inflammatory pathology (reviewed in Maloy and Powrie, 2011; Saleh and Trinchieri, 2011). There is currently no cure for IBD, and hence the diseases are primarily managed by alleviation of the symptoms (Saleh and Trinchieri, 2011). Thus a better understanding of the underlying molecular or genetic factors that contribute to IBD will enhance and facilitate development of better treatment options for these diseases.

The gut-associated lymphoid tissue is the largest component of the immune system and plays a critical role in the modulation of the intestinal microbiota and in the maintenance of intestinal tissue homeostasis (Fournier and Parkos, 2012; Saleh and Trinchieri, 2011). Neutrophils are the most abundant immune cell in the blood and form the first line of defense against invading microbes, thus playing a pivotal role in antimicrobial host defense (Németh and Mócsai, 2012). Neutrophils are recruited rapidly to sites of inflammation where their primary role is to kill invading bacteria through phagocytosis. Neutrophils release preformed granular enzymes and proteins like **m**yelo**p**er**o**xidase (MPO) and produce a range of oxygen species that kill invading bacterial species (reviewed in Mócsai, 2013). However, the highly destructive capacity of neutrophils also raises the potential for neutrophils to damage healthy tissues, as occurs in

many inflammatory diseases such as IBD, acute respiratory distress syndrome, and rheumatoid arthritis (reviewed in Summers *et al.*, 2010).

During our recent characterization of a novel transgenic mouse overexpressing the POZ-ZF protein Kaiso (*Kaiso*$^{Tg/+}$), we observed significant neutrophil infiltration and intestinal inflammation (Chaudhary *et al.*, 2013). These mice phenocopied phenotypes observed upon limited ablation of Kaiso's binding partner p120^{ctn} in murine intestines (Smalley-Freed *et al.*, 2010; Smalley-Freed *et al.*, 2011). Kaiso is a POZ-ZF transcription factor that regulates target genes by binding to either methylated CpG dinucleotides or a non-methylated DNA sequence called the **K**aiso **b**inding **s**ite (KBS) (Daniel *et al.*, 2002; Prokhortchouk *et al.*, 2001). Kaiso's DNA-binding ability and transcriptional activity is negatively regulated by nuclear p120^{ctn} (Daniel and Reynolds, 1999; Jiang *et al*., 2012; Kelly *et al*., 2004b; Liu *et al*., 2014; Ogden *et al*., 2008; Prokhortchouk *et al*., 2001). Kaiso plays key roles in vertebrate development and tumourigenesis (Dai et al., 2011; Jones et al., 2014; Kim et al., 2004; Lopes et al., 2008; Park et al., 2005; Prokhortchouk et al., 2006) but a thorough understanding of its mechanism of action in these events remain unknown.

Since Kaiso's binding partner has been implicated as an anti-inflammatory factor, and intestinal-specific Kaiso expression induced an inflammatory phenotype that mimicked mice with limited p120^{ctn}-ablation, we hypothesized that the Kaiso-induced inflammatory phenotype involved a misregulation of the p120^{ctn}-mediated anti-inflammatory pathway. To address this hypothesis and gain insight into the mechanism of

Kaiso-induced intestinal inflammation (Chaudhary *et al.*, 2013), we characterized two independent mouse transgenic lines with differing Kaiso expression levels, Line A (moderate Kaiso) and Line E (high Kaiso). Both mouse lines exhibited thickening of the intestinal mucosa and the underlying muscularis concomitant with the presence of crypt abscesses, which are a classical feature of active IBD and result from extensive neutrophil transmigration across the intestinal epithelial (Xavier and Podolsky, 2007). Interestingly, the higher Kaiso expressing transgenic line (Line E) developed more crypt abscesses and an earlier onset of inflammation than the low Kaiso expressing line, Line A, suggesting that high Kaiso expression increases the susceptibility to inflammation. Both transgenic lines show a significant increase in neutrophils in the lamina propria upon induction of inflammation. The $Kaiso^{Tg/+}$ mice also had increased intestinal expression of the pro-inflammatory adhesion molecule, ICAM-1, and matrilysin (MMP7), which play roles in neutrophil adhesion and recruitment (Swee *et al.*, 2008; Thomas and Baumgart, 2012). Together our data suggests that the tissue damage observed following ectopic Kaiso expression in the intestines is a consequence of excessive neutrophil infiltration into the tissue, which may be in response to increased ICAM-1 and MMP7 expression induced by Kaiso.

4.3 RESULTS

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

Kaiso transgenic mice display an inflammatory phenotype in the small and large intestines after 8 months of age as evaluated via myeloperoxidase (MPO) activity (Chaudhary *et al.*, 2013). To further characterize the Kaiso inflammatory phenotype, two

Kaiso transgenic lines were examined: Line A and the higher Kaiso expressing Line E (Chaudhary *et al.*, 2013). Both Line A and E mice display a thickening of the underlying muscle layer (*muscularis externa*) at ~8 months of age (Figure 4.1A). Additionally, both Line A and E mice have "lesions" in the small intestine (SI) that are comprised of blunted and fused villi with high leukocyte infiltrates. These lesions were dispersed throughout the length of the SI tissue with normal villi in between (Figure 4.1A). Additionally, both transgenic lines develop crypt abscesses at ~8 months of age (n=3 per genotype), though Line E mice develop more crypt abscesses compared to Line A mice (Figure 4.1B). Interestingly, thickening of the *muscularis externa*, discontinuous lesions and crypt abscesses are hallmarks of inflammatory-related tissue damage that is common in IBD, specifically Crohn's Disease and intermediate IBD that has traits of both Crohn's Disease and Ulcerative Colitis (Actis *et al.*, 2014).

4.3.2 High Kaiso expression induces neutrophil infiltration and activation.

Since the *Kaiso*$^{Tg/+}$ mice display chronic inflammation and tissue damage with a high level of leukocyte infiltrates, we examined the immune cells in the lamina propria in the SI using flow cytometry. Lamina propria cells were freshly isolated using collagenase and DNase digestion steps and purified using a Percoll gradient as previously described (Chu *et al.*, 2014). These initial analyses were performed on ~8 month old Line A mice (n=8/genotype). The innate immune cell population including neutrophils, eosinophils, dendritic cells, mast cells, and tissue resident macrophages were assessed in the *Kaiso*$^{Tg/+}$ mice. After identifying single viable cells, the cells were gated for CD45^{+} to analyze leukocytes. Within the leukocyte population, the cells were further gated for the various

leukocyte immune cell subtypes. Line A mice showed a significant increase in neutrophils (Figure 4.2A) with minimal change in other immune cell types (data not shown). Macrophage quantities are shown for reference.

To determine the earliest age at which intestinal inflammation can be detected in the *Kaiso*$^{Tg/+}$ mice, MPO activity, which is a surrogate marker for neutrophil activity (Brazil *et al.*, 2013), was measured in mice at approximately 8, 14 and 24 weeks of age (Figure 4.2B). In addition to determining when the intestinal inflammation begins, we also assessed if the bacterial and pathogenic load from the environment had an effect on the development of intestinal inflammation in the *Kaiso*$^{Tg/+}$ mice. Thus, mice were housed in two conditions: the Barrier (an ultraclean room with lower bacterial and pathogen load), or a common specific pathogen free (SPF) room (Figure 4.2B). MPO activity was measured in the SI homogenates as previously described (Chaudhary *et al.*, 2013). In both housing conditions, Line E mice exhibited significantly increased MPO activity at all ages tested (8, 14, 24 weeks) compared to the non-Tg siblings, but there was no significant change in MPO activity in Line A mice at any of the ages tested (Figure 4.2B). A similar inflammatory pattern was observed in the colon (large intestine) of both lines (data not shown). Interestingly, both transgenic lines show similar MPO levels in both environments tested (Figure 4.2B), suggesting that Kaiso-induced inflammation is independent of the environment. The earlier manifestation of MPO activity in Line E mice suggests that the higher Kaiso expression enhances the susceptibility to inflammation.

We next sought to compare the neutrophil population in the lamina propria at 14 weeks when Line E, and not Line A, mice have active inflammation. Since MPO activity is a good indicator of neutrophil activity, and the other immune cell types were unaltered in ~8 month old mice, we used flow cytometry to assess the neutrophil population (n=9/genotype). In addition to neutrophils, the tissue-resident macrophage population was also examined as they are common and abundant inhabitants of the intestines, and are actively involved in clearing neutrophils (Däbritz, 2014). At 14 weeks of age, Line E mice had a significant infiltration of neutrophils into the lamina propria while Line A showed a slight but non-significant increase (Figure 4.3C). Interestingly, macrophage numbers were lower in both lines, though not significant in Line E mice (Figure 4.3D). Together, this data suggests that higher Kaiso expression induces an early onset of inflammation that is neutrophil-specific and independent of the environment.

4.3.3 Kaiso overexpression increases intestinal expression of MMP7

Neutrophils are relatively short-lived leukocytes and, as part of the innate immune system, are the first responders to inflammation (Summers *et al.*, 2010). However, excessive recruitment and accumulation of neutrophils can lead to mucosal injury (Fournier and Parkos, 2012). One factor that regulates the influx of neutrophils to the mucosa is the release of a matrix metalloproteinase, *matrilysin* (MMP7), by the intestinal epithelium (Swee *et al.*, 2008). Since *MMP7* is a known Kaiso target gene (Spring *et al.*, 2005), we sought to determine if MMP7 expression levels were altered in the *Kaiso*$^{Tg/+}$ mice. Surprisingly, using immunohistochemistry and immunoblot, we detected an increase in MMP7 expression in the SI of *Kaiso*$^{Tg/+}$ mice (Figure 4.3). MMP7 expression

is localized throughout the crypts in the *Kaiso*$^{Tg/+}$ mice rather than concentrated at the bottom of the crypts as seen in the non-Tg siblings (Figure 4.3). Since MMP7 expression is increased in the *Kaiso*$^{Tg/+}$ mice and MMP7 is known to regulate the influx of neutrophils, MMP7 may be contributing to the recruitment of neutrophils into the SI in the *Kaiso*$^{Tg/+}$ mice. However, as we had previously reported that Kaiso transcriptionally repressed MMP7 expression (Spring *et al.*, 2005), the increased MMP7 levels observed in the *Kaiso*$^{Tg/+}$ mice may not be a direct transcriptional effect of Kaiso overexpression.

4.3.4 *Kaiso*$^{Tg/+}$ mice have enhanced intestinal permeability

The increased MMP7 expression, MPO activity and neutrophil infiltration within the SI are strong indicators of inflammation. Intestinal inflammation is often correlated with an increase in permeability through the intestine that leads to bacterial influx into the tissue (Pastorelli *et al.*, 2013). Indeed, intestinal epithelial cells provide a physical barrier between intestinal microbiota and lamina propria, and regulate intestinal permeability (Däbritz, 2014). Thus we tested the integrity of the intestinal epithelial barrier of the *Kaiso*$^{Tg/+}$ mice by administering **f**luorescein **i**so**t**hio**c**yanate (FITC)-Dextran (4000 Da) by intra-gastric gavage and measuring systemic uptake in the blood. At 14 weeks of age, Line E mice show a significant increase in plasma FITC-Dextran suggesting an enhanced gut permeability that is indicative of a defective epithelial barrier (Figure 4.4A). Interestingly, Line A mice do not show any change in permeability implying that the severe inflammation observed in Line E mice correlates with permeability defects.

To further characterize the gut permeability in the *Kaiso*$^{Tg/+}$ mice, we assessed the expression and subcellular localization of selected protein components of the *adherens*

and tight junctions. The main *adherens* junction component, E-cadherin, when disrupted in murine intestines leads to a compromised epithelial barrier (Bondow *et al.*, 2012). The tight junction protein, Claudin-1, is one of the proteins known to regulate paracellular permeability in the intestine and is a key regulator of the epithelial barrier (Furuse *et al.*, 2002). Isolated intestinal epithelial cells from *Kaiso*$^{Tg/+}$ show no detectable change in the total amount of E-cadherin, but there was increased Claudin-1 expression (Figure 4.4B). However, immunofluorescence staining revealed changes in the localization of E-cadherin and Claudin-1 in the *Kaiso*$^{Tg/+}$ compared to non-Tg (Figure 4.4C and D). Though there did not appear to be a statistically significant difference in E-cadherin levels in non-Tg (Figure 4.4Ci) compared to *Kaiso*$^{Tg/+}$ mice (Figure 4.4Cii), the *Kaiso*$^{Tg/+}$ display strong E-cadherin expression within the crypts and was localized closer to the basal side compared to the more apical cell-cell contacts as seen in the non-Tg villi (Figure 4.4C i vs ii). In contrast, we detected an overall increase in claudin-1 expression in the *Kaiso*$^{Tg/+}$ mice (Figure 4.4D). The non-Tg mice show a weak apical localization of Claudin-1 in the villi with no detectable staining within the crypts (Figure 4.4Di), while the *Kaiso*$^{Tg/+}$ mice have varying expression patterns between normal villi (Figure 4.4Dii) and inflamed villi (Figure 4.4Diii). Within normal villi of the *Kaiso*$^{Tg/+}$, Claudin-1 is localized to the apical region of the villi with low/negligible staining in the crypts (Figure 4.4Dii). However, within inflamed villi in *Kaiso*$^{Tg/+}$ mice, Claudin-1 expression is concentrated within the crypts (Figure 4.4Diii). The epithelial barrier is highly regulated and dependent on appropriate expression and localization of junctional proteins. The

observed changes in E-cadherin localization and the increase in Claudin-1 expression could exacerbate the inflammatory phenotype of the *Kaiso*$^{Tg/+}$ mice.

4.3.5 *Kaiso*$^{Tg/+}$ mice have increased expression of inflammatory markers

Since the *Kaiso*$^{Tg/+}$ mice show a strong inflammatory phenotype, we examined the expression of the hallmark inflammatory marker NFκB (Wullaert *et al.*, 2011). The NFκB family of transcription factors consist of five members in mammals, including the p65/RelA protein, which translocates to the nucleus upon activation and induce transcription of inflammatory response genes (Wullaert *et al.*, 2011). NFκB expression and activity is regulated by many signalling pathways, but of most interest to us was the pathway involving Kaiso's binding partner p120^{ctn} which was shown to regulate NFκB activity in multiple tissues including the epidermis and the lung (Perez-Moreno *et al.*, 2006; Qin *et al.*, 2014). Not surprisingly, isolated epithelial cells from the SI of *Kaiso*$^{Tg/+}$ mice had increased NFκB p65 expression as measured by immunoblot (Figure 4.6A).

In addition to regulating NFκB activity, p120^{ctn}'s anti-inflammatory role also negatively correlates with the neutrophil adhesion protein, intercellular adhesion molecule-1 (ICAM-1), in endothelial cells (Alcaide *et al.*, 2012; O'Donnell *et al.*, 2011). ICAM-1 is important for neutrophil adhesion and their transepithelial migration (Thomas and Baumgart, 2012). Our *Kaiso*$^{Tg/+}$ mice have an infiltration of neutrophils within the intestines and a mislocalization of p120^{ctn} (Chaudhary *et al.*, 2013), thus we proceeded to investigate the expression of ICAM-1 within the intestinal tissues. Isolated intestinal epithelial cells showed greater ICAM-1 expression in the *Kaiso*$^{Tg/+}$ mice as compared to non-Tg siblings (Figure 4.5A). To further study the Kaiso-ICAM-1 relationship in

intestinal epithelial cells, we assessed Kaiso, p120^{ctn}, and ICAM-1 expression in Kaiso or p120^{ctn} knockdown HCT116 colon cancer cell lines. Compared to the shRNA control, Kaiso knockdown resulted in decreased ICAM-1 expression, while p120^{ctn} knockdown restored ICAM-1 expression (Figure 4.5B). Though it is difficult to decipher the mechanism of Kaiso-ICAM-1 regulation, the ICAM-1 promoter does contain four (4) core-KBS sites and multiple CpGs (Figure 4.6C), which may make it a direct target gene of Kaiso. Together, our data suggests that Kaiso may regulate neutrophil infiltration in the intestines by multiple mechanisms.

4.4 DISCUSSION

Previously, we reported that Kaiso transgenic mice have inflamed intestines, however the precise mechanism underlying the inflammation in this model was unknown (Chaudhary *et al.*, 2013). Thus, to further characterize this inflammation, we characterized two independent transgenic lines to ensure the phenotype was not an off-target effect of the transgene insertion. Both Kaiso transgenic lines used in this study developed crypt abscesses at ~8 months of age. Crypt abscesses are the result of massive numbers of activated neutrophils transmigrating and accumulating within the intestinal epithelial layer and are a hallmark of chronic inflammation (Xavier and Podolsky, 2007). Formation of crypt abscesses results in the deformation of the crypt architecture and subsequently leads to intestinal tissue damage (Fournier and Parkos, 2012). Kaiso transgenic mice had more crypt abscesses than non-Tg siblings and the numbers correlated with the transgene expression levels, i.e. the moderate expressing Kaiso transgenic line, Line A, exhibited fewer crypt abscesses compared to the higher

expressing line, Line E. The transgenic lines develop intestinal inflammation and exhibit increased neutrophil numbers in the SI, though the onset of inflammation depends upon the Kaiso expression levels. Since the higher expression Kaiso leads to an earlier onset of inflammation, the development of more crypt abscesses in Line E mice could be due to a longer duration of inflammation.

Chronic inflammation of the intestine can lead to IBD which is sub-classified as UC or CD (Maloy and Powrie, 2011). CD involves inflammation of any part of the gastrointestinal system that extends into the underlying *muscularis*, while UC is frequently classified as inflammation of the colon confined within the mucosa (reviewed in Maloy and Powrie, 2011). Additionally, the ulcerations seen in CD are discontinuous with normal villi in between lesions (reviewed in Maloy and Powrie, 2011). The Kaiso transgenic mice develop discontinuous lesions and thickening of the *muscularis* similar to tissues from patients with CD compared to UC. The persistent migration of neutrophils into the intestine results in tissue damage and thus the thickening of the *muscularis* could be downstream of the neutrophilic infiltration. In fact, as assessed by MPO activity, we detected activated neutrophils as early as 8 weeks in Line E mice. The prolonged exposure of the intestine to activated neutrophils could induce the tissue damage observed and the greater number of crypt abscesses. Tissue-resident macrophages typically remove neutrophils from the intestinal tissue (Däbritz, 2014). However, if there is aberrant or inefficient macrophage clearing, neutrophils can cause tissue damage (Fournier and Parkos, 2012). Macrophages also release the immune suppressive cytokine IL-10 and protect the tissues from innate immune damage (Däbritz, 2014). The decrease

in tissue-resident macrophage numbers in the *Kaiso*$^{Tg/+}$ mice suggests that neutrophil clearing is downregulated, thus further contributing to the increase in the neutrophil population within the intestine.

One mechanism whereby neutrophils are recruited into tissues is via matrix metalloproteases such as MMP7 (Fournier and Parkos, 2012). *MMP7*$^{-/-}$ mice show reduced inflammation and bacterial damage (Vandenbroucke *et al.*, 2014), and impaired transepithelial neutrophil influx by decreasing neutrophil chemoattractants (Swee *et al.*, 2008). Together, these data suggest that MMP7 plays a pro-inflammatory role in the intestine. The *Kaiso*$^{Tg/+}$ mice exhibit an increase in MMP7 expression within the intestines which may act to induce neutrophil attracting chemokines, thus increasing neutrophil influx into the intestines. The increased MMP7 expression in the *Kaiso*$^{Tg/+}$ mice also correlates with the increase in Paneth cell reported in the *Kaiso*$^{Tg/+}$ mice (Chaudhary *et al.*, 2013) as MMP7 is responsible for α-defensins activation in Paneth cells (Vandenbroucke *et al.*, 2014). Our detection of increased MMP7 in *Kaiso*$^{Tg/+}$ mice was unexpected since we previously reported that Kaiso repressed MMP7 gene expression (Spring *et al.*, 2005). However, the increase in numbers of Paneth cells may mask the Kaiso-mediated repression of MMP7 thus inducing an increase in MMP7 in the Kaiso transgenics. Alternatively, since Kaiso-mediated transcriptional repression of MMP7 is relieved by nuclear p120^{ctn} (Ogden *et al.*, 2008), the increased nuclear p120^{ctn} in *Kaiso*$^{Tg/+}$ mice (Chaudhary *et al.*, 2013) may be inhibiting Kaiso-mediated transcriptional repression and contribute to the increase in MMP7 expression. This would result in augmented recruitment of neutrophils in these tissues.

Kaiso's binding partner, $p120^{ctn}$, has been shown to regulate NFκB pro-inflammatory activity in epithelial tissues, thus giving $p120^{ctn}$ an anti-inflammatory role (Perez-Moreno *et al.*, 2006; Qin *et al.*, 2014). Kaiso transgenic mice display decreased $p120^{ctn}$ expression at the membrane but increased nuclear $p120^{ctn}$ (Chaudhary *et al.*, 2013). This suggests that $p120^{ctn}$'s anti-inflammatory role requires its cytoplasmic localization. Not surprisingly, NFκB p65 expression was increased in the Kaiso transgenic mice, which likely promotes the active inflammation within these mice. NFκB's pro-inflammatory function has been well documented in multiple tissues (reviewed in Wullaert *et al.*, 2011), and one of NFκB's target genes, ICAM-1, is also inversely correlated with $p120^{ctn}$ expression levels (Alcaide *et al.*, 2012; O'Donnell *et al.*, 2011). ICAM-1 is a known ligand of neutrophils and is expressed in intestinal epithelial cells under inflammatory conditions (Parkos *et al.*, 1996). Thus another mechanism for Kaiso-mediated inflammation may be through the modulation of ICAM-1 expression in the intestinal epithelial cells allowing for greater neutrophilic accumulation. Though most studies have reported on Kaiso's transcriptional activity in the context of transcriptional repression of target genes, Kaiso has also been implicated as a transcriptional activator (Rodova *et al.*, 2004). However, Kaiso's mechanism of transcriptional activation is currently unknown and to date no other target genes have been reported that are transcriptionally activated by Kaiso. Since the ICAM-1 promoter contains four core KBS's, one exciting possibility is that Kaiso may be activating ICAM-1 expression. Alternately, the nuclear localization of $p120^{ctn}$ in the $Kaiso^{Tg/+}$ mice (Chaudhary *et al.*, 2013) may relieve Kaiso-mediated transcriptional repression of ICAM-1, which would

manifest as increased ICAM-1 expression. Further studies to decipher Kaiso's transcriptional regulation of ICAM-1, if any, are required.

Within the intestine, ICAM-1 expression not only enhances neutrophil adhesion, but also increases neutrophil recruitment, which compromises the epithelial barrier function (Sumagin *et al.*, 2014). Contrary to our observations in the Line A mice, intestinal permeability in Line E mice is significantly higher than non-Tg siblings at 14 weeks of age. At 14 weeks, Line A mice do not show signs of inflammation while the Line E mice develop inflammation as early as 8 weeks, and subsequently develop chronic inflammation. Since the Line A mice did not show any intestinal barrier defects, it is difficult to determine cause and effect: did Kaiso induce inflammation which then induced the barrier defect or is the intestinal inflammation a secondary effect of the barrier defect? Claudin-1 is a key regulator of epithelial barrier function, and is increased in active IBD and correlated with inflammatory activity (Furuse *et al.*, 2002; Pope *et al.*, 2014; Weber *et al.*, 2008). *Kaiso*$^{Tg/+}$ mice show an overall increase in Claudin-1 within the epithelial cells supporting the hypothesis that the *Kaiso*$^{Tg/+}$ mice undergo chronic active inflammation inducing a barrier defect and increase in Claudin-1.

Overall, *Kaiso*$^{Tg/+}$ mice have neutrophil infiltration that may be due to an increase in MMP7 and ICAM-1 expression (Figure 4.6). We propose that the increased levels of ICAM-1 allow the infiltrated activated neutrophils to adhere and remain within the intestinal epithelium where they promote inflammation and subsequent tissue damage. The chronic inflammation induces a leaky barrier that further exacerbates the

inflammatory phenotype. Hence, the development of intestinal inflammation in the *Kaiso*$^{Tg/+}$ mice makes it a novel genetic model to study the development of IBD and to address important questions regarding intestinal homeostasis and immunity.

Figure 4.1: Kaiso transgenic mice display hallmarks of chronic inflammation.

(A) Kaiso transgenic mice (*Kaiso*$^{Tg/+}$) display discontinuous lesions (crypt abscesses) through the small intestine (red asterisks) that are interspersed with normal villi. *Kaiso*$^{Tg/+}$ also exhibit a thickening of the *muscularis externa* (black asterisks) compared to the Non-Tg siblings. **(B)** Crypt abscesses are a hallmark of chronic inflammation and active IBD and are characterized by the presence of numerous active neutrophils that have migrated across the epithelium and deform the crypt architecture. Line A *Kaiso*$^{Tg/+}$ mice have less crypt abscesses compared to Line E transgenics (with higher Kaiso expression) at ~8 months of age. * represents $p<0.05$.

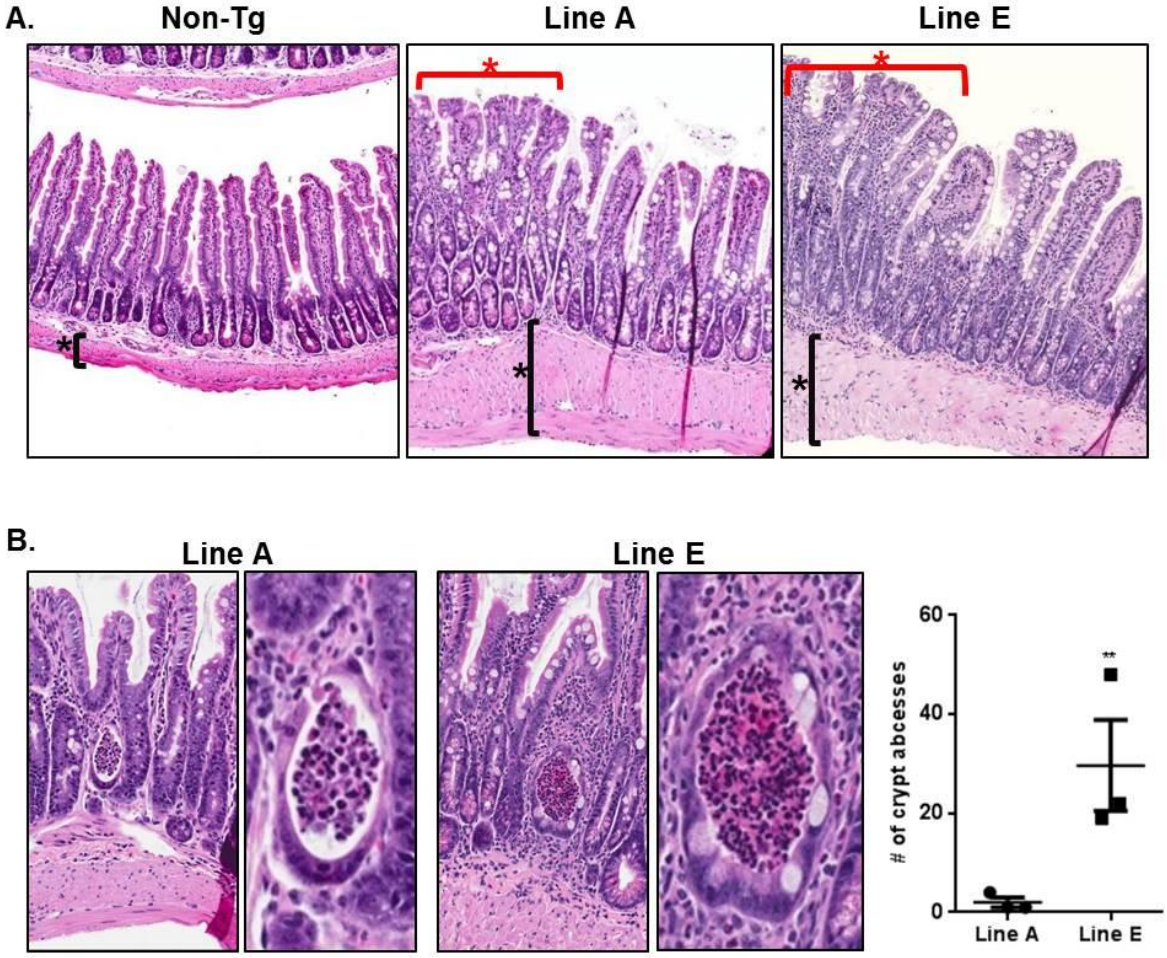

A.
Non-Tg
Line A
Line E
B.
Line A
Line E
# of crypt abcesses
60
40
20
0
**
Line A
Line E

Figure 4.2: Ectopic Kaiso expression induces neutrophilia in the murine intestine independent of the environment.

(A) At ~8 months, Line A mice exhibit a greater intestinal neutrophil population with a slight decrease in macrophages. **(B)** MPO analysis of age-matched Line A and E mice show an increase in inflammation in Line E (VKE) mice compared to Line A (VKA) and non-Tg mice in both Barrier as well as SPF room (n=5-7 per genotype per age). **(C)** At 14 weeks of age, Line E mice show a significant increase in neutrophil numbers compared to age-matched non-Tg mice and Line A mice. **(D)** At 14 weeks of age, Line E show no change in macrophages, while Line A mice show decrease numbers of macrophages. Live (PI^-) leukocytes ($CD45^+$) were gated first, and analyzed for neutrophils (F $7/4^+$ and Ly-$6G^+$) **(A, C)** or macrophages ($F4/80^+$) **(A, D)**. ** represents p-value < 0.05.

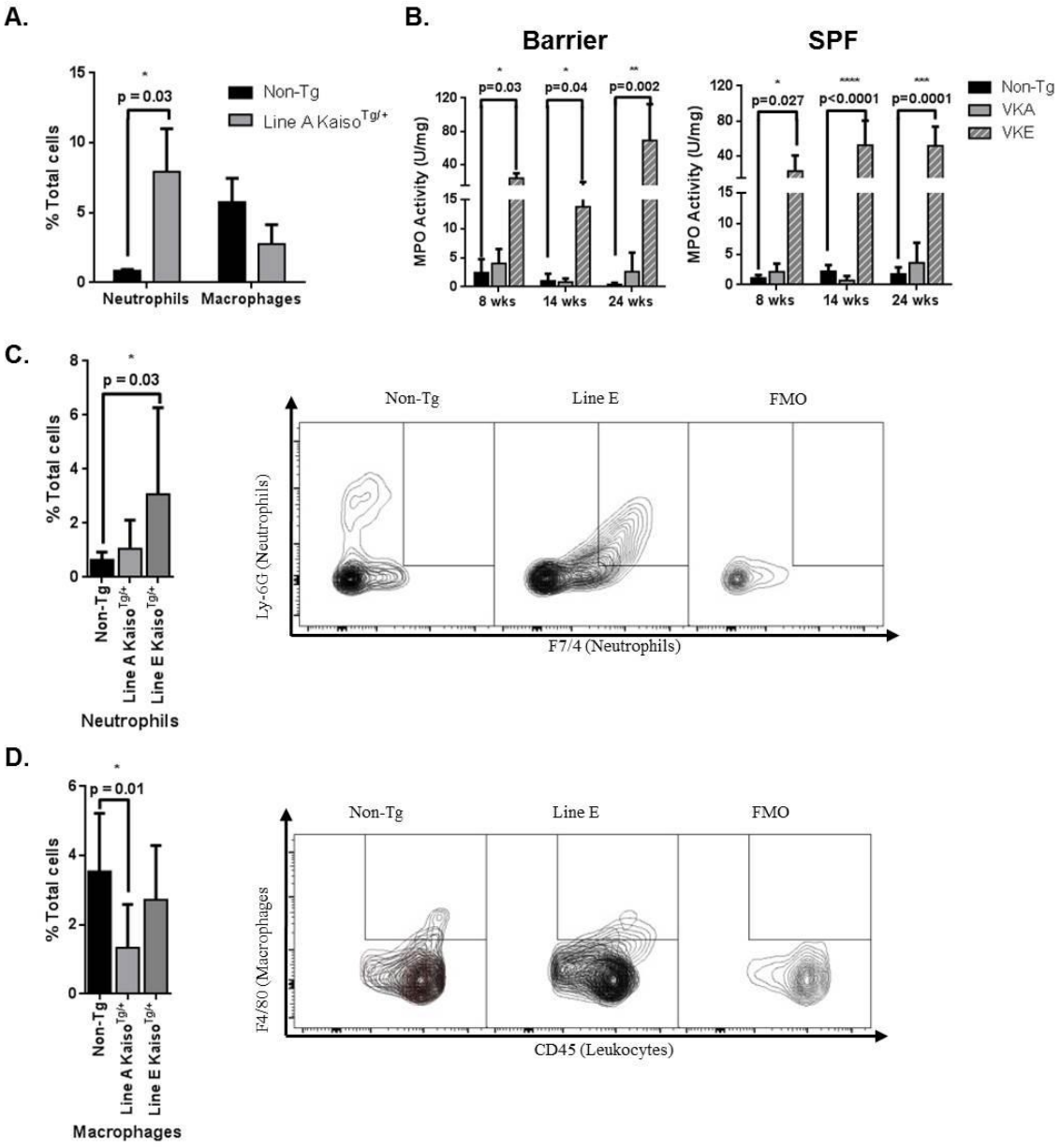

A.
% Total cells
p = 0.03
Non-Tg
Line A Kaiso Tg/+
Neutrophils
Macrophages
B.
Barrier
SPF
MPO Activity (U/mg)
p=0.03
p=0.04
p=0.002
p=0.027
p<0.0001
p=0.0001
8 wks
14 wks
24 wks
Non-Tg
VKA
VKE
C.
% Total cells
p = 0.03
Non-Tg
Line A Kaiso Tg/+
Line E Kaiso Tg/+
Neutrophils
Non-Tg
Line E
FMO
Ly-6G (Neutrophils)
F7/4 (Neutrophils)
D.
% Total cells
p = 0.01
Non-Tg
Line A Kaiso Tg/+
Line E Kaiso Tg/+
Macrophages
Non-Tg
Line E
FMO
F4/80 (Macrophages)
CD45 (Leukocytes)

Figure 4.3 Ectopic Kaiso expression increases MMP7 expression levels.

(A) Immunohistochemistry shows increased MMP7 expression in intestinal crypts of Line A *Kaiso*$^{Tg/+}$ mice, and some expression in the villi. **(B)** Immunoblot analysis also shows increased MMP7 expression in Kaiso overexpressing tissues of Line A mice at ~6 months of age. A similar increase was observed in *Kaiso*$^{Tg/+}$ Line E mice.

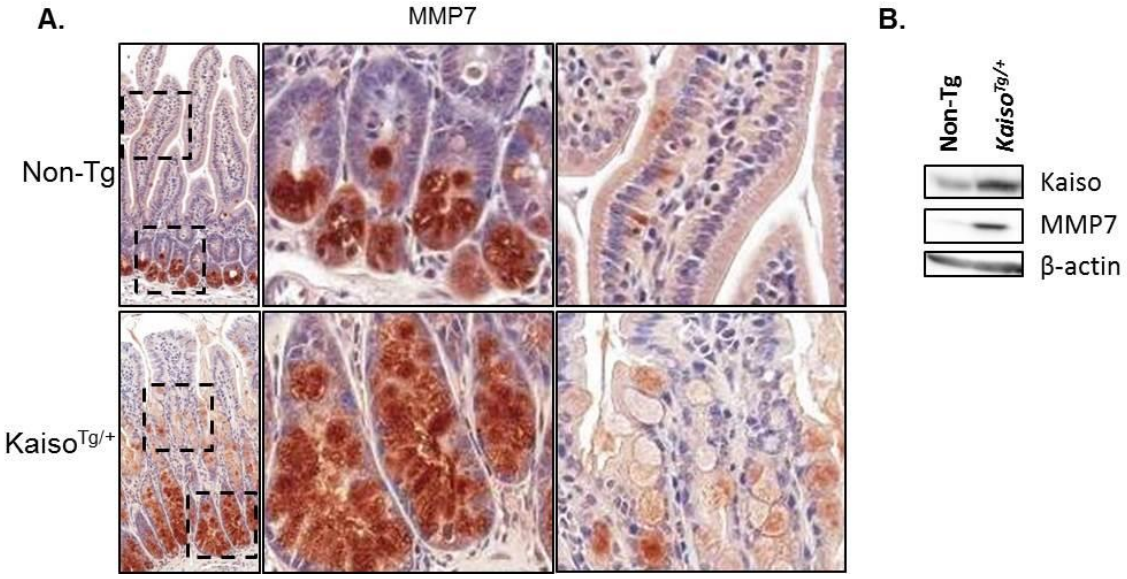

A.
MMP7
Non-Tg
Kaiso^Tg/+
B.
Non-Tg
Kaiso^Tg/+
Kaiso
MMP7
β-actin

Figure 4.4 Kaiso expression levels influences intestinal epithelial permeability.

(A) FITC-Dextran administration to Line E mice shows a significant intestinal barrier defect at 14 weeks of age, compared to Line A or non-Tg mice. * represents p-value <0.05. **(B)** *Kaiso*$^{Tg/+}$ mice show no change in total E-cadherin (*adherens* junction) but display an increase in Claudin-1 (tight junction) expression. **(C)** E-cadherin expression is localized to the membrane in the non-Tg (i) and *Kaiso*$^{Tg/+}$ (ii) mice. The non-Tg mice display strong E-cadherin expression in the villi (i') compared to the crypts (i''), while *Kaiso*$^{Tg/+}$ mice show strong E-cadherin expression in both the villi (ii') and crypts (ii''). The villar E-cadherin expression in the *Kaiso*$^{Tg/+}$ is stronger at the basal side of the cells (ii') than at the cell-cell junctions as seen in non-Tg tissues (i'). **(D)** Non-Tg mice have weak apical Claudin-1 expression in the villi (i') and negligible expression in the crypts (i''). Conversely, *Kaiso*$^{Tg/+}$ intestinal tissues have stronger Claudin-1 expression (ii, iii). *Kaiso*$^{Tg/+}$ have an overall increase in Claudin-1 expression in normal villi (ii') with low detection in the crypts (ii''); while inflamed lesion tissue shows lower Claudin-1 expression in the villi (iii') and stronger expression in the crypts (iii''). White boxes indicate magnified sections.

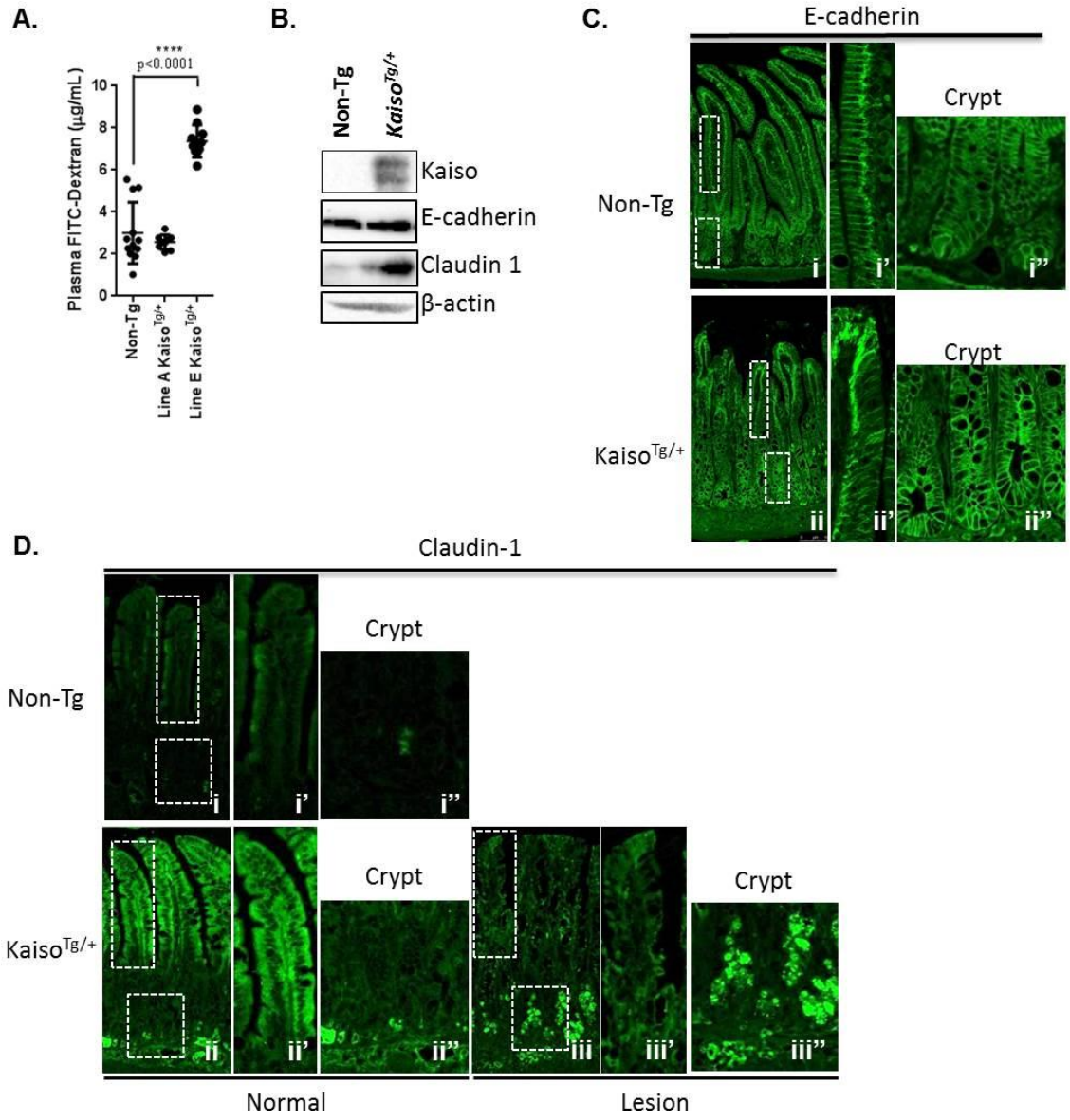

A.
****
p<0.0001
Plasma FITC-Dextran (µg/mL)
10
8
6
4
2
0
Non-Tg
Line A Kaiso Tg/+
Line E Kaiso Tg/+
B.
Non-Tg
Kaiso Tg/+
Kaiso
E-cadherin
Claudin 1
β-actin
C.
E-cadherin
Non-Tg
Crypt
i
i'
i''
Kaiso Tg/+
Crypt
ii
ii'
ii''
D.
Claudin-1
Non-Tg
Crypt
i
i'
i''
Kaiso Tg/+
Crypt
Crypt
ii
ii'
ii''
iii
iii'
iii''
Normal
Lesion

Figure 4.5 Kaiso transgenic mice express high levels of NFκB and ICAM-1.

(A) *Kaiso*$^{Tg/+}$ mice express more inflammatory markers such as ICAM-1 and NFκB in intestinal epithelial cells. **(B)** In Kaiso-depleted HCT116 colon cancer cell lines, decreased Kaiso expression correlated with decreased ICAM-1 expression, while p120^{ctn} expression inversely correlates with ICAM-1 expression.

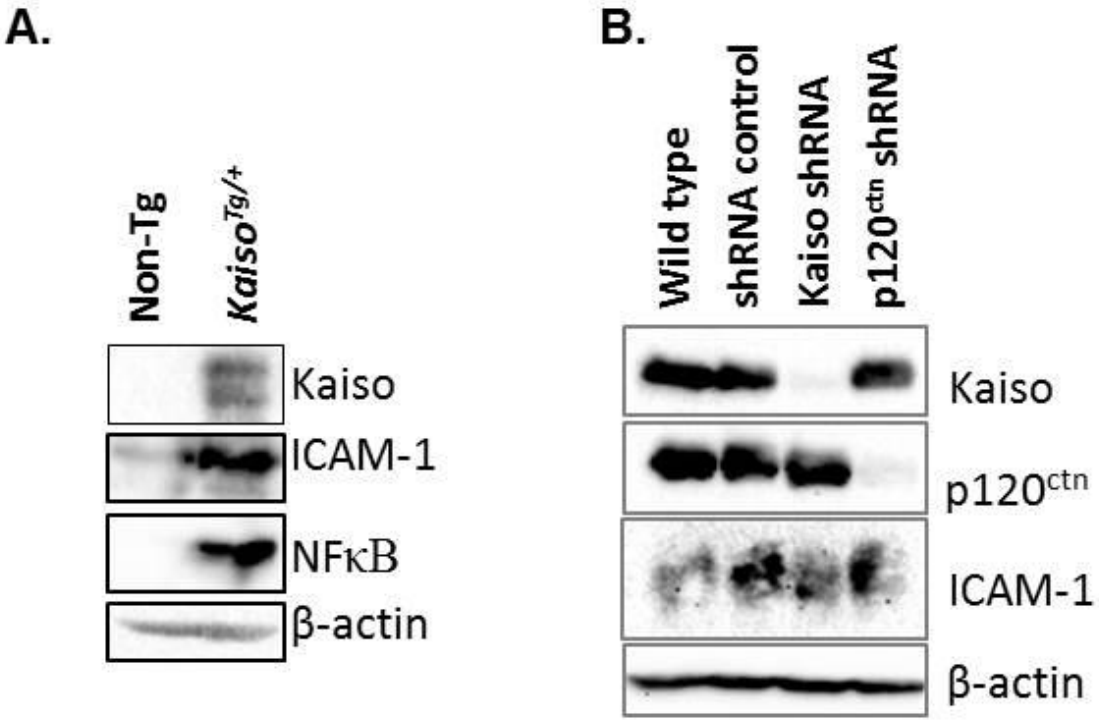
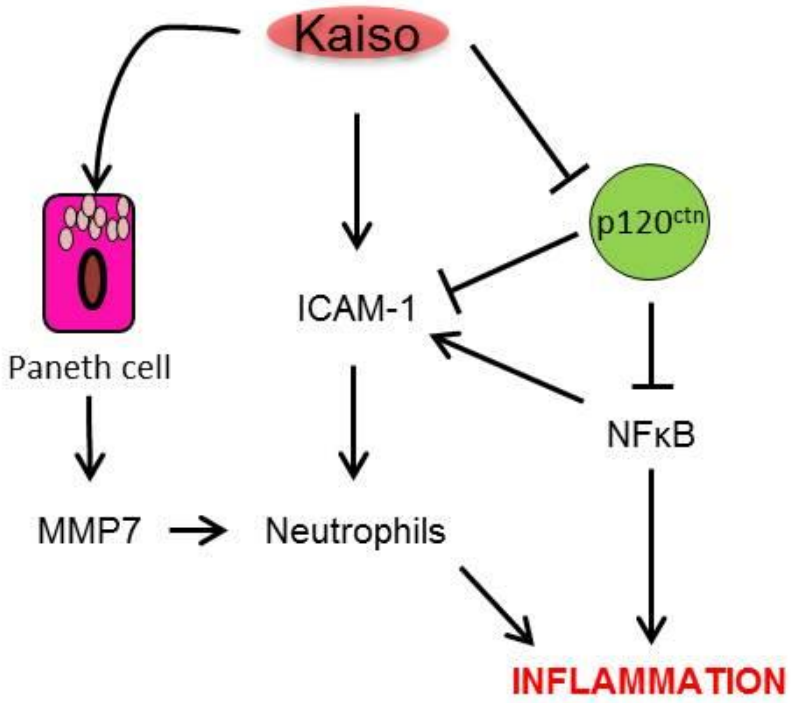

A.
Non-Tg
Kaiso^Tg/+
Kaiso
ICAM-1
NFκB
β-actin
B.
Wild type
shRNA control
Kaiso shRNA
p120ctn shRNA
Kaiso
p120ctn
ICAM-1
β-actin

Figure 4.6: Schematic model depicting possible Kaiso-mediated pro-inflammatory effects.

MMP7 and ICAM-1 play pro-inflammatory roles in murine intestines. Kaiso-mediated increase in Paneth cells induces MMP7 expression, while Kaiso may have direct or indirect regulation (via $p120^{ctn}$) of ICAM-1 expression. Additionally, $p120^{ctn}$'s anti-inflammatory role is also inhibited by Kaiso overexpression as $Kaiso^{Tg/+}$ mice have decreased $p120^{ctn}$ expression at the membranes.


Kaiso
p120ctn
ICAM-1
Paneth cell
NFκB
MMP7
Neutrophils
INFLAMMATION

CHAPTER 5: DISCUSSION

5.1 Villin-mediated Kaiso overexpressing mouse model

The Kaiso overexpressing transgenic mouse model is the first of its kind developed to study the tissue-specific role of Kaiso *in vivo*. Several independent studies demonstrated a role for Kaiso in *Xenopus* development via the regulation of the Wnt signalling pathway (Park *et al.*, 2005; Ruzov *et al.*, 2004). In mice, however, where Kaiso is expressed in multiple murine tissues including the skin, intestines and lungs (Shumskaya *et al.*, 2014), Kaiso-null mice developed normally and had viable offspring (Prokhortchouk *et al.*, 2006). Thus, it was postulated that the Kaiso-like proteins, ZBTB4 and ZBTB38, may be functionally redundant with Kaiso and compensate for the absence of Kaiso during murine development in that model (Prokhortchouk *et al.*, 2006).

Since Kaiso plays a role in regulating Wnt target genes and Wnt signalling is crucial in intestinal development and maintenance, we generated multiple Kaiso overexpressing transgenic lines using the *villin* promoter to study the intestinal-specific effects of Kaiso overexpression (Chapter 3; Chaudhary *et al.*, 2013). Villin is a calcium-regulated actin binding protein that is expressed in undifferentiated and differentiated cells of the intestines. Thus, its promoter is suitable to control the expression of transgenes specifically in mammalian intestines (reviewed in Friederich *et al.*, 1990; Robine *et al.*, 1997). The use of the *villin* promoter to drive the intestinal-specific *Kaiso* expression allowed for successful tissue-specific expression within Kaiso transgenic

(*Kaiso*$^{Tg/+}$) mice. Kaiso expression is localized in the nuclei of the villi and crypt cells throughout the murine intestines (Chapter 3; Chaudhary *et al.*, 2013).

The *Kaiso*$^{Tg/+}$ mice develop elongated crypts that may be explained by increased Wnt signalling in the intestines. Upregulation of Wnt signalling via homozygous deletion of *Apc* gene leads to dramatically enlarged crypts in murine intestines (Sansom *et al.*, 2004). The Kaiso-null mice, when bred to the *Apc*$^{Min/+}$ mouse model (with constitutive Wnt) developed fewer and smaller polyps suggesting a positive regulation of Wnt signalling by Kaiso (Prokhortchouk *et al.*, 2006). While this was unexpected based on previous findings in *Xenopus* (Park *et al.*, 2006; Park *et al.*, 2005; Ruzov *et al.*, 2004), it is consistent with the recent finding that Kaiso transgenic mice exhibit increased β-galactosidase staining when bred to Axin2-LacZ mice (Pierre *et al*., 2015; manuscript in revision). The increased β-galactosidase staining suggests an overall increase in the Wnt signalling pathway, which may contribute to the enlarged cryptal structures.

The inflammatory phenotype seen in the Kaiso transgenic mice is reminiscent of the **s**enescence **a**ccelerated **m**ouse (SAM) model, specifically the SAMP1/Yit substrain (reviewed in Pizarro *et al.*, 2011). The SAMP1/Yit mouse is a model for spontaneous ileitis and was originally generated by >20 generations of sibling mating of the SAM mice (reviewed in Pizarro *et al.*, 2011). The earliest histological alterations within these mice include crypt elongation, villous blunting and an increase in secretory cells at the expense of absorptive enterocytes (reviewed in Pizarro *et al.*, 2011), a phenotype similar to the *Kaiso*$^{Tg/+}$ mice. The SAMP1/Yit mice develop both acute and chronic intestinal

inflammation with inflammatory infiltrates comprised of macrophages and neutrophils, and develop crypt abscesses with age (Matsumoto *et al.*, 1998), again similar to the *Kaiso*$^{Tg/+}$ mice which exhibit neutrophil infiltration and crypt abscess development (Chapter 4). Despite these similarities, one key difference is that the SAMP1/Yit mouse model exhibits chronic intestinal inflammation localized to the terminal ileum, making it a model of Crohn's disease-like ileitis (Matsumoto *et al.*, 1998; reviewed in Pizarro *et al.*, 2011), while the *Kaiso*$^{Tg/+}$ mice develop intestinal inflammation throughout the small intestine. The *Kaiso*$^{Tg/+}$ mice could thus be used as a spontaneous model for IBD, specifically chronic Crohn's-related inflammation.

The *villin* gene is also expressed in the epithelial cells of the kidney proximal tubules, though *villin*-transgene expression within the kidneys is sensitive to the site of integration (Pinto *et al.*, 1999). Though slight differences in Kaiso expression were observed in the kidneys of the *Kaiso*$^{Tg/+}$ mice and their non-Tg littermates (Appendix 2), the characterization of kidneys from *Kaiso*$^{Tg/+}$ mice was beyond the scope of this study.

5.2 Kaiso overexpression decreases Notch signalling and affects epithelial cell differentiation

The Kaiso transgenic mice show an increase in the differentiation of secretory cells (goblet cells, EEC and Paneth cells) in both the small and large intestines. It is well established that constitutive activation of Notch signalling promotes differentiation of the absorptive enterocytes (Fre *et al.*, 2005; Stanger *et al.*, 2005), while inhibition of Notch signalling supports differentiation of the secretory cell lineage (van Es *et al.*, 2005;

VanDussen *et al*., 2012). Indeed, within the *Kaiso*$^{Tg/+}$ mice, the number of secretory cells in increased, and this correlated with the decrease in the Notch effector protein HES1 (Chapter 3; Chaudhary *et al.*, 2013). Since the *Kaiso*$^{Tg/+}$ mice have a decrease in *Hes1* mRNA, it would be interesting to perform **ch**romatin **i**mmuno**p**recipitation (ChIP) analysis on the *Hes1* promoter to evaluate whether Kaiso directly represses its gene expression.

Though *Hes1* is decreased in the *Kaiso*$^{Tg/+}$ mice, it has been shown that the decrease in *Hes1* alone is not sufficient to maintain an increase in the number of secretory cells in adult mice (Ueo *et al.*, 2012). Ueo and colleagues showed that two other Notch effectors, HES3 and HES5, are involved in maintaining the increase of secretory cell formation in adult mice (Ueo *et al.*, 2012). Indeed, *Hes3* and *Hes5* expression were previously shown to be upregulated in the absence of *Hes1* in embryonic intestines suggesting that HES3 and HES5 expression could compensate for the *Hes1* deficiency (Jensen *et al.*, 2000). Indeed, *Hes1/3/5* triple knockout mice develop an increase in secretory cells which was maintained up to 2 months of age (Ueo *et al.*, 2012). The *Kaiso*$^{Tg/+}$ mice have decreased *Hes1* expression that is consistent with the augmentation of secretory cells observed in mice as young as 90 days of age (Pierre *et al*., 2015; manuscript in revision), and is maintained up to 12 months of age (Chapter 3; Chaudhary *et al.*, 2013). Since the phenotype is maintained well into adulthood, it is possible that the *Kaiso*$^{Tg/+}$ mice have a decrease in the other two Notch effectors, HES3 and HES5.

In addition to regulating cell differentiation, Notch signaling also regulates cell proliferation within the intestines (Ueo *et al*., 2012; VanDussen *et al*., 2012). The *Hes1/3/5* triple knockout mice exhibit decreased numbers of $Ki67^+$ proliferative cells in intestinal crypts up to 2 months of age, however *Hes1* deficient mice did not show any such change in $Ki67^+$ cell numbers (Ueo *et al.*, 2012). The $Kaiso^{Tg/+}$ mice have a reduction in the number of $Ki67^+$ cells, and the phenotype is maintained into adulthood (12 months of age) (Chapter 3; Chaudhary *et al.*, 2013). The decline in proliferation within the $Kaiso^{Tg/+}$ intestines may be due to all three Notch effectors, HES1, 3 and 5, being inhibited in the $Kaiso^{Tg/+}$ mice. Thus, it would be of interest to measure the *Hes1*, *3*, and *5* expression levels in the $Kaiso^{Tg/+}$ mice.

The decrease in the Notch effector(s) in the $Kaiso^{Tg/+}$ mice may be due to a direct gene repression by Kaiso, or an indirect effect via the negative regulation of the Notch signalling pathway. The Notch-regulated transcription factor, MATH1, a HES target gene, is known to be required (Shroyer *et al.*, 2007; Yang *et al.*, 2001) and sufficient (VanDussen and Samuelson, 2010) for the differentiation of all three secretory cell types. Thus it would be of interest to see if the MATH1 expression is altered in the $Kaiso^{Tg/+}$ mice as a further indication of Kaiso's regulation of the Notch pathway.

Dysregulation of the Notch signalling pathway has been implicated in the pathogenesis of IBD, though the exact role of Notch signalling is complex and requires clarification (reviewed in Noah and Shroyer, 2013). Depletion of goblet cells in active UC and misregulation of secretory cell differentiation in CD have been associated with

increased Notch signalling (Wehkamp *et al.*, 2005; Zheng *et al.*, 2011). Additionally, Notch activity is enhanced in hyperplastic crypts from inflamed tissues of UC patients, and promotes the regeneration of the epithelium in late phases of colitis (Okamoto *et al.*, 2009). Levels of cleaved Notch-1, indicating Notch activation, are also increased in colonic epithelium of CD patients (Dahan *et al.*, 2011). During colitis development, impairing Notch signalling reduced the intestinal damage by preventing the loss of goblet cells that play a protective role in the initial phases (Shinoda *et al.*, 2010). However independent studies suggest that inhibition of Notch signalling in intestinal epithelial cells induces spontaneous chronic colitis due to increased intestinal permeability and bacterial translocation (Obata et al., 2012). Decreased Paneth cell maturation and expression of α-defensins has also been associated with CD and may contribute to IBD susceptibility (Koslowski *et al.*, 2010; Wehkamp *et al.*, 2005). Thus, Notch signalling's role in IBD, whether contributing to preventing intestinal inflammation, remains controversial.

The Kaiso-mediated inflammatory phenotype supports a preventative role for Notch in the development of inflammation. Since goblet cell numbers are increased as early as 90 days after birth, which is before the onset of inflammation (Pierre *et al*., 2015; manuscript in revision), it may be that early inhibition of Notch signalling contributes to the development of chronic inflammation. It would be of interest to see if the inflammatory index is lowered in the $Kaiso^{Tg/+}$ mice by breeding them with mice constitutively expressing the **N**otch **i**ntra**c**ellular **d**omain (NICD) (as used in (Fre *et al.*, 2005)), and if the secretory cell numbers are restored to normal levels. If the $Kaiso^{Tg/+}$

mice with activated Notch decreases the inflammatory index, it would suggest the regulation of Notch signalling is pertinent for inhibiting inflammation. Nevertheless, though Kaiso has been shown to affect proliferation in *Xenopus* studies via the Wnt pathway (Ruzov *et al.*, 2004), this is the first study relating Kaiso to the Notch signalling pathway.

5.3 Intrinsic effects of Kaiso overexpression leads to spontaneous intestinal inflammation

The *Kaiso*$^{Tg/+}$ mice display decreased expression of Kaiso's binding partner, p120^{ctn}, at the cell membrane but an increase in its nuclear localization (Chapter 3; Chaudhary *et al.*, 2013). Conditional knockdown of p120^{ctn} in mice results in pro-inflammatory cytokine release and immune cell infiltration into the intestines, suggesting an anti-inflammatory role for p120^{ctn} (Smalley-Freed *et al.*, 2010; Smalley-Freed *et al.*, 2011). The p120^{ctn}-deficient intestinal inflammation is characterized by massive neutrophil infiltration similar to that observed in some IBD patients (Smalley-Freed *et al.*, 2010; Smalley-Freed *et al.*, 2011). The neutrophil infiltration observed in the *Kaiso*$^{Tg/+}$ mice may thus be a consequence of the decrease of p120^{ctn} at the cell membrane. Interestingly, p120^{ctn} also influences the transcription of the pro-inflammatory adhesion molecule, ICAM-1 (Alcaide *et al.*, 2012; O'Donnell *et al.*, 2011; Wang *et al.*, 2011). The *Kaiso*$^{Tg/+}$ mice have increased ICAM-1 expression which may further potentiate the inflammatory phenotype by increasing neutrophil adhesion within the epithelial cells of the intestine (Chapter 4).

The anti-inflammatory role of p120^{ctn} has been demonstrated to be initially cell autonomous upon the loss of p120^{ctn} mediated by an intrinsic mechanism (reviewed in). This may shed light upon the underlying mechanism of inflammation in the *Kaiso*$^{Tg/+}$ mice. p120^{ctn} null epidermal cells show an increase in NFκB activity and cytokine production via the activation of the Rho GTPase pathway (Perez-Moreno *et al.*, 2006). In p120^{ctn} knockout mice, Rho GTPase activity is markedly reduced (Smalley-Freed *et al.*, 2010). It would be of interest to determine if the loss of p120^{ctn} from the cell membrane upon Kaiso overexpression leads to an increase in Rho GTPase activity and the induction of the NFκB pro-inflammatory cascade in the *Kaiso*$^{Tg/+}$ mice. The NFκB activity in response to p120^{ctn} misregulation in murine lungs was found to be partially regulated by Rho activity (Qin *et al.*, 2014) and partially by TLR4 (Wang *et al.*, 2011). Activation of the TLR4-mediated pathway induces an inflammatory gene expression cascade by activating NFκB (reviewed in Wullaert *et al.*, 2011). As such, TLR4 signaling should also be evaluated in the *Kaiso*$^{Tg/+}$ mice to provide an explanation for the inflammatory phenotype.

The *Kaiso*$^{Tg/+}$ mice have an increased intestinal permeability (Chapter 4) similar to p120^{ctn} deficient mice (Smalley-Freed *et al.*, 2010). Interestingly, inhibition of Notch signalling also yields an enhancement in intestinal permeability leading to an influx of bacteria that contributes to the development of colitis (Obata *et al.*, 2012). The intestinal permeability defect observed in the *Kaiso*$^{Tg/+}$ mice could thus be due to a combination of loss of p120^{ctn} at the membrane and a decrease in Notch signalling, which would further contribute to the active intestinal inflammation.

Claudin-1, despite being a tight junction protein that regulates intestinal permeability (Lu *et al.*, 2013), is increased in active IBD as a consequence of the inflammation (Weber *et al.*, 2008). This paradox can be explained by the increase in β-catenin transcriptional activity in chronic inflammation (Weber *et al.*, 2008). Claudin-1 is a Wnt target gene that is upregulated in colorectal cancers (Miwa *et al.*, 2001). Since the *Kaiso*$^{Tg/+}$ mice exhibit increased Wnt signalling (Pierre *et al.*, 2015; manuscript in revision), it is possible that the increased claudin-1 expression may be a result of active Wnt signalling. The increase in claudin-1 expression observed in the *Kaiso*$^{Tg/+}$ mice may also be a consequence of the active intestinal inflammation within these mice.

Apart from claudin-1, claudin-2 is also increased in active IBD (Weber *et al.*, 2008), and SAMP1/Yit mice with chronic inflammation show an increase in claudin-2 expression (Reuter and Pizarro, 2009). Thus, it would be beneficial to investigate the expression levels of other claudins and their influence on ion and molecular influx from the lumen into the intestines. In addition to the claudins, investigating the expression of the other tight junction proteins, like ZO-1 and occludin, would also give insight into the paracellular permeability of the *Kaiso*$^{Tg/+}$ mice within the intestines.

The *Kaiso*$^{Tg/+}$ mice also exhibit increased MMP7 expression within the intestines (Chapter 4). This was an unexpected finding since it was previously demonstrated that Kaiso repressed MMP7 expression in cultured cell lines (Spring *et al.*, 2005). However, since MMP7 is also a Wnt target gene (Spring *et al.*, 2005), the increase in MMP7 in the *Kaiso*$^{Tg/+}$ mice may be a result of the increase in Wnt signalling. However, as MMP7 is

expressed by Paneth cells and is responsible for α-defensin activation (Vandenbroucke *et al.*, 2014), the increased Paneth cell numbers in *Kaiso*$^{Tg/+}$ mice may explain the increase in MMP7 expression levels in the *Kaiso*$^{Tg/+}$ mice. One consequence of increased MMP7 within the intestines is intestinal leakage and increased susceptibility to spontaneous inflammation (Vandenbroucke *et al.*, 2014). Since MMP7 is also suggested to regulate the recruitment of neutrophils by modulating gradients of chemokines within the intestines (Swee *et al.*, 2008), the increased MMP7 levels in *Kaiso*$^{Tg/+}$ mice may regulate pro-inflammatory cytokines leading to a neutrophil influx in the intestines, and further potentiate the inflammatory phenotype.

Collectively our data demonstrates a pro-inflammatory role for Kaiso in murine intestines. Though the mechanism of the inflammation is not yet elucidated, it is clear that the *Kaiso*$^{Tg/+}$ mice are a fascinating model to study spontaneous development of chronic inflammation.

5.4 Kaiso transgenic mice are predisposed to develop colon cancer

Patients with IBD have a greatly increased risk of developing colorectal cancer (CRC) as a result of the chronic inflammation and genetic mutations (Ahmadi *et al.*, 2009). As previously mentioned, when Kaiso-null mice were bred to the *Apc*$^{Min/+}$ mouse model of CRC, the progeny developed fewer and smaller polyps (Prokhortchouk *et al.*, 2006). However, when *Kaiso*$^{Tg/+}$ mice were mated to the *Apc*$^{Min/+}$ mice, the progeny had a significantly greater tumour burden compared to the *Apc*$^{Min/+}$ siblings (Pierre *et al*., 2015; manuscript in revision). The *Kaiso*$^{Tg/+}$, *Apc*$^{Min/+}$ mice also have enhanced intestinal

inflammation compared to their *Apc*$^{Min/+}$ counterparts, concomitant with an increase in pro-inflammatory cytokine production (Pierre *et al*., 2015; manuscript in revision). Though the *Kaiso*$^{Tg/+}$ mice themselves do not develop polyps, it is possible that the inflammatory phenotype of the *Kaiso*$^{Tg/+}$ mice may prime them to develop CRC as they age.

The observed pro-inflammatory changes (increased MMP7 & claudin-1 expression and decreased p120^{ctn} expression) in *Kaiso*$^{Tg/+}$ mice also correlated with CRC development in both human and murine tissues, which would further prime the *Kaiso*$^{Tg/+}$ mice into developing CRC. In fact, MMP7 overexpression occurs in pre-malignant and malignant gastric lesions, and increases cancer susceptibility (Swee *et al.*, 2008; Vandenbroucke *et al.*, 2014), while conditional p120^{ctn} knockdown mice developed adenomas within 18 months in the intestines (Smalley-Freed *et al.*, 2011). In addition, claudin-1 is highly increased and dysregulated in colon cancer cells, which affects the cells' differentiation, transformation and metastatic abilities (Dhawan *et al.*, 2005). Increased claudin-1 is also postulated to contribute to the development of colitis-associated cancer (Kinugasa *et al.*, 2010). Thus, the pro-inflammatory changes within the *Kaiso*$^{Tg/+}$ mice may increase tumour susceptibility within this mouse model.

5.5 Future studies

The characterization of the *Kaiso*$^{Tg/+}$ mice yielded very interesting results including increased secretory cell types, villar fusion and blunting, and neutrophilic-specific inflammation within the intestines. To further study the Kaiso-Notch relationship,

chromatin **i**mmuno**p**recipitation (ChIP) analysis on the *Hes1* promoter should be performed to evaluate whether Kaiso directly represses its gene expression. Additionally, Kaiso's regulation of the other Notch effectors, HES3 and 5 should be evaluated.

The influx of active neutrophils within tissues can induce tissue damage (Summers *et al.*, 2010) as that seen in the *Kaiso*$^{Tg/+}$ mice. To evaluate if the inflammatory phenotype is exacerbated by the influx of neutrophils, the *Kaiso*$^{Tg/+}$ mice should be treated with resorcinol, which is a drug that is absorbed by the small intestine and inhibits MPO activity (Schneider and Issekutz, 1996). Upon treatment with resorcinol, if the tissue damage observed (villar fusion, crypt hyperplasia, permeability defect) in the *Kaiso*$^{Tg/+}$ mice is decreased, it can be concluded that neutrophil activity is responsible for the accumulation of tissue damage. This would help further understand the consequence of activated neutrophils contribution to the inflammatory phenotype in the *Kaiso*$^{Tg/+}$ mice.

The barrier defect within the *Kaiso*$^{Tg/+}$ mice should be evaluated in further detail. The transepithelial resistance across the intestine can be measured using an Ussing chamber (Li *et al.*, 2004) which would increase our knowledge about the location of the barrier defect and the ability of bacterial translocation through the epithelial barrier. An increase in bacterial influx intensified the chronic inflammatory phenotype observed upon Notch inhibition in the intestines (Obata et al., 2012). Bacterial influx can be gauged by measuring the levels of **l**ipo**p**oly**s**accharide (LPS) in the *Kaiso*$^{Tg/+}$ mice. Treatment of the *Kaiso*$^{Tg/+}$ mice with broad-range antibiotics would decrease the bacterial

influx across the barrier, and may potentially improve the inflammatory index of these mice.

Cytokines are signals produced by epithelial and innate immune cells upon interaction with bacteria on the luminal side of the intestines. Released cytokines interact with and activate the adaptive immune cells in response to bacterial influx. Various cytokines prevent neutrophilic apoptosis including GM-CSF (Fournier and Parkos, 2012). Interestingly, GM-CSF is increased in *Kaiso*$^{Tg/+}$, *Apc*$^{Min/+}$ mice which may explain the accumulation of neutrophils in the *Kaiso*$^{Tg/+}$ mice. Nevertheless, a complete cytokine analysis of the intestines from the *Kaiso*$^{Tg/+}$ mice should be performed to understand the dynamics of the interactions of the epithelial, innate and adaptive immune cells.

Chronic inflammation in IBD patients increases their risk to developing CRC. In addition, *Kaiso*$^{Tg/+}$, *Apc*$^{Min/+}$ mice develop greater number of polyps, which may be a consequence of increased intestinal inflammation. To investigate the tumour-susceptibility potential of Kaiso-mediated inflammation, the *Kaiso*$^{Tg/+}$ mice should be treated with DSS and **a**z**o**xy**m**ethane (AOM), and evaluated for polyp development. DSS/AOM together provides a chemically induced CRC model in mice (De Robertis *et al*., 2011), which may complement the increased polyps phenotype observed in the *Kaiso*$^{Tg/+}$, *Apc*$^{Min/+}$ mice. Increased polyp numbers in DSS/AOM treated *Kaiso*$^{Tg/+}$ mice would also support a role for Kaiso-mediated inflammation contributing to the development of CAC.

5.6 Concluding Remarks

Most recent studies have focused on elucidating Kaiso's tumourigenic contributions (Cofre *et al*., 2012; Jiang *et al*., 2012; Jones *et al*., 2014; Jones *et al.,* 2012). Here, we performed the first *in vivo* study to elucidate the role of Kaiso in a mammalian model using tissue-specific Kaiso overexpression. We show that Kaiso overexpression has a multifaceted effect on the intestines; Kaiso alters epithelial differentiation by increasing the commitment to the secretory cell lineage possibly via regulation of the Notch signalling pathway. This study is the first to link Kaiso and the Notch signalling pathway. Moreover, the overexpression of Kaiso induced an inflammatory phenotype with intestinal mucosal injury, increased permeability and neutrophilic infiltration. The $Kaiso^{Tg/+}$ mice are a fascinating model that may be beneficial for further investigation of molecular mechanisms that contribute to IBD and to the progression of IBD to CRC.

REFERENCES

Actis, G.C., Pellicano, R., Rosina, F., 2014. Inflammatory bowel diseases: Current problems and future tasks. World J Gastrointest Pharmacol Ther 5, 169-174.

Ahmadi, A., Polyak, S., Draganov, P.V., 2009. Colorectal cancer surveillance in inflammatory bowel disease: the search continues. World J Gastroenterol 15, 61-66.

Ahmadian, M., Suh, J.M., Hah, N., Liddle, C., Atkins, A.R., Downes, M., Evans, R.M., 2013. PPARγ signaling and metabolism: the good, the bad and the future. Nat Med 19, 557-566.

Alcaide, P., Martinelli, R., Newton, G., Williams, M.R., Adam, A., Vincent, P.A., Luscinskas, F.W., 2012. p120-Catenin prevents neutrophil transmigration independently of RhoA inhibition by impairing Src dependent VE-cadherin phosphorylation. Am J Physiol Cell Physiol 303, C385-395.

Annese, V., Rogai, F., Settesoldi, A., Bagnoli, S., 2012. PPARγ in Inflammatory Bowel Disease. PPAR Res 2012, 620839.

Barak, Y., Nelson, M.C., Ong, E.S., Jones, Y.Z., Ruiz-Lozano, P., Chien, K.R., Koder, A., Evans, R.M., 1999. PPAR gamma is required for placental, cardiac, and adipose tissue development. Mol Cell 4, 585-595.

Bienz, M., Clevers, H., 2000. Linking colorectal cancer to Wnt signaling. Cell 103, 311-320.

Blattler, A., Yao, L., Wang, Y., Ye, Z., Jin, V.X., Farnham, P.J., 2013. ZBTB33 binds unmethylated regions of the genome associated with actively expressed genes. Epigenetics Chromatin 6, 13.

Bondow, B.J., Faber, M.L., Wojta, K.J., Walker, E.M., Battle, M.A., 2012. E-cadherin is required for intestinal morphogenesis in the mouse. Dev Biol 371, 1-12.

Brabletz, T., Jung, A., Dag, S., Hlubek, F., Kirchner, T., 1999. beta-catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. Am J Pathol 155, 1033-1038.

Brazil, J.C., Louis, N.A., Parkos, C.A., 2013. The role of polymorphonuclear leukocyte trafficking in the perpetuation of inflammation during inflammatory bowel disease. Inflamm Bowel Dis 19, 1556-1565.

Canada, Crohn's and Colitis Foundation of Canada, 2013. Ideas in Action-2013 Annual Report.

Canada, Government of Canada, 2014. Canadian Cancer Statistics.

Chaudhary, R., Pierre, C.C., Nanan, K., Wojtal, D., Morone, S., Pinelli, C., Wood, G.A., Robine, S., Daniel, J.M., 2013. The POZ-ZF transcription factor Kaiso (ZBTB33) induces inflammation and progenitor cell differentiation in the murine intestine. PLoS One 8, e74160.

Chu, D.K., Jimenez-Saiz, R., Verschoor, C.P., Walker, T.D., Goncharova, S., Llop-Guevara, A., Shen, P., Gordon, M.E., Barra, N.G., Bassett, J.D., Kong, J., Fattouh, R., McCoy, K.D., Bowdish, D.M., Erjefalt, J.S., Pabst, O., Humbles, A.A., Kolbeck, R., Waserman, S., Jordana, M., 2014. Indigenous enteric eosinophils control DCs to initiate a primary Th2 immune response in vivo. Journal of Experimental Medicine 211, 1657-1672.

Clevers, H., Nusse, R., 2012. Wnt/β-catenin signaling and disease. Cell 149, 1192-1205.

Cofre, J., Menezes, J.R., Pizzatti, L., Abdelhay, E., 2012. Knock-down of Kaiso induces proliferation and blocks granulocytic differentiation in blast crisis of chronic myeloid leukemia. Cancer Cell Int 12, 28.

Crawford, H.C., Fingleton, B.M., Rudolph-Owen, L.A., Goss, K.J., Rubinfeld, B., Polakis, P., Matrisian, L.M., 1999. The metalloproteinase matrilysin is a target of beta-catenin transactivation in intestinal tumors. Oncogene 18, 2883-2891.

Crosnier, C., Stamataki, D., Lewis, J., 2006. Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. Nat Rev Genet 7, 349-359.

Dahan, S., Rabinowitz, K.M., Martin, A.P., Berin, M.C., Unkeless, J.C., Mayer, L., 2011. Notch-1 signaling regulates intestinal epithelial barrier function, through interaction with CD4+ T cells, in mice and humans. Gastroenterology 140, 550-559.

Dai, S.D., Wang, Y., Jiang, G.Y., Zhang, P.X., Dong, X.J., Wei, Q., Xu, H.T., Li, Q.C., Zhao, C., Wang, E.H., 2010. Kaiso is expressed in lung cancer: its expression and localization is affected by p120ctn. Lung Cancer 67, 205-215.

Dai, S.D., Wang, Y., Miao, Y., Zhao, Y., Zhang, Y., Jiang, G.Y., Zhang, P.X., Yang, Z.Q., Wang, E.H., 2009. Cytoplasmic Kaiso is associated with poor prognosis in non-small cell lung cancer. BMC Cancer 9, 178.

Dai, S.D., Wang, Y., Zhang, J.Y., Zhang, D., Zhang, P.X., Jiang, G.Y., Han, Y., Zhang, S., Cui, Q.Z., Wang, E.H., 2011. Upregulation of δ-catenin is associated with poor prognosis and enhances transcriptional activity through Kaiso in non-small-cell lung cancer. Cancer Sci 102, 95-103.

Daniel, J.M., 2007. Dancing in and out of the nucleus: p120(ctn) and the transcription factor Kaiso. Biochim Biophys Acta 1773, 59-68.

Daniel, J.M., Reynolds, A.B., 1999. The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor. Mol Cell Biol 19, 3614-3623.

Daniel, J.M., Spring, C.M., Crawford, H.C., Reynolds, A.B., Baig, A., 2002. The p120(ctn)-binding partner Kaiso is a bi-modal DNA-binding protein that recognizes both a sequence-specific consensus and methylated CpG dinucleotides. Nucleic Acids Res 30, 2911-2919.

Davis, M.A., Reynolds, A.B., 2006. Blocked acinar development, E-cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland. Dev Cell 10, 21-31.

De Mey, J.R., Freund, J.N., 2013. Understanding epithelial homeostasis in the intestine: An old battlefield of ideas, recent breakthroughs and remaining controversies. Tissue Barriers 1, e24965.

De Robertis, M., Massi, E., Poeta, M.L., Carotti, S., Morini, S., Cecchetelli, L., Signori, E., Fazio, V.M., 2011. The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. J Carcinog 10, 9.

Dhawan, P., Singh, A.B., Deane, N.G., No, Y., Shiou, S.R., Schmidt, C., Neff, J., Washington, M.K., Beauchamp, R.D., 2005. Claudin-1 regulates cellular transformation and metastatic behavior in colon cancer. J Clin Invest 115, 1765-1776.

Dieleman, L.A., Ridwan, B.U., Tennyson, G.S., Beagley, K.W., Bucy, R.P., Elson, C.O., 1994. Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. Gastroenterology 107, 1643-1652.

Donaldson, N.S., Pierre, C.C., Anstey, M.I., Robinson, S.C., Weerawardane, S.M., Daniel, J.M., 2012. Kaiso represses the cell cycle gene cyclin D1 via sequence-specific and methyl-CpG-dependent mechanisms. PLoS One 7, e50398.

Dubuquoy, L., Rousseaux, C., Thuru, X., Peyrin-Biroulet, L., Romano, O., Chavatte, P., Chamaillard, M., Desreumaux, P., 2006. PPARgamma as a new therapeutic target in inflammatory bowel diseases. Gut 55, 1341-1349.

Däbritz, J., 2014. Granulocyte macrophage colony-stimulating factor and the intestinal innate immune cell homeostasis in Crohn's disease. Am J Physiol Gastrointest Liver Physiol 306, G455-465.

Ek, W.E., D'Amato, M., Halfvarson, J., 2014. The history of genetics in inflammatory bowel disease. Ann Gastroenterol 27, 294-303.

Fanning, A.S., Anderson, J.M., 2009. Zonula occludens-1 and -2 are cytosolic scaffolds that regulate the assembly of cellular junctions. Ann N Y Acad Sci 1165, 113-120.

Farmer, S.R., 2005. Regulation of PPARgamma activity during adipogenesis. Int J Obes (Lond) 29 Suppl 1, S13-16.

Fevr, T., Robine, S., Louvard, D., Huelsken, J., 2007. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. Mol Cell Biol 27, 7551-7559.

Filion, G.J., Zhenilo, S., Salozhin, S., Yamada, D., Prokhortchouk, E., Defossez, P.A., 2006. A family of human zinc finger proteins that bind methylated DNA and repress transcription. Mol Cell Biol 26, 169-181.

Fournier, B.M., Parkos, C.A., 2012. The role of neutrophils during intestinal inflammation. Mucosal Immunol 5, 354-366.

Fre, S., Huyghe, M., Mourikis, P., Robine, S., Louvard, D., Artavanis-Tsakonas, S., 2005. Notch signals control the fate of immature progenitor cells in the intestine. Nature 435, 964-968.

Friederich, E., Pringault, E., Arpin, M., Louvard, D., 1990. From the structure to the function of villin, an actin-binding protein of the brush border. Bioessays 12, 403-408.

Furuse, M., Hata, M., Furuse, K., Yoshida, Y., Haratake, A., Sugitani, Y., Noda, T., Kubo, A., Tsukita, S., 2002. Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice. J Cell Biol 156, 1099-1111.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S., 1993. Occludin: a novel integral membrane protein localizing at tight junctions. J Cell Biol 123, 1777-1788.

Furuse, M., Sasaki, H., Fujimoto, K., Tsukita, S., 1998. A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. J Cell Biol 143, 391-401.

Gassler, N., Rohr, C., Schneider, A., Kartenbeck, J., Bach, A., Obermüller, N., Otto, H.F., Autschbach, F., 2001. Inflammatory bowel disease is associated with changes of enterocytic junctions. Am J Physiol Gastrointest Liver Physiol 281, G216-228.

Goldstein, N.S., 2006. Isolated ileal erosions in patients with mildly altered bowel habits. A follow-up study of 28 patients. Am J Clin Pathol 125, 838-846.

Gopalakrishnan, N., Saravanakumar, M., Madankumar, P., Thiyagu, M., Devaraj, H., 2014. Colocalization of β-catenin with Notch intracellular domain in colon cancer: a possible role of Notch1 signaling in activation of CyclinD1-mediated cell proliferation. Mol Cell Biochem.

Gregorieff, A., Clevers, H., 2005. Wnt signaling in the intestinal epithelium: from endoderm to cancer. Genes Dev 19, 877-890.

Guruharsha, K.G., Kankel, M.W., Artavanis-Tsakonas, S., 2012. The Notch signalling system: recent insights into the complexity of a conserved pathway. Nat Rev Genet 13, 654-666.

Hartsock, A., Nelson, W.J., 2008. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. Biochim Biophys Acta 1778, 660-669.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., Kinzler, K.W., 1998. Identification of c-MYC as a target of the APC pathway. Science 281, 1509-1512.

Hermiston, M.L., Wong, M.H., Gordon, J.I., 1996. Forced expression of E-cadherin in the mouse intestinal epithelium slows cell migration and provides evidence for nonautonomous regulation of cell fate in a self-renewing system. Genes Dev 10, 985-996.

.

.

Ireton, R.C., Davis, M.A., van Hengel, J., Mariner, D.J., Barnes, K., Thoreson, M.A., Anastasiadis, P.Z., Matrisian, L., Bundy, L.M., Sealy, L., Gilbert, B., van Roy, F., Reynolds, A.B., 2002. A novel role for p120 catenin in E-cadherin function. J Cell Biol 159, 465-476.

Ivanov, A.I., Nusrat, A., Parkos, C.A., 2004. The epithelium in inflammatory bowel disease: potential role of endocytosis of junctional proteins in barrier disruption. Novartis Found Symp 263, 115-124; discussion 124-132, 211-118.

Jacques, P., Elewaut, D., 2008. Joint expedition: linking gut inflammation to arthritis. Mucosal Immunol 1, 364-371.

Jensen, J., Pedersen, E.E., Galante, P., Hald, J., Heller, R.S., Ishibashi, M., Kageyama, R., Guillemot, F., Serup, P., Madsen, O.D., 2000. Control of endodermal endocrine development by Hes-1. Nat Genet 24, 36-44.

Jiang, G., Wang, Y., Dai, S., Liu, Y., Stoecker, M., Wang, E., Wang, E., 2012. P120-catenin isoforms 1 and 3 regulate proliferation and cell cycle of lung cancer cells via beta-catenin and Kaiso respectively. PloS one 7, e30303.

Jones, J., Wang, H., Karanam, B., Theodore, S., Dean-Colomb, W., Welch, D.R., Grizzle, W., Yates, C., 2014. Nuclear localization of Kaiso promotes the poorly differentiated phenotype and EMT in infiltrating ductal carcinomas. Clin Exp Metastasis 31, 497-510.

Jones, J., Wang, H., Zhou, J., Hardy, S., Turner, T., Austin, D., He, Q., Wells, A., Grizzle, W.E., Yates, C., 2012. Nuclear Kaiso indicates aggressive prostate cancers and promotes migration and invasiveness of prostate cancer cells. Am J Pathol 181, 1836-1846.

Kapoor, A., Li, H.J., Leiter, A.B., 2007. Intestinal development: the many faces of Wnt signaling. Gastroenterology 133, 710-712.

Kelly, K.F., Daniel, J.M., 2006. POZ for effect--POZ-ZF transcription factors in cancer and development. Trends Cell Biol 16, 578-587.

Kelly, K.F., Otchere, A.A., Graham, M., Daniel, J.M., 2004a. Nuclear import of the BTB/POZ transcriptional regulator Kaiso. J Cell Sci 117, 6143-6152.

Kelly, K.F., Spring, C.M., Otchere, A.A., Daniel, J.M., 2004b. NLS-dependent nuclear localization of p120ctn is necessary to relieve Kaiso-mediated transcriptional repression. J Cell Sci 117, 2675-2686.

Khounlotham, M., Kim, W., Peatman, E., Nava, P., Medina-Contreras, O., Addis, C., Koch, S., Fournier, B., Nusrat, A., Denning, T.L., Parkos, C.A., 2012. Compromised intestinal epithelial barrier induces adaptive immune compensation that protects from colitis. Immunity 37, 563-573.

Kim, S.W., Fang, X., Ji, H., Paulson, A.F., Daniel, J.M., Ciesiolka, M., van Roy, F., McCrea, P.D., 2002. Isolation and characterization of XKaiso, a transcriptional repressor that associates with the catenin Xp120(ctn) in Xenopus laevis. J Biol Chem 277, 8202-8208.

Kim, S.W., Park, J.I., Spring, C.M., Sater, A.K., Ji, H., Otchere, A.A., Daniel, J.M., McCrea, P.D., 2004. Non-canonical Wnt signals are modulated by the Kaiso transcriptional repressor and p120-catenin. Nat Cell Biol 6, 1212-1220.

Kinugasa, T., Akagi, Y., Yoshida, T., Ryu, Y., Shiratuchi, I., Ishibashi, N., Shirouzu, K., 2010. Increased claudin-1 protein expression contributes to tumorigenesis in ulcerative colitis-associated colorectal cancer. Anticancer Res 30, 3181-3186.

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

Kosinski, C., Stange, D.E., Xu, C., Chan, A.S., Ho, C., Yuen, S.T., Mifflin, R.C., Powell, D.W., Clevers, H., Leung, S.Y., Chen, X., 2010. Indian hedgehog regulates intestinal stem cell fate through epithelial-mesenchymal interactions during development. Gastroenterology 139, 893-903.

Koslowski, M.J., Beisner, J., Stange, E.F., Wehkamp, J., 2010. Innate antimicrobial host defense in small intestinal Crohn's disease. Int J Med Microbiol 300, 34-40.

Kucharzik, T., Walsh, S.V., Chen, J., Parkos, C.A., Nusrat, A., 2001. Neutrophil transmigration in inflammatory bowel disease is associated with differential expression of epithelial intercellular junction proteins. Am J Pathol 159, 2001-2009.

Kuhnert, F., Davis, C.R., Wang, H.T., Chu, P., Lee, M., Yuan, J., Nusse, R., Kuo, C.J., 2004. Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. Proc Natl Acad Sci U S A 101, 266-271.

Kundu, P., Ling, T.W., Korecka, A., Li, Y., D'Arienzo, R., Bunte, R.M., Berger, T., Arulampalam, V., Chambon, P., Mak, T.W., Wahli, W., Pettersson, S., 2014. Absence of intestinal PPARγ aggravates acute infectious colitis in mice through a lipocalin-2-dependent pathway. PLoS Pathog 10, e1003887.

Kühl, A.A., Kakirman, H., Janotta, M., Dreher, S., Cremer, P., Pawlowski, N.N., Loddenkemper, C., Heimesaat, M.M., Grollich, K., Zeitz, M., Farkas, S., Hoffmann, J.C., 2007. Aggravation of different types of experimental colitis by depletion or adhesion blockade of neutrophils. Gastroenterology 133, 1882-1892.

Laukoetter, M.G., Nava, P., Lee, W.Y., Severson, E.A., Capaldo, C.T., Babbin, B.A., Williams, I.R., Koval, M., Peatman, E., Campbell, J.A., Dermody, T.S., Nusrat, A., Parkos, C.A., 2007. JAM-A regulates permeability and inflammation in the intestine in vivo. J Exp Med 204, 3067-3076.

Li, H., Sheppard, D.N., Hug, M.J., 2004. Transepithelial electrical measurements with the Ussing chamber. J Cyst Fibros 3 Suppl 2, 123-126.

Liu, Y., Dong, Q.Z., Wang, S., Xu, H.T., Miao, Y., Wang, L., Wang, E.H., 2014. Kaiso interacts with p120-catenin to regulate β-catenin expression at the transcriptional level. PLoS One 9, e87537.

Lopes, E.C., Valls, E., Figueroa, M.E., Mazur, A., Meng, F.G., Chiosis, G., Laird, P.W., Schreiber-Agus, N., Greally, J.M., Prokhortchouk, E., Melnick, A., 2008. Kaiso contributes to DNA methylation-dependent silencing of tumor suppressor genes in colon cancer cell lines. Cancer Res 68, 7258-7263.

Lu, Z., Ding, L., Lu, Q., Chen, Y.H., 2013. Claudins in intestines: Distribution and functional significance in health and diseases. Tissue Barriers 1, e24978.

Lutgens, M.W., van Oijen, M.G., van der Heijden, G.J., Vleggaar, F.P., Siersema, P.D., Oldenburg, B., 2013. Declining risk of colorectal cancer in inflammatory bowel disease: an updated meta-analysis of population-based cohort studies. Inflamm Bowel Dis 19, 789-799.

Maloy, K.J., Powrie, F., 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature 474, 298-306.

Martin Caballero, I., Hansen, J., Leaford, D., Pollard, S., Hendrich, B.D., 2009. The methyl-CpG binding proteins Mecp2, Mbd2 and Kaiso are dispensable for mouse embryogenesis, but play a redundant function in neural differentiation. PLoS One 4, e4315.

Matsumoto, S., Okabe, Y., Setoyama, H., Takayama, K., Ohtsuka, J., Funahashi, H., Imaoka, A., Okada, Y., Umesaki, Y., 1998. Inflammatory bowel disease-like enteritis and caecitis in a senescence accelerated mouse P1/Yit strain. Gut 43, 71-78.

Milano, J., McKay, J., Dagenais, C., Foster-Brown, L., Pognan, F., Gadient, R., Jacobs, R.T., Zacco, A., Greenberg, B., Ciaccio, P.J., 2004. Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. Toxicol Sci 82, 341-358.

Miwa, N., Furuse, M., Tsukita, S., Niikawa, N., Nakamura, Y., Furukawa, Y., 2001. Involvement of claudin-1 in the beta-catenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers. Oncol Res 12, 469-476.

Mohapatra, S.K., Guri, A.J., Climent, M., Vives, C., Carbo, A., Horne, W.T., Hontecillas, R., Bassaganya-Riera, J., 2010. Immunoregulatory actions of epithelial cell PPAR gamma at the colonic mucosa of mice with experimental inflammatory bowel disease. PLoS One 5, e10215.

Mócsai, A., 2013. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. J Exp Med 210, 1283-1299.

Nakamura, T., Tsuchiya, K., Watanabe, M., 2007. Crosstalk between Wnt and Notch signaling in intestinal epithelial cell fate decision. J Gastroenterol 42, 705-710.

Noah, T.K., Donahue, B., Shroyer, N.F., 2011. Intestinal development and differentiation. Exp Cell Res 317, 2702-2710.

Noah, T.K., Shroyer, N.F., 2013. Notch in the intestine: regulation of homeostasis and pathogenesis. Annu Rev Physiol 75, 263-288.

Németh, T., Mócsai, A., 2012. The role of neutrophils in autoimmune diseases. Immunol Lett 143, 9-19.

O'Donnell, J.J., Zhuge, Y., Holian, O., Cheng, F., Thomas, L.L., Forsyth, C.B., Lum, H., 2011. Loss of p120 catenin upregulates transcription of pro-inflammatory adhesion molecules in human endothelial cells. Microvasc Res 82, 105-112.

Obata, Y., Takahashi, D., Ebisawa, M., Kakiguchi, K., Yonemura, S., Jinnohara, T., Kanaya, T., Fujimura, Y., Ohmae, M., Hase, K., Ohno, H., 2012. Epithelial cell-intrinsic Notch signaling plays an essential role in the maintenance of gut immune homeostasis. J Immunol 188, 2427-2436.

Ogaki, S., Shiraki, N., Kume, K., Kume, S., 2013. Wnt and notch signals guide embryonic stem cell differentiation into the intestinal lineages. Stem Cells 31, 1086-1096.

Ogden, S.R., Wroblewski, L.E., Weydig, C., Romero-Gallo, J., O'Brien, D.P., Israel, D.A., Krishna, U.S., Fingleton, B., Reynolds, A.B., Wessler, S., Peek, R.M., 2008. p120 and Kaiso regulate Helicobacter pylori-induced expression of matrix metalloproteinase-7. Mol Biol Cell 19, 4110-4121.

Okamoto, R., Tsuchiya, K., Nemoto, Y., Akiyama, J., Nakamura, T., Kanai, T., Watanabe, M., 2009. Requirement of Notch activation during regeneration of the intestinal epithelia. Am J Physiol Gastrointest Liver Physiol 296, G23-35.

Ostanin, D.V., Pavlick, K.P., Bharwani, S., D'Souza, D., Furr, K.L., Brown, C.M., Grisham, M.B., 2006. T cell-induced inflammation of the small and large intestine in immunodeficient mice. Am J Physiol Gastrointest Liver Physiol 290, G109-119.

Park, J.I., Ji, H., Jun, S., Gu, D., Hikasa, H., Li, L., Sokol, S.Y., McCrea, P.D., 2006. Frodo links Dishevelled to the p120-catenin/Kaiso pathway: distinct catenin subfamilies promote Wnt signals. Dev Cell 11, 683-695.

Park, J.I., Kim, S.W., Lyons, J.P., Ji, H., Nguyen, T.T., Cho, K., Barton, M.C., Deroo, T., Vleminckx, K., Moon, R.T., McCrea, P.D., 2005. Kaiso/p120-catenin and TCF/beta-catenin complexes coordinately regulate canonical Wnt gene targets. Dev Cell 8, 843-854.

Parkos, C.A., Colgan, S.P., Diamond, M.S., Nusrat, A., Liang, T.W., Springer, T.A., Madara, J.L., 1996. Expression and polarization of intercellular adhesion molecule-1 on human intestinal epithelia: consequences for CD11b/CD18-mediated interactions with neutrophils. Mol Med 2, 489-505.

Pastorelli, L., De Salvo, C., Mercado, J.R., Vecchi, M., Pizarro, T.T., 2013. Central Role of the Gut Epithelial Barrier in the Pathogenesis of Chronic Intestinal Inflammation: Lessons Learned from Animal Models and Human Genetics. Front Immunol 4, 280.

Perez-Moreno, M., Davis, M.A., Wong, E., Pasolli, H.A., Reynolds, A.B., Fuchs, E., 2006. p120-catenin mediates inflammatory responses in the skin. Cell 124, 631-644.

Perez-Moreno, M., Song, W., Pasolli, H.A., Williams, S.E., Fuchs, E., 2008. Loss of p120 catenin and links to mitotic alterations, inflammation, and skin cancer. Proc Natl Acad Sci U S A 105, 15399-15404.

Peterson, L.W., Artis, D., 2014. Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat Rev Immunol 14, 141-153.

Pierre, C., Longo, J., Mavor, M., Milosavljevic, S., Chaudhary, R., Gilbreath, E., Yates, C., Daniel, J., 2015. Kaiso potentiates intestinal tumorigenesis in *Apc*$^{Min/+}$ mice and promotes canonical Wnt signalling activity and inflammation in the intestine. (in revision), BBA - Molecular Basis of Disease.

Pinto, D., Robine, S., Jaisser, F., El Marjou, F.E., Louvard, D., 1999. Regulatory sequences of the mouse villin gene that efficiently drive transgenic expression in immature and differentiated epithelial cells of small and large intestines. J Biol Chem 274, 6476-6482.

Pizarro, T.T., Pastorelli, L., Bamias, G., Garg, R.R., Reuter, B.K., Mercado, J.R., Chieppa, M., Arseneau, K.O., Ley, K., Cominelli, F., 2011. SAMP1/YitFc mouse strain: a spontaneous model of Crohn's disease-like ileitis. Inflamm Bowel Dis 17, 2566-2584.

Pope, J.L., Bhat, A.A., Sharma, A., Ahmad, R., Krishnan, M., Washington, M.K., Beauchamp, R.D., Singh, A.B., Dhawan, P., 2014. Claudin-1 regulates intestinal epithelial homeostasis through the modulation of Notch-signalling. Gut 63, 622-634.

Prokhortchouk, A., Hendrich, B., Jørgensen, H., Ruzov, A., Wilm, M., Georgiev, G., Bird, A., Prokhortchouk, E., 2001. The p120 catenin partner Kaiso is a DNA methylation-dependent transcriptional repressor. Genes Dev 15, 1613-1618.

Prokhortchouk, A., Sansom, O., Selfridge, J., Caballero, I.M., Salozhin, S., Aithozhina, D., Cerchietti, L., Meng, F.G., Augenlicht, L.H., Mariadason, J.M., Hendrich, B.,

Melnick, A., Prokhortchouk, E., Clarke, A., Bird, A., 2006. Kaiso-deficient mice show resistance to intestinal cancer. Mol Cell Biol 26, 199-208.

Qin, L., Qin, S., Zhang, Y., Zhang, C., Ma, H., Li, N., Liu, L., Wang, X., Wu, R., 2014. p120 Modulates LPS-Induced NF- κ B Activation Partially through RhoA in Bronchial Epithelial Cells. Biomed Res Int 2014, 932340.

Raghav, S.K., Waszak, S.M., Krier, I., Gubelmann, C., Isakova, A., Mikkelsen, T.S., Deplancke, B., 2012. Integrative genomics identifies the corepressor SMRT as a gatekeeper of adipogenesis through the transcription factors C/EBPbeta and KAISO. Mol Cell 46, 335-350.

Rao, T.P., Kühl, M., 2010. An updated overview on Wnt signaling pathways: a prelude for more. Circ Res 106, 1798-1806.

Reuter, B.K., Pizarro, T.T., 2009. Mechanisms of tight junction dysregulation in the SAMP1/YitFc model of Crohn's disease-like ileitis. Ann N Y Acad Sci 1165, 301-307.

Reynolds, A.B., Daniel, J., McCrea, P.D., Wheelock, M.J., Wu, J., Zhang, Z., 1994. Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. Mol Cell Biol 14, 8333-8342.

Robine, S., Jaisser, F., Louvard, D., 1997. Epithelial cell growth and differentiation. IV. Controlled spatiotemporal expression of transgenes: new tools to study normal and pathological states. Am J Physiol 273, G759-762.

Rockman, S.P., Currie, S.A., Ciavarella, M., Vincan, E., Dow, C., Thomas, R.J., Phillips, W.A., 2001. Id2 is a target of the beta-catenin/T cell factor pathway in colon carcinoma. J Biol Chem 276, 45113-45119.

Rodilla, V., Villanueva, A., Obrador-Hevia, A., Robert-Moreno, A., Fernández-Majada, V., Grilli, A., López-Bigas, N., Bellora, N., Albà, M.M., Torres, F., Duñach, M., Sanjuan, X., Gonzalez, S., Gridley, T., Capella, G., Bigas, A., Espinosa, L., 2009. Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. Proc Natl Acad Sci U S A 106, 6315-6320.

Rodova, M., Kelly, K.F., VanSaun, M., Daniel, J.M., Werle, M.J., 2004. Regulation of the rapsyn promoter by kaiso and delta-catenin. Mol Cell Biol 24, 7188-7196.

Rubin, D.C., Shaker, A., Levin, M.S., 2012. Chronic intestinal inflammation: inflammatory bowel disease and colitis-associated colon cancer. Front Immunol 3, 107.

Ruzov, A., Dunican, D.S., Prokhortchouk, A., Pennings, S., Stancheva, I., Prokhortchouk, E., Meehan, R.R., 2004. Kaiso is a genome-wide repressor of transcription that is essential for amphibian development. Development 131, 6185-6194.

Ruzov, A., Hackett, J.A., Prokhortchouk, A., Reddington, J.P., Madej, M.J., Dunican, D.S., Prokhortchouk, E., Pennings, S., Meehan, R.R., 2009a. The interaction of xKaiso with xTcf3: a revised model for integration of epigenetic and Wnt signalling pathways. Development 136, 723-727.

Ruzov, A., Savitskaya, E., Hackett, J.A., Reddington, J.P., Prokhortchouk, A., Madej, M.J., Chekanov, N., Li, M., Dunican, D.S., Prokhortchouk, E., Pennings, S., Meehan, R.R., 2009b. The non-methylated DNA-binding function of Kaiso is not required in early Xenopus laevis development. Development 136, 729-738.

Saitou, M., Furuse, M., Sasaki, H., Schulzke, J.D., Fromm, M., Takano, H., Noda, T., Tsukita, S., 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. Mol Biol Cell 11, 4131-4142.

Saleh, M., Trinchieri, G., 2011. Innate immune mechanisms of colitis and colitis-associated colorectal cancer. Nat Rev Immunol 11, 9-20.

Sansom, O.J., Reed, K.R., Hayes, A.J., Ireland, H., Brinkmann, H., Newton, I.P., Batlle, E., Simon-Assmann, P., Clevers, H., Nathke, I.S., Clarke, A.R., Winton, D.J., 2004. Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev 18, 1385-1390.

Sasai, N., Nakao, M., Defossez, P.A., 2010. Sequence-specific recognition of methylated DNA by human zinc-finger proteins. Nucleic Acids Res 38, 5015-5022.

Schneider, M.R., Dahlhoff, M., Horst, D., Hirschi, B., Trülzsch, K., Müller-Höcker, J., Vogelmann, R., Allgäuer, M., Gerhard, M., Steininger, S., Wolf, E., Kolligs, F.T., 2010. A key role for E-cadherin in intestinal homeostasis and Paneth cell maturation. PLoS One 5, e14325.

Schneider, T., Issekutz, A.C., 1996. Quantitation of eosinophil and neutrophil infiltration into rat lung by specific assays for eosinophil peroxidase and myeloperoxidase - Application in a Brown Norway rat model of allergic pulmonary inflammation. Journal of Immunological Methods 198, 1-14.

Shinoda, M., Shin-Ya, M., Naito, Y., Kishida, T., Ito, R., Suzuki, N., Yasuda, H., Sakagami, J., Imanishi, J., Kataoka, K., Mazda, O., Yoshikawa, T., 2010. Early-stage blocking of Notch signaling inhibits the depletion of goblet cells in dextran sodium sulfate-induced colitis in mice. J Gastroenterol 45, 608-617.

Shroyer, N.F., Helmrath, M.A., Wang, V.Y., Antalffy, B., Henning, S.J., Zoghbi, H.Y., 2007. Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis. Gastroenterology 132, 2478-2488.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., Ben-Ze'ev, A., 1999. The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway. Proc Natl Acad Sci U S A 96, 5522-5527.

Shumskaya, V.S., Zhigalova, N.A., Prokhorchouk, A.V., Prokhorchouk, E.B., 2014. Distribution of Kaiso protein in mouse tissues. Histochem Cell Biol.

Smalley-Freed, W.G., Efimov, A., Burnett, P.E., Short, S.P., Davis, M.A., Gumucio, D.L., Washington, M.K., Coffey, R.J., Reynolds, A.B., 2010. p120-catenin is essential for maintenance of barrier function and intestinal homeostasis in mice. J Clin Invest 120, 1824-1835.

Smalley-Freed, W.G., Efimov, A., Short, S.P., Jia, P., Zhao, Z., Washington, M.K., Robine, S., Coffey, R.J., Reynolds, A.B., 2011. Adenoma formation following limited ablation of p120-catenin in the mouse intestine. PLoS One 6, e19880.

Solanas, G., Batlle, E., 2011. Control of cell adhesion and compartmentalization in the intestinal epithelium. Exp Cell Res 317, 2695-2701.

Spring, C.M., Kelly, K.F., O'Kelly, I., Graham, M., Crawford, H.C., Daniel, J.M., 2005. The catenin p120ctn inhibits Kaiso-mediated transcriptional repression of the beta-catenin/TCF target gene matrilysin. Exp Cell Res 305, 253-265.

Stanger, B.Z., Datar, R., Murtaugh, L.C., Melton, D.A., 2005. Direct regulation of intestinal fate by Notch. Proc Natl Acad Sci U S A 102, 12443-12448.

Su, L., Shen, L., Clayburgh, D.R., Nalle, S.C., Sullivan, E.A., Meddings, J.B., Abraham, C., Turner, J.R., 2009. Targeted epithelial tight junction dysfunction causes immune activation and contributes to development of experimental colitis. Gastroenterology 136, 551-563.

Sumagin, R., Robin, A.Z., Nusrat, A., Parkos, C.A., 2014. Transmigrated neutrophils in the intestinal lumen engage ICAM-1 to regulate the epithelial barrier and neutrophil recruitment. Mucosal Immunol 7, 905-915.

Summers, C., Rankin, S.M., Condliffe, A.M., Singh, N., Peters, A.M., Chilvers, E.R., 2010. Neutrophil kinetics in health and disease. Trends Immunol 31, 318-324.

Suzuki, T., 2013. Regulation of intestinal epithelial permeability by tight junctions. Cell Mol Life Sci 70, 631-659.

Swee, M., Wilson, C.L., Wang, Y., McGuire, J.K., Parks, W.C., 2008. Matrix metalloproteinase-7 (matrilysin) controls neutrophil egress by generating chemokine gradients. J Leukoc Biol 83, 1404-1412.

Tang, Y., Clayburgh, D.R., Mittal, N., Goretsky, T., Dirisina, R., Zhang, Z., Kron, M., Ivancic, D., Katzman, R.B., Grimm, G., Lee, G., Fryer, J., Nusrat, A., Turner, J.R., Barrett, T.A., 2010. Epithelial NF-kappaB enhances transmucosal fluid movement by altering tight junction protein composition after T cell activation. Am J Pathol 176, 158-167.

Terzic, J., Grivennikov, S., Karin, E., Karin, M., 2010. Inflammation and colon cancer. Gastroenterology 138, 2101-2114 e2105.

Tetsu, O., McCormick, F., 1999. Beta-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature 398, 422-426.

Thomas, S., Baumgart, D.C., 2012. Targeting leukocyte migration and adhesion in Crohn's disease and ulcerative colitis. Inflammopharmacology 20, 1-18.

Thoreson, M.A., Anastasiadis, P.Z., Daniel, J.M., Ireton, R.C., Wheelock, M.J., Johnson, K.R., Hummingbird, D.K., Reynolds, A.B., 2000. Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. J Cell Biol 148, 189-202.

Tsuchiya, K., Nakamura, T., Okamoto, R., Kanai, T., Watanabe, M., 2007. Reciprocal targeting of Hath1 and beta-catenin by Wnt glycogen synthase kinase 3beta in human colon cancer. Gastroenterology 132, 208-220.

Tunggal, J.A., Helfrich, I., Schmitz, A., Schwarz, H., Günzel, D., Fromm, M., Kemler, R., Krieg, T., Niessen, C.M., 2005. E-cadherin is essential for in vivo epidermal barrier function by regulating tight junctions. EMBO J 24, 1146-1156.

Turner, J.R., 2009. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol 9, 799-809.

Ueo, T., Imayoshi, I., Kobayashi, T., Ohtsuka, T., Seno, H., Nakase, H., Chiba, T., Kageyama, R., 2012. The role of Hes genes in intestinal development, homeostasis and tumor formation. Development 139, 1071-1082.

van Dop, W.A., Uhmann, A., Wijgerde, M., Sleddens-Linkels, E., Heijmans, J., Offerhaus, G.J., van den Bergh Weerman, M.A., Boeckxstaens, G.E., Hommes, D.W., Hardwick, J.C., Hahn, H., van den Brink, G.R., 2009. Depletion of the colonic epithelial precursor cell compartment upon conditional activation of the hedgehog pathway. Gastroenterology 136, 2195-2203.e2191-2197.

van Es, J.H., van Gijn, M.E., Riccio, O., van den Born, M., Vooijs, M., Begthel, H., Cozijnsen, M., Robine, S., Winton, D.J., Radtke, F., Clevers, H., 2005. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. Nature 435, 959-963.

van Roy, F.M., McCrea, P.D., 2005. A role for Kaiso-p120ctn complexes in cancer? Nat Rev Cancer 5, 956-964.

Vandenbroucke, R.E., Vanlaere, I., Van Hauwermeiren, F., Van Wonterghem, E., Wilson, C., Libert, C., 2014. Pro-inflammatory effects of matrix metalloproteinase 7 in acute inflammation. Mucosal Immunol 7, 579-588.

VanDussen, K.L., Carulli, A.J., Keeley, T.M., Patel, S.R., Puthoff, B.J., Magness, S.T., Tran, I.T., Maillard, I., Siebel, C., Kolterud, Å., Grosse, A.S., Gumucio, D.L., Ernst, S.A., Tsai, Y.H., Dempsey, P.J., Samuelson, L.C., 2012. Notch signaling modulates proliferation and differentiation of intestinal crypt base columnar stem cells. Development 139, 488-497.

VanDussen, K.L., Samuelson, L.C., 2010. Mouse atonal homolog 1 directs intestinal progenitors to secretory cell rather than absorptive cell fate. Dev Biol 346, 215-223.

Vanuytsel, T., Senger, S., Fasano, A., Shea-Donohue, T., 2013. Major signaling pathways in intestinal stem cells. Biochim Biophys Acta 1830, 2410-2426.

Vaziri, N.D., Yuan, J., Rahimi, A., Ni, Z., Said, H., Subramanian, V.S., 2012. Disintegration of colonic epithelial tight junction in uremia: a likely cause of CKD-associated inflammation. Nephrol Dial Transplant 27, 2686-2693.

Vereecke, L., Beyaert, R., van Loo, G., 2011. Enterocyte death and intestinal barrier maintenance in homeostasis and disease. Trends Mol Med 17, 584-593.

Vermeulen, J.F., van de Ven, R.A., Ercan, C., van der Groep, P., van der Wall, E., Bult, P., Christgen, M., Lehmann, U., Daniel, J., van Diest, P.J., Derksen, P.W., 2012. Nuclear Kaiso expression is associated with high grade and triple-negative invasive breast cancer. PLoS One 7, e37864.

Wada, K., Nakajima, A., Blumberg, R.S., 2001. PPARgamma and inflammatory bowel disease: a new therapeutic target for ulcerative colitis and Crohn's disease. Trends Mol Med 7, 329-331.

Wahli, W., Michalik, L., 2012. PPARs at the crossroads of lipid signaling and inflammation. Trends Endocrinol Metab 23, 351-363.

Wallace, K.L., Zheng, L.B., Kanazawa, Y., Shih, D.Q., 2014. Immunopathology of inflammatory bowel disease. World J Gastroenterol 20, 6-21.

Wang, Y., Li, L., Li, Q., Xie, C., Wang, E., 2012. Expression of P120 catenin, Kaiso, and metastasis tumor antigen-2 in thymomas. Tumour Biol 33, 1871-1879.

Wang, Y.L., Malik, A.B., Sun, Y., Hu, S., Reynolds, A.B., Minshall, R.D., Hu, G., 2011. Innate immune function of the adherens junction protein p120-catenin in endothelial response to endotoxin. J Immunol 186, 3180-3187.

Weber, C.R., Nalle, S.C., Tretiakova, M., Rubin, D.T., Turner, J.R., 2008. Claudin-1 and claudin-2 expression is elevated in inflammatory bowel disease and may contribute to early neoplastic transformation. Lab Invest 88, 1110-1120.

Wehkamp, J., Salzman, N.H., Porter, E., Nuding, S., Weichenthal, M., Petras, R.E., Shen, B., Schaeffeler, E., Schwab, M., Linzmeier, R., Feathers, R.W., Chu, H., Lima, H., Fellermann, K., Ganz, T., Stange, E.F., Bevins, C.L., 2005. Reduced Paneth cell alpha-defensins in ileal Crohn's disease. Proc Natl Acad Sci U S A 102, 18129-18134.

Wullaert, A., Bonnet, M.C., Pasparakis, M., 2011. NF-κB in the regulation of epithelial homeostasis and inflammation. Cell Res 21, 146-158.

Xavier, R.J., Podolsky, D.K., 2007. Unravelling the pathogenesis of inflammatory bowel disease. Nature 448, 427-434.

Xiao, K., Oas, R.G., Chiasson, C.M., Kowalczyk, A.P., 2007. Role of p120-catenin in cadherin trafficking. Biochim Biophys Acta 1773, 8-16.

Yamada, S., Pokutta, S., Drees, F., Weis, W.I., Nelson, W.J., 2005. Deconstructing the cadherin-catenin-actin complex. Cell 123, 889-901.

Yang, Q., Bermingham, N.A., Finegold, M.J., Zoghbi, H.Y., 2001. Requirement of Math1 for secretory cell lineage commitment in the mouse intestine. Science 294, 2155-2158.

Yang, R., Bie, W., Haegebarth, A., Tyner, A.L., 2006. Differential regulation of D-type cyclins in the mouse intestine. Cell Cycle 5, 180-183.

Yoon, H.G., Chan, D.W., Reynolds, A.B., Qin, J., Wong, J., 2003. N-CoR mediates DNA methylation-dependent repression through a methyl CpG binding protein Kaiso. Mol Cell 12, 723-734.

Zanello, G., Kevans, D., Goethel, A., Silverberg, M., Tyler, A., Croitoru, K., 2014. Genetics and innate and adaptive immunity in IBD. Nestle Nutr Inst Workshop Ser 79, 41-55.

Zecchini, V., Domaschenz, R., Winton, D., Jones, P., 2005. Notch signaling regulates the differentiation of post-mitotic intestinal epithelial cells. Genes Dev 19, 1686-1691.

Zhang, Y.Z., Li, Y.Y., 2014. Inflammatory bowel disease: pathogenesis. World J Gastroenterol 20, 91-99.

Zheng, X., Tsuchiya, K., Okamoto, R., Iwasaki, M., Kano, Y., Sakamoto, N., Nakamura, T., Watanabe, M., 2011. Suppression of hath1 gene expression directly regulated by hes1 via notch signaling is associated with goblet cell depletion in ulcerative colitis. Inflamm Bowel Dis 17, 2251-2260.

APPENDIX 1: KAISO OVEREXPRESSION LEADS TO LOWER WEIGHT GAIN AND FAT:BODY WEIGHT RATIO IN MICE.

Preface:

This appendix summarizes other preliminary studies performed during our initial characterization of Kaiso transgenic mice. I performed all the experiments, and Dr. Daniel and I contributed intellectually towards the experimental design.

Upon characterization of the Kaiso transgenic mice, *Kaiso*$^{Tg/+}$, it was observed that they were smaller and weighed less compared to their non-Tg siblings. Further examination revealed that the *Kaiso*$^{Tg/+}$ males had a lower abdominal fat:body weight ratio, suggesting that the mice may have a nutrient uptake defect. Since we subsequently found that *Kaiso*$^{Tg/+}$ mice had increased intestinal secretory cells, this may be contributing to the low weight gain. Since Kaiso has been implicated in mediating the adipocyte differentiation factor, PPARγ, we examined PPARγ expression levels in this mouse model to determine if that could explain the decreased fat:body weight ratio observed. However, we obtained inconsistent results between PPARγ mRNA and protein levels, and thus Kaiso's regulation of PPARγ in the murine intestines of our mouse model remains unclear and requires further clarification.

Revisions were made to the appendix to maintain formatting consistency with other chapters. Significant revisions include elimination of methods section (details in Chapter 2), and removal of reference list (included in combined thesis reference list).

A.1.1 Abstract:

Recently, the unique BTB/POZ transcription factor Kaiso was shown to regulate adipogenesis via interaction with the co-repressor SMRT *in vitro*. One pro-adipogenesis gene regulated was PPARγ, which, in addition to regulating adipogenesis, is also known to regulate intestinal inflammation. Interestingly, our recently generated Kaiso overexpressing transgenic mice (*Kaiso*$^{Tg/+}$) exhibited increased intestinal inflammation and this raised the possibility that the Kaiso inflammatory phenotype was mediated via PPARγ. To further elucidate the mechanism of inflammation in the *Kaiso*$^{Tg/+}$ mice, PPARγ expression levels were evaluated. *Kaiso*$^{Tg/+}$ mice weighed less and had a lower fat:body weight ratio than non-transgenic mice, supporting a role for Kaiso in regulating adipogenesis. PPARγ protein expression was also decreased in *Kaiso*$^{Tg/+}$ mice, though *PPARγ* mRNA expression suggests an increase in the *Kaiso*$^{Tg/+}$ mice. Due to the discrepancy between PPARγ protein and mRNA expression levels, it is currently difficult to make conclusions about PPARγ's role in regulating inflammation and adipogenesis in the *Kaiso*$^{Tg/+}$ mice.

A.1.2 Introduction:

Kaiso, a BTB-POZ family transcription factor, plays an important role in vertebrate development, as best depicted in the *Xenopus* model system (Kim *et al*., 2002; Park *et al*., 2005; Ruzov *et al*., 2004). To elucidate Kaiso's role in mammalian development, Kaiso-null mice were created and found to be viable and fertile with no gross morphological defects (Prokhortchouk *et al.*, 2006) despite Kaiso expression normally being present in multiple tissues (Shumskaya *et al.*, 2014). The lack of developmental defects in Kaiso-null mice was attributed to expression of the Kaiso-like proteins, ZBTB4 and ZBTB38, which may have redundant roles (Prokhortchouk *et al.*, 2006). Thus, Kaiso overexpressing transgenic (*Kaiso*$^{Tg/+}$) mice were created to study Kaiso's role in development and WNT signalling in the context of murine intestines (Chaudhary *et al.*, 2013). *Kaiso*$^{Tg/+}$ mice exhibited enlarged crypts and increased numbers of secretory cells, reminiscent of inhibition of Notch signalling (Chaudhary *et al.*, 2013). Additionally, *Kaiso*$^{Tg/+}$ mice also developed spontaneous intestinal inflammation but the mechanism is yet to be elucidated (Chaudhary *et al.*, 2013).

Kaiso, like other BTB/POZ transcription factors, possesses an N-terminal POZ domain that mediates homo and heterodimeric protein-protein interactions (Kelly and Daniel, 2006). Kaiso functions primarily as a transcriptional repressor that recruits co-repressor complexes like HDAC, SMRT, and NCoR (Daniel and Reynolds, 1999; Kelly and Daniel, 2006; Park *et al*., 2005; Prokhortchouk *et al*., 2001; Spring *et al*., 2005; Yoon *et al*., 2003). A recent study suggests a role for Kaiso in adipogenesis via interactions with SMRT in 3T3-L1 preadipocytes *in vitro* (Raghav *et al.*, 2012). Using ChIP-seq and

bioinformatics, it was found that Kaiso tethers SMRT to promoter proximal sites of active genes in 3T3-L1 cells (Raghav *et al.*, 2012). Depletion of both Kaiso and SMRT resulted in enhanced adipogenesis via upregulation of adipogenesis genes, including **p**eroxisome **p**roliferator-**a**ctivated **r**eceptor-γ (PPARγ) (Raghav *et al.*, 2012).

PPARγ is deemed the master regulator of adipocyte differentiation and function (Ahmadian *et al.*, 2013; Barak *et al.*, 1999; Farmer, 2005). In 3T3-L1 cells, **C**CAAT/**e**nhancer **b**inding **p**rotein (C/EBP) expression increases upon adipocyte differentiation, increasing the transcription of *PPARγ*, which consequently activates C/EBPs expression in a positive feedback loop (Farmer, 2005). Together, PPARγ and C/EBPs modulate expression of multiple pro-adipogentic target genes (Ahmadian *et al.*, 2013; Farmer, 2005).

In addition to its role in adipocyte differentiation, PPARγ also plays a significant role in modulating inflammatory responses (Annese *et al.*, 2012; Dubuquoy *et al.*, 2006; Wahli and Michalik, 2012). PPARγ binds to the NFκB transcription factor and inhibits the NFκB downstream pro-inflammatory signalling cascade (Dubuquoy *et al.*, 2006; Wada *et al.*, 2001). In the intestines, depletion of PPARγ in epithelial cells results in increased susceptibility to DSS-induced colitis (Mohapatra *et al.*, 2010). Interestingly, these mice also exhibited greater leukocyte infiltration and mucosal wall thickening (Mohapatra *et al.*, 2010). Microbial infection induces acute colitis by inhibiting PPARγ expression in intestinal epithelial cells and elevates the host innate immune response

(Kundu *et al.*, 2014). Together, these data demonstrate a role for PPARγ in regulating intestinal inflammation.

Since Kaiso transgenic mice elicited an inflammatory bowel disease phenotype, we sought to further understand the underlying mechanism of the inflammation. During initial characterization, the *Kaiso*$^{Tg/+}$ mice were observed to be smaller than their age-matched non-Tg siblings. Indeed, *Kaiso*$^{Tg/+}$ mice were born with similar weights as their non-Tg siblings; however they gained less weight over time. Additionally, *Kaiso*$^{Tg/+}$ mice had lower fat:body weight ratios, suggesting that Kaiso overexpression affected nutrient uptake. *Kaiso*$^{Tg/+}$ mice expressed less PPARγ protein was observed in the intestines, although *PPARγ* mRNA expression was modestly increased. Together, these data support an *in vivo* role of Kaiso in regulating inflammation perhaps via the regulation of PPARγ.

A.1.3 Results

A.1.3.1 Kaiso overexpression in the intestine yields smaller mice

During our phenotypic analysis of *Kaiso*$^{Tg/+}$ mice, we observed that Line A mice were smaller compared to their non-Tg siblings. However this difference was not evident in Line B mice (Figure A1.1 A). Indeed, weights at death of Line A *Kaiso*$^{Tg/+}$ mice were significantly lower than their age-matched non-Tg siblings, but Line B *Kaiso*$^{Tg/+}$ mice exhibited no such difference (Figure A1.1 B). The discrepancy of weight difference between the two lines may be attributed to the difference in their Kaiso expression levels; Line A *Kaiso*$^{Tg/+}$ mice have a higher copy number of *Kaiso* transgene and thus express more Kaiso compared to Line B mice (Chaudhary *et al.*, 2013).

In an attempt to determine whether this weight difference was the result of a lower birth weight, Line A pups were weighed monthly from birth up to ~11 months (330 days) of age (Figure A1.1C). No difference in birth weights was observed between *Kaiso*$^{Tg/+}$ mice (n=8) and their non-Tg siblings (n=5), and both genotypes gained weight at a similar rate up to 120 days. After 120 days, the growth rate of the *Kaiso*$^{Tg/+}$ mice slowed significantly when compared to either age-matched non-Tg siblings (Figure A1.1C). Interestingly, gross morphological analysis of lower abdominal fat pads in male *Kaiso*$^{Tg/+}$ mice revealed a lower fat:body weight ratio compared to their non-Tg siblings (Figure A1.1D). No significant change in fat:body weight ratio was observed in *Kaiso*$^{Tg/+}$ females, and this was likely due to additional fat in the female mammary fat pads.

A.1.3.2 Kaiso overexpression results in altered PPARγ expression

Kaiso was recently shown to inhibit terminal adipogenesis in conjunction with the SMRT corepressor via its regulation of PPARγ (Raghav *et al.*, 2012). As PPARγ also plays a protective role in intestinal inflammation and in murine models of IBD (reviewed in Annese *et al.*, 2012) and the *Kaiso*$^{Tg/+}$ mice also exhibit intestinal inflammation, we investigated PPARγ expression levels to determine if PPARγ dysfunction could explain the Kaiso-induced intestinal inflammation and low body weight. We detected reduced PPARγ expression in the small and large intestines of both Line A and Line B *Kaiso*$^{Tg/+}$ mice via IHC (Figure A1.2A). However, qRT-PCR analysis revealed an increase in *PPARγ* transcripts in *Kaiso*$^{Tg/+}$ tissues, though not significant (Figure A1.2B). More experiments need to be performed to clarify whether PPARγ contributes to the observed *Kaiso*$^{Tg/+}$ weight and inflammatory phenotypes.

A.1.4 Discussion

Previous characterization of *Kaiso*$^{Tg/+}$ mice with intestinal-specific Kaiso overexpression revealed gross morphological changes including enlarged crypts, increased number of secretory cells and increased inflammation (Chaudhary *et al.*, 2013). Further characterization of the *Kaiso*$^{Tg/+}$ mice revealed smaller mice with lower body weights in Line A *Kaiso*$^{Tg/+}$ although Line B mice showed no such differences. Since *Kaiso*$^{Tg/+}$ mice have an increased number of secretory cells, the number of enterocytes involved in nutrient uptake may be reduced and cause a decrease in weight gain upon Kaiso overexpression. The lower weight gain cannot be attributed to the inflammatory phenotype of the *Kaiso*$^{Tg/+}$ mice, as inflammation is not evident in Line A mice until after 6 months of age (Chapter 4).

Our finding that *Kaiso*$^{Tg/+}$ mice weighed significantly less than their non-Tg siblings, with a lower fat to body ratio provides *in vivo* support to the findings of Raghav *et al.* that Kaiso may regulate adipogenesis via regulation of *PPARγ* (Raghav *et al.*, 2012). The *Kaiso*$^{Tg/+}$ mice also develop intestinal inflammation (Chaudhary *et al.*, 2013), and PPARγ has been shown to play a protective role in intestinal inflammation and in murine models of IBD (Annese *et al.*, 2012). In fact, PPARγ, when activated, inhibits the NFκB pathway resulting in an inhibition of cytokines, and inflammatory cell recruitment into inflamed tissues (Dubuquoy *et al.*, 2006). This is in support of the observation that *Kaiso*$^{Tg/+}$ mice exhibit increased inflammation similar to that seen in IBD patients including massive leukocyte infiltration into the intestinal lamina propria (Chapter 4). Indeed, PPARγ expression is reduced in both the large and small intestines of the

Kaiso$^{Tg/+}$ mice, supporting PPARγ's protective function. However, *PPARγ* mRNA levels are increased, albeit not significantly, in *Kaiso*$^{Tg/+}$ mice. The increase in transcript levels may not necessarily translate to an increase in protein levels, though further studies are needed to understand PPARγ's functionality (effects on PPARγ targets) in the *Kaiso*$^{Tg/+}$ mice.

Overall, given that Kaiso negatively regulates adipogenesis, a PPARγ-mediated process, and that PPARγ protects against inflammation, it is tempting to hypothesize that Kaiso's pro-inflammatory effects may also be regulated via inhibition of PPARγ activity in the gut.

Figure A1.1: Line A Kaiso transgenic mice are smaller at 6-12 months of age.

(A) Kaiso transgenic (VK) from Line A are smaller in size when compared to non-transgenic (NT) siblings at 6-12 months, while Line B VK mice show no difference. **(B)** Line A *Kaiso*$^{Tg/+}$ mice (n=10) weigh significantly less compared to their Non-Tg siblings (n=12), while Line B mice (n=10) show no difference. **(C)** Line A *Kaiso*$^{Tg/+}$ mice (n=8) were weighed from birth and compared to their Non-Tg siblings (n=5). *Kaiso*$^{Tg/+}$ mice gained weight at a similar rate as their Non-Tg siblings; however they have less weight gain after 120 days. **(D)** *Kaiso*$^{Tg/+}$ males (n=8) from Line A have decreased abdominal fat:body weight ratio compared to non-Tg males (n=6), while no differences were observed in females of both genotypes (n=7).

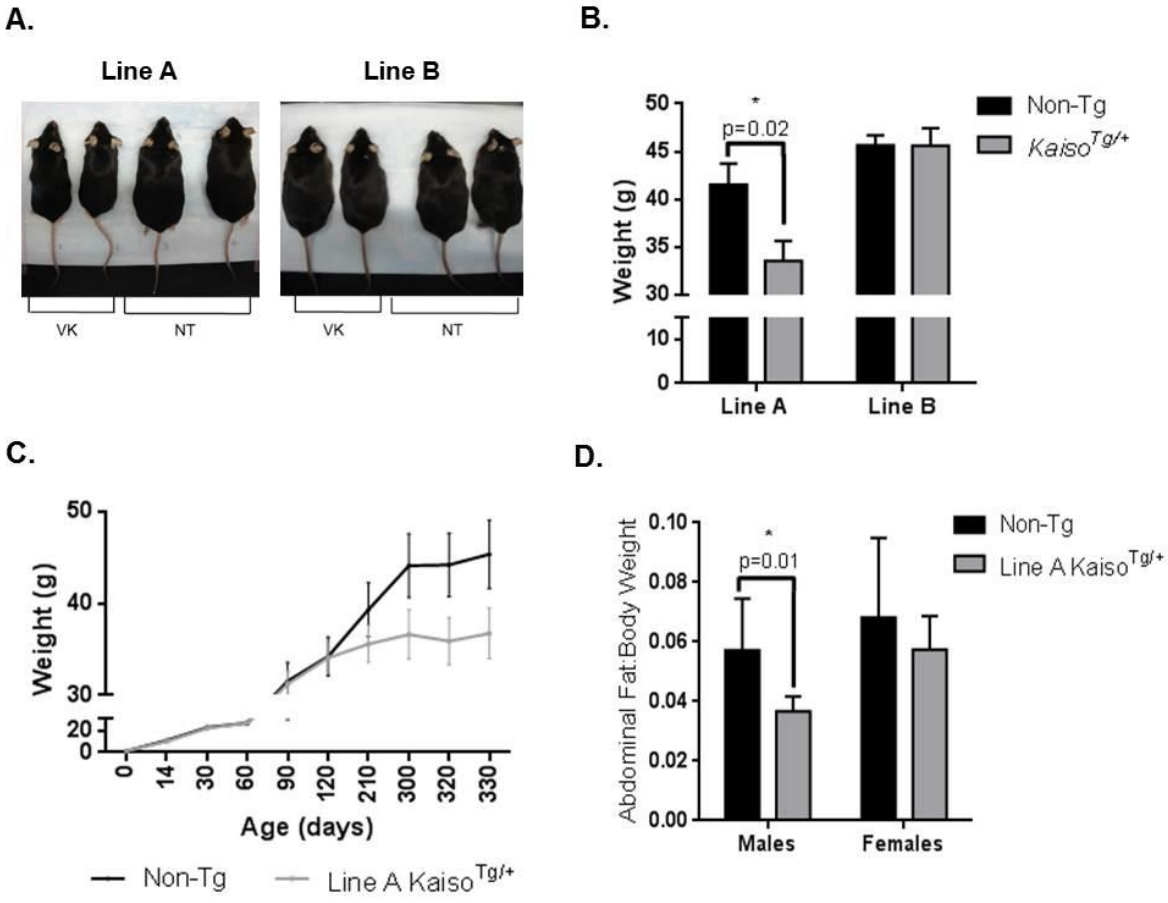
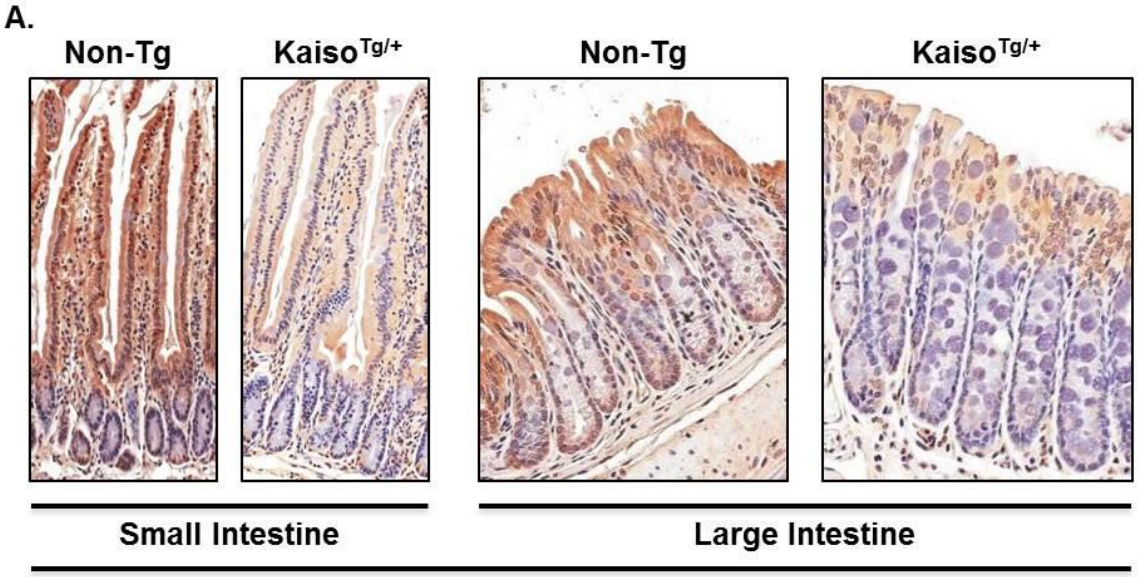
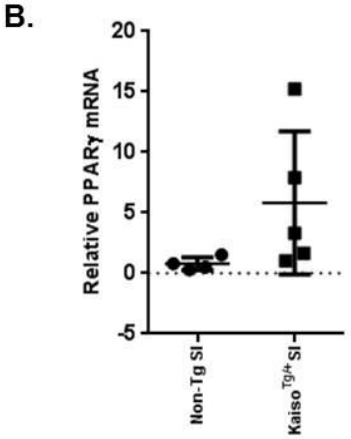

A.
Line A
Line B
VK
NT
VK
NT
B.
Weight (g)
p=0.02
Non-Tg
Kaiso Tg/+
Line A
Line B
C.
Weight (g)
Age (days)
Non-Tg
Line A Kaiso Tg/+
D.
Abdominal Fat:Body Weight
p=0.01
Non-Tg
Line A Kaiso Tg/+
Males
Females

Figure A1.2: PPARγ protein expression is decreased while PPARγ mRNA is increased in Kaiso transgenic mice.

(A) PPARγ expression was detected in both small and large intestines, with nuclear expression evident in the villi of the small intestine and the crypts of the large intestines in the Non-Tg siblings. On the other hand, *Kaiso*$^{Tg/+}$ mice display an overall reduction of PPARγ staining in both small and large intestines. **(B)** PPARγ mRNA levels are modestly increased in *Kaiso*$^{Tg/+}$ mice.


A.
Non-Tg
Kaiso Tg/+
Non-Tg
Kaiso Tg/+
Small Intestine
Large Intestine
PPARγ


B.
Relative PPARγ mRNA
20
15
10
5
0
-5
Non-Tg SI
Kaiso Tg/+ SI

APPENDIX 2: KAISO OVEREXPRESSION IN MURINE KIDNEY TISSUES

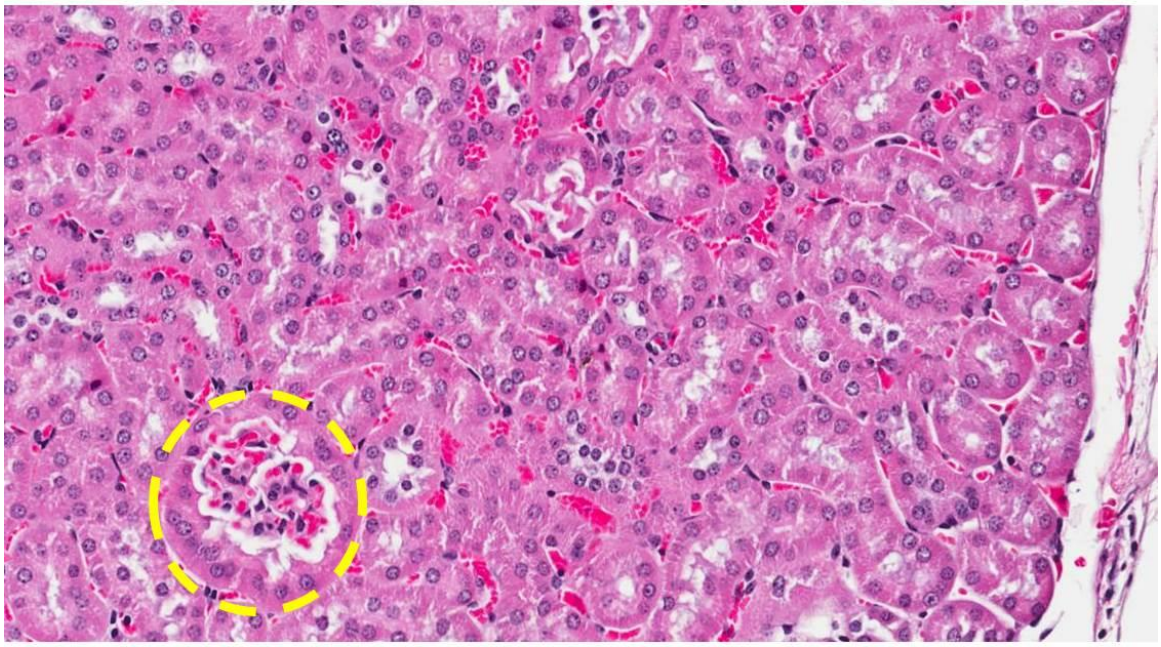
Preface:

The villin promoter used to target Kaiso expression to murine intestines also results in transgene expression in the kidney (Robine *et al.*, 1997). Thus we examined our *Kaiso*$^{Tg/+}$ mice to confirm specificity of the villin promoter in the kidneys. Kaiso overexpression in kidneys was confirmed using IHC and Kaiso-specific antibodies. Gross morphological analysis of *Kaiso*$^{Tg/+}$ kidneys revealed no differences compared to non-Tg siblings (Figure A2.1) although Kaiso expression was increased in some cells (Figure A2.2). The complete characterization of Kaiso overexpression in kidneys was however beyond the scope of this study.

Figure A2.1: Kaiso overexpression does not alter gross morphology of murine kidneys

The Kaiso transgenic mice do not show any gross morphological differences in the cortex of the kidneys compared to the non-Tg siblings. (glomerulus = yellow dotted circle).

Non-Tg Kidney



Kaiso$^{Tg/+}$ Kidney

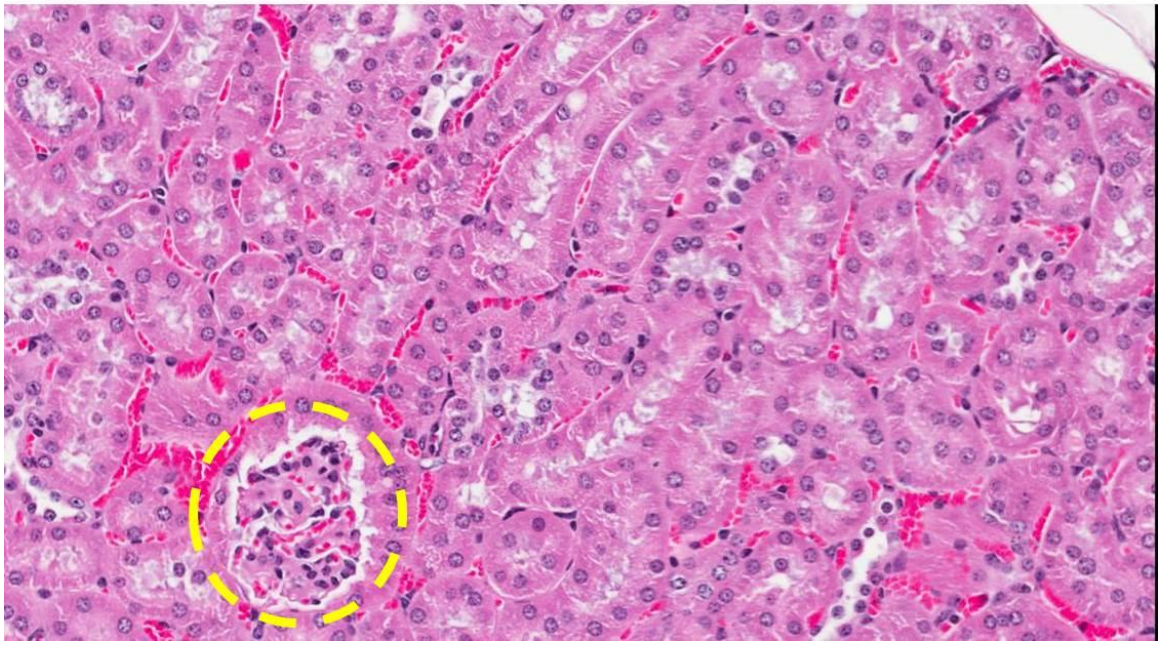
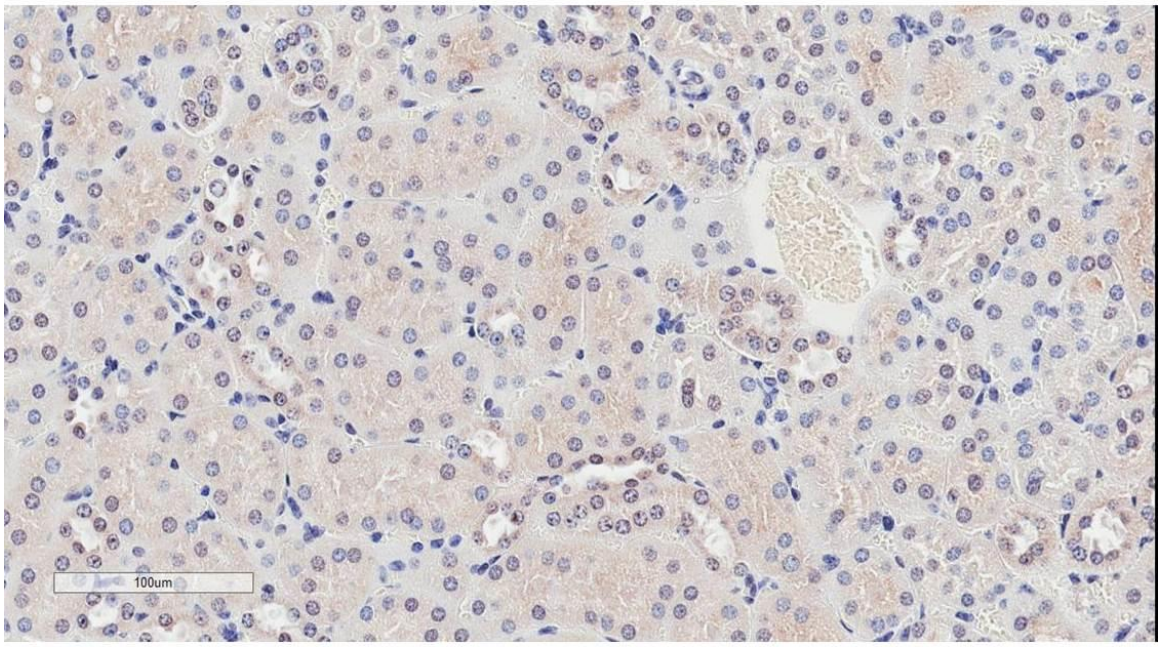


Figure A2.2: Kaiso is not overexpressed in all epithelial cells of the kidney cortex

Kaiso transgenic mice display a strong nuclear Kaiso expression in kidney epithelial cells of the cortex (black arrow) compared to the non-Tg siblings. However, this Kaiso expression was not observed in all cells of the Kaiso transgenic mice (yellow arrowhead).

Non-Tg Kidney



Kaiso$^{Tg/+}$ Kidney