# THE CHARACTERIZATION OF KAISO TRANSGENIC MICE

**Characterization of Carcinogen-treated Kaiso Transgenic ($K^{Tg}$) Mice**

**By**

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Abstract:

Colitis-associated cancer (CAC) is a poorly characterized subgroup of colorectal cancers (CRC) that afflicts ~20% patients suffering from inflammatory bowel disease (IBD). The limited understanding of CAC stems from the lack of suitable mammalian model systems, as well as a general gap in knowledge regarding the molecular mechanisms of this disease. Currently, colitis is modelled by the use of the detergent dextran sodium sulfate (DSS) to induce inflammation in the intestines of mice. Studies have shown that increased expression of the transcription factor Kaiso causes intestinal inflammation and early-stage tumorigenesis in mice, even without additional intestinal insult. This inflammatory progression mimics the beginnings of CAC in humans, and we postulate that with a "second-hit" caused by a carcinogen such as azoxymethane (AOM), the mice will cross the threshold from inflammation to carcinogenesis.

Wildtype (WT), *Kaiso*$^{Tg}$ mice, and APC$^{min/+}$/*Kaiso*$^{Tg}$ crossed mice were exposed to various combinations of the pro-inflammatory detergent DSS, the carcinogen AOM and the general anti-inflammatory, aspirin. Intestinal tissues were collected for gross morphological assessment, polyp quantification and Immunohistochemistry (IHC) analysis, in order to determine the relative expression level and localization of pro-inflammatory and tumorigenic proteins. We hypothesized that exposure to DSS or AOM will exacerbate Kaiso-mediated intestinal inflammation and lead to colitis-associated cancer (i.e. polyp formation), while aspirin will rescue the APC$^{min/+}$/*Kaiso*$^{Tg}$ accelerated tumour forming phenotype. *Kaiso*$^{Tg}$ treated with AOM or DSS exhibited an impeded weight gain phenotype, extensive intestinal hyperplasia and altered gene expression. IHC analysis revealed that two key adhesion proteins, p120$^{ctn}$ and E-Cadherin exhibit aberrant expression and localization in *Kaiso*$^{Tg}$, independent of treatment. Additionally, it was observed that AOM treatment and Kaiso overexpression work synergistically to produce an ectopic expression profile for the proliferation marker, Ki67. Together these finding suggest a role for Kaiso in intestinal inflammation, cancer initiation via altered proliferation, and the destabilization of adherens junctions, leading to a compromised intestinal epithelial barrier.

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Hebrews 6:19: "We have this hope, as an anchor for the soul"

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ACF | **Aberrant Crypt Foci** |
| AOM | **Azoxymethane** |
| APC | **Adenomatous Polyposis Coli** |
| BSA | **Bovine Serum Albumin** |
| BTB/POZ-ZF | **Zinc finger and broad-complex tramtrack and bric-a-brac/poxvirus and zinc finger** |
| CD | **Crohn's Disease** |
| CRC | **Colorectal Cancer** |
| CAC | **Colitis-Associated Cancer** |
| DAB | **3'-Diaminobenzidine** |
| DSH | **Dishevelled** |
| DSS | **Dextran Sodium Sulfate** |
| EMT | **Epithelial-to-Mesenchymal Transition** |
| IHC | **Immunohistochemistry** |
| IBD | **Inflammatory Bowel Disease** |
| ISC | **Intestinal Stem Cell** |
| LI | **Large Intestine** |
| LP | **Lamina Propria** |
| LSB | **Laemmli Sample Buffer** |
| PBS | **Phosphate-Buffered Saline** |
| ROS | **Reactive Oxygen Species** |
| RNI | **Reactive Nitrogen Intermediates** |
| SI | **Small intestine** |
| TBS | **Tris-Buffered Saline** |
| TCF | **Transcription Factor** |
| UC | **Ulcerative Colitis** |
| VK | **Villin-Kaiso** |
| WT | **Wildtype** |

# 1. INTRODUCTION

## 1.1.1 The Intestinal Tract

The mammalian intestinal tract consists of four main sections: the duodenum, the jejunum, the ileum and the colon [1, 2]. The duodenum, jejunum and ileum comprise the small intestine, while the colon makes up the majority of the large intestine. The small intestine and the large intestine (colon) serve separate and distinct functions, but both share the important general responsibility of digesting food, as well as absorbing nutrients and preparing the waste for excretion [3, 4]. Comparatively, the small intestine is 3-4 times longer than the colon on average and acts as an enzyme reservoir which facilitates much of the food digestion and nutrient absorption. In contrast the large intestine is mainly responsible for waste storage, fluid re-absorption and salt recovery [1, 2, 5, 6].

Morphologically, the small and large intestines are similar; both consist of deep folds known as crypts of Lieberkühn. However, the small intestine utilizes finger-like protrusions known as villi, which serve to greatly increase the intestinal surface area, and facilitate efficient nutrient absorption [1, 3]. These villi vary in size throughout the intestinal tract, starting very long and flexible in the duodenum, and becoming shorter and hardier towards the terminal ilium. Both large and small intestinal tissues regenerate rapidly from crypt-housed **I**ntestinal **S**tem **C**ells (ISC), which duplicate to produce a transit-amplifying cell that will differentiate into one of 4 major cell types as it migrates up to the villi tips [2, 7]. The original ISC remains at the base of the crypt for further proliferation and will divide approximately once per day [5]. The progenitor cells that migrate up the villus will differentiate into either absorptive cells, known as enterocytes, or one of three secretory cell types: goblet, enteroendocrine or Paneth cells [7, 8]. Paneth cells are the only differentiated cell type that migrates downward towards the base of the crypt and they help contribute to immune responses by releasing anti-microbial and regulatory compounds against foreign antigens [1, 3].

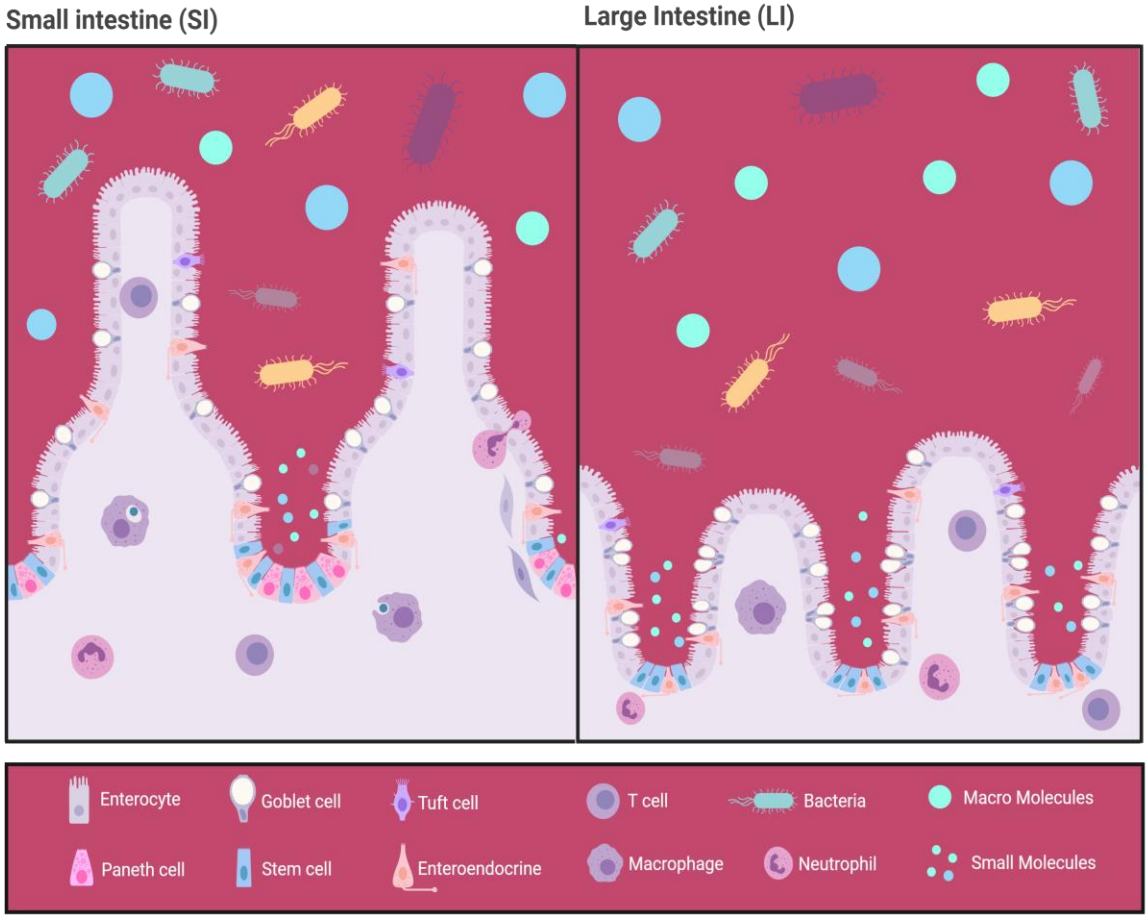



***Figure 1: Intestinal Morphology.*** *Both the small intestine and colon utilize crypts for housing intestinal stem cells, which duplicate and ascend upward towards the luminal surface. Midway up the crypt, one stem cell becomes a transit-amplifying cell while the other daughter cell returns to the crypt base for further replication. The transit-amplifying cell differentiates into one of the 4 cell fates and continues to rise in a conveyer belt fashion as more and more cells are born, pushing the older cells up towards the lumen. In the colon, cells die once they reach the lumen, but in the small intestine, cells continue to ascend the villi, until they eventually reach the top and undergo cell death when they shed from the epithelium. These cells undergo the highest turnover of any fixed cells in the body, living only 5-7 days per cell at the longest.*

Enterocytes are intestinal absorptive cells and function as reuptake vessels that may also secrete immunoglobulins [2]. Goblet cells are glandular epithelial cells that secrete mucin, which is a major component of intestinal mucus. Enteroendocrine cells release hormones and signal peptides in response to specific stimuli [3]. As these cells migrate towards the apical surface, they eventually reach the villus tip and are sloughed off into the lumen, to be replaced every 3-4 days by the constantly proliferating stem cells in the crypt base. In situations of de-regulated intestinal homeostasis, there is an increased risk of areas of uncontrolled cell proliferation (polyps) forming, which are the precursors for intestinal carcinoma [3, 9, 10].

### 1.1.2 Intestinal Inflammation

The intestinal epithelium serves an additional purpose in that it forms a physical barrier between the ***L****amina* ***P****ropria* (LP) and the intestinal lumen. This semi-permeable epithelium prevents foreign pathogens and environmental toxins from entering the body through the gut, but it allows fluids, nutrients and hormone signals to pass freely through the epithelial barrier [9, 11, 12]. In cases of dysfunctional cell-cell adhesion, the barrier can become "leaky", allowing unfamiliar pathogens and other intestinal irritants into the lamina propria and ultimately the interior of the body, where they can cause infection and inflammation. Inflammation is a natural immune response that causes multiple homeostatic processes in response to certain stimuli, often mediated by both innate and adaptive immune cells (e.g. neutrophils and T-cells respectively) [11, 13-15]. Inflamed tissues may present in the form of hot, red, and/or disfigured flesh. These symptoms are typically caused by secreted cytokines that recruit neutrophils to the site, and facilitate angiogenesis so that additional blood vessels may be utilized to aid repair of the damaged tissue [16, 17]. Infection, physical insult, and chemical imbalance can all lead to inflammation throughout the body. When the epithelium of the colon is inflamed, it is called colitis, and is often accompanied by several tell-tale cellular and physiological phenomena. In an inflamed patch of tissue in the intestinal tract

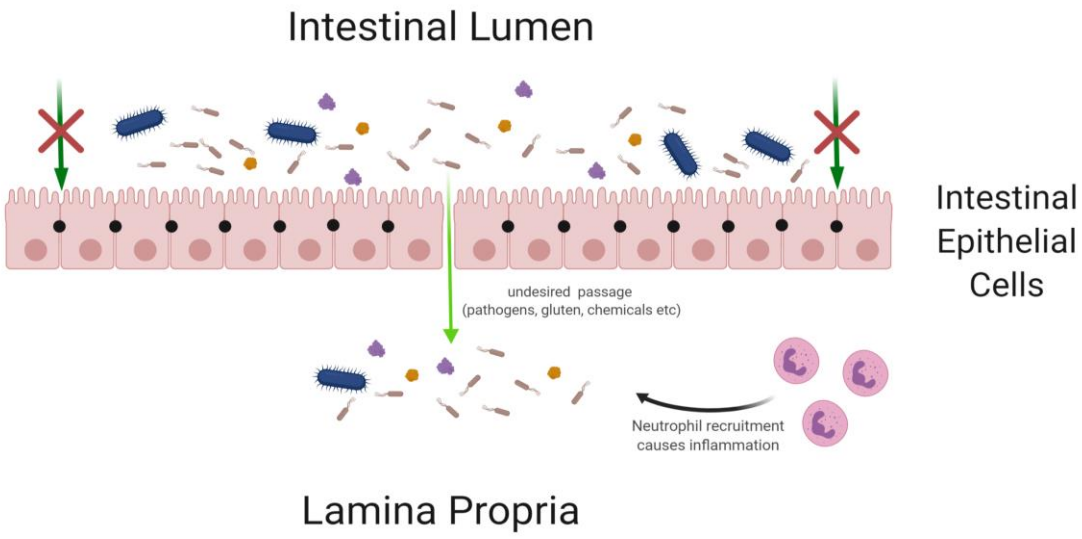



***Figure 2: Intestinal epithelial barrier leakage allows for microbial infection.*** *The intestinal barrier typically forms an impenetrable wall, preventing invasion of undesired pathogens and particles from the intestinal lumen into the Lamina Propria (LP)*

(i.e. an ulcer), there are significantly more secretory cells present than in healthy intestines, with a lower abundance of epithelial cells in the crypts and villi.

This shift in cell fate is driven by inhibition of the Notch signaling pathway, and leads to an overabundance of goblet, enteroendocrine and Paneth cells, preventing absorptive cells from populating the epithelium, and reducing nutrient retention [1, 2, 12]. Additionally, the LP of the ulcer often displays signs of neutrophil invasion, which can lead to Peyer's patches and other forms of tissue modification [11, 18]. When intestinal tissues undergo intense inflammation, several physiological structures begin to break down; the villi will become especially fragile and begin to blunt and fuse with their neighbours. Additionally, crypts begin to expand and extend, forming masses of inflamed tissue that cannot perform their normal epithelial function [3, 5, 10, 11, 18-20].

Sustained inflammation can cause chronic inflammatory diseases, such as Inflammatory Bowel Disease (IBD). This disease is a catch-all term for chronic inflammatory afflictions of the gastrointestinal (GI) tract and includes disorders such as **C**rohn's **D**isease (CD) and **U**lcerative **C**olitis (UC) [14, 18, 20]. UC presents as continuous areas of inflamed tissue throughout the distal colon, whereas CD presents as discontinuous inflamed patches throughout the entirety of the GI tract [21]. The specific etiology of IBD largely remains a mystery, but researchers and clinicians agree that contributing factors include a multitude of environmental and genetic factors, e.g. diet/lifestyle, gut microbiome homeostasis and the presence of mutations in key tumour suppressor encoding genes such as APC, E-Cadherin and multiple anti-inflammatory cytokines [18, 20, 22]. Importantly, IBD predisposes the patient to a specific form of inflammation-initiated cancer known as **c**olitis-**a**ssociated **c**ancer (CAC). IBD is an ailment most commonly diagnosed in young Caucasian people, with over 25% of diagnoses occurring in adolescents below the age of 16, but can occur at any age, in any cohort [23, 24]. Additionally, IBD is grossly over-represented in the Western world, with Canada and the U.S.A suffering the highest penetrance of 0.7% and 1.3% of their populations

respectively [24]. With increased prevalence of the Western lifestyle (high fat, sugar, salt, protein and alcohol in the diet combined with a sedentary way of life), IBD pervasiveness has consistently increased throughout the developed and developing worlds at an alarming rate, applying pressure to IBD researchers in their pursuit of therapeutics. While little is known regarding the etiology of IBD, even less is known about the transition between IBD and CAC in affected individuals [5, 10, 11, 18, 20, 21, 23, 24].

### 1.2.1 Colorectal Cancer and the Colon

Individuals who have suffered from IBD for more than 30 years have an ~20% higher chance of developing **C**olo**r**ectal **c**ancer (CRC) and more specifically CAC [18]. CRC is the third most commonly diagnosed cancer worldwide, with the majority of cases appearing in developed and developing countries, especially in the Western world; CRC is the third most common cause of cancer death in women, and the second most common in men [9, 18]. CRC progression is heavily influenced by environmental risk factors such as age and lifestyle, with a weak genetic component. CRC can be promoted in humans who are exposed to a Western diet, have a sedentary lifestyle, or abuse alcohol and tobacco [18]. Although only 2% of all CRC cases can be linked with previous IBD, the rate of death resulting from CRC in IBD patients can be as high as 15%.

The colon's primary function is to extract water and any remaining nutrients from feces that are being stored and eventually transported to the rectum, imposing a harsh environment on the epithelial cells [2, 3]. Due to constant damage and dehydration, the intestinal lining (epithelial cell layer) is continuously shed and renewed via stem cells housed at the base of the crypts [25]. When these ascending cells reach the top or luminal side of the intestinal epithelial layer, they generally undergo a specific type of apoptosis, known as anoikis, to prevent an over-accumulation of cells [26, 27]. However, in CRC, the cells do not enter cell cycle arrest and continue to multiply without properly migrating up the crypts and into the villi, causing an over-accumulation of

proliferating epithelial cells which may ultimately result in formation of **a**berrant **c**rypt **f**oci (ACF). ACFs are thought to be precursors to intestinal polyps known as colonic adenomas, which may undergo additional mutations that lead to the development of full-blown colon carcinoma [28]. In general, CRC follows a multi-stage tumour progression that is marked by the loss, or activation, of certain cancer-related proteins. For example, a healthy intestinal epithelium will progress to a hyperplastic epithelium following a loss of the adenomatous polyposis coli (APC) protein [22, 29, 30]. Upon DNA hypomethylation of several key oncogenes, the hyperplasia progresses further to an early adenoma. Cells in an early adenoma may experience additional activating mutations in the *K-ras* oncogene and evolve further into an intermediate adenoma. Finally, once the p53 tumour suppressor is lost, an adenoma will progress to a carcinoma, and may become free to metastasize and invade distant tissues [28, 31, 32].

### 1.2.2 Colitis-Associated Cancer

An inflammatory microenvironment in the intestines may accelerate the mutational events that lead to cancer, and promotes substantial DNA hypomethylation and histone acetylation in order to increase transcription pro-inflammatory genes [14, 33]. In fact, while inflammation is not considered a decisive initiator of tumorigenesis, it is still a well-known genotoxin [3, 5, 9, 10, 18, 20, 33]. Inflammatory cells such as neutrophils produce copious amounts of **r**eactive **o**xygen **s**pecies (ROS) and **r**eactive **n**itrogen **i**ntermediates (RNI), which can interact with cellular DNA and cause severe damage and mutations, although it is widely believed that these mutagens account for a very small percentage of tumour initiators [5, 10, 20].

Currently it remains unknown what specifically causes the increased cancer rate in IBD patients but it is postulated that the damaged intestinal tissue leads to an abundance of neutrophils that are recruited to ward off infection, which release interleukins and other cytokines that cause an inflammatory immune response [11, 13, 14, 19, 33]. The inflammation itself is governed by inappropriate regulation of the innate

and adaptive immune systems. These regulations may lead to the disequilibrium of certain cytokines and signaling proteins such as those produced by the T-helper immune cells: Th1, Th2 and Th17 [3, 11]. These signals may elicit numerous responses depending on the specific cytokine released, but both Th1 and Th2 cells have the ability to activate themselves, while inhibiting each other using auto-regulatory cytokines and proteins such as IL4 and interferon-β [11]. These T-helper secretions are often related with IBD; UC usually presents with increased levels of Th2 cytokines, such as IL4 and IL25, while CD presents with an overabundance of Th1 stimulated cytokines, such as IL10 and IL27, two inflammatory cytokines regulated by the transcription factor Kaiso (Daniel Lab, Unpublished RNASeq Data), implying Kaiso plays a potentially integral role in CD and CAC development [3].

### 1.3 The Transcription Factor Kaiso

Kaiso is a POZ-ZF transcription factor with an N-terminus BTB/POZ domain that facilitates protein-protein interactions such as homo- and heterodimerization with other POZ proteins, or the recruitment of histone deacetylases and nuclear receptor repressors [34]. This heterochromatin formation induced by histone deacetylases prevents transcription at specific promoter sites, allowing Kaiso to act as a transcriptional repressor [35, 36]. Kaiso also contains a C-terminal Zinc Finger (ZF) domain that mediates its DNA binding [34]. The Kaiso ZF domain recognizes a specific consensus sequence known as the Kaiso binding site (KBS), TCCTGCNA where N is any nucleotide. Additionally, Kaiso binds to methylated CpG dinucleotides as well as to the palindromic sequence: TCTCGCGAGA [35, 36]. To date most studies have implicated Kaiso as a transcriptional repressor, however there are some studies that have identified Kaiso as a transcriptional activator, for example, in *rapsyn* promotor regulation [37]. In fact, it was recently discovered that Kaiso's transcriptional role is determined by post-translational modifications, specifically by covalent linkages of small ubiquitin-like modifier (SUMO) polypeptides that, when present, designate Kaiso as a transcriptional


***Figure 3: The transcription factor Kaiso.*** *Kaiso binds to both DNA and other proteins. Kaiso binds to proteins such as p120ctn using its N-terminus POZ domain, and binds to methylated promoter DNA, or to its specific consensus sequences using its ZF DNA binding motif.*

activator, and when SUMO is absent, Kaiso functions as a transcriptional repressor [38]. Interestingly despite Kaiso's reputation as a transcriptional repressor, under normal conditions the protein is usually SUMOylated, implying that in healthy cells (or during normal cell homeostasis) Kaiso generally functions as a transcription activator. Notably, under hyper-osmotic stress, Kaiso becomes deSUMOylated, molecularly switching its function to transcriptional repressor, which we often see in malignant cells [38].

Although Kaiso's mechanism of action is yet to be fully established, Kaiso has been implicated in several biological processes including cell adhesion, apoptosis, tumour metastasis and inflammation [39-41]. In previous studies, it was found that high Kaiso expression accelerates tumour formation and leads to poor prognosis and mortality in experimental breast cancer mice models [39]. In transgenic mouse models, intestinal Kaiso-overexpression increases neutrophil recruitment and activity, reduces expression of the cell adhesion protein E-cadherin, stimulates inflammatory pathways and inhibits epithelial repair mechanisms via promoter binding and protein-protein interactions [12, 40, 41].

Kaiso typically localizes to the nucleus in tumour tissues, although Kaiso may be found in the cytoplasm in some malignant tissues. Kaiso's nuclear localization leads to altered gene expression; some target genes are positively regulated while others are repressed, which can in turn affect oncoprotein expression and function [42]. Kaiso utilizes multiple methods to regulate cellular processes, in addition to binding to promoter DNA using its ZF domain, Kaiso may also form complexes with other proteins (even homodimerizing with other Kaiso molecules) using its N-terminus POZ domain [35]. Kaiso often forms complexes with other transcriptional binding partners to inhibit or activate Kaiso's transcription factors capabilities depending on the provided stimuli; Kaiso will also bind to proteins in the cytoplasm where it moderates protein stability [43, 44]. For example, Kaiso alters the ability of p300 acetyltransferase to modify numerous

sites on the tumour suppressor p53 protein, which promotes stability and increased p53 expression in cultured cells [43, 44].

Kaiso was originally discovered as a nuclear binding partner of the cell adhesion protein p120 catenin ($p120^{ctn}$) ~ 20 years ago, and this interaction remains one of the most important interactions for a thorough understanding of Kaiso's role in mammalian cells and vertebrates. In addition to interacting with $p120^{ctn}$, Kaiso also interacts with other adhesion proteins such as E-Cadherin and ZO-1 that are integral components of adherens and tight junctions respectively [12]. Kaiso's interactions with these adhesion molecules allow Kaiso to disrupt intercellular adhesion, which then contribute to physiological perturbations such as intestinal barrier leakage, which in turn inevitably leads to intestinal inflammation. Additionally, malfunctioning cell adhesion proteins allow tumour cells to lift from the basement membrane and metastasize throughout the body, implicating Kaiso as a major contributor in tumour metastasis [36, 39, 45, 46].

$p120^{ctn}$ has multiple roles in addition to its role in cell adhesion. These include acting as an anti-inflammatory protein through the Rho-ROCK pathway, a cascade involved in cytoskeletal dynamics; when in the cytoplasm $p120^{ctn}$ inhibits RhoA through the guanine exchange factor (GEF) Vav2, which dissociates bound GDP from RhoA [47]. RhoA functions as an activator of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a potent and ubiquitous inflammatory protein [13, 16, 47, 48]. When RhoA is bound to GDP, it facilitates degradation of NF-κB inhibitors, allowing the nuclear factor to migrate into the nucleus and promote expression of immune system cytokines [13, 48]. When RhoA is unbound from GDP, NF-κB remains inactive and the immune response is attenuated [47]. This interaction allows $p120^{ctn}$ to regulate inflammatory responses while it is localized in the cytoplasm. However, when Kaiso binds to $p120^{ctn}$, both proteins are translocated to the nucleus where the Kaiso-$p120^{ctn}$ complex regulates target gene promoter sequences [42]. When $p120^{ctn}$ translocates to the nucleus it effectively becomes sequestered, forcing the anti-inflammatory and cell

adhesion functions of $p120^{ctn}$ (that are made possible through cytoplasmic protein-protein interactions) to halt; meaning that higher levels of Kaiso-mediated transcription lead to aberrant localization and subsequently reduced levels of $p120^{ctn}$ facilitated anti-inflammatory and cell adhesion mechanisms [35, 47, 49].

### 1.4 Kaiso and Cell Adhesion

All epithelial cells in the human body are anchored to a basement membrane extracellular matrix, and/or to neighbouring cells through intercellular adhesion complexes [26]. There are three main types of intercellular adhesion machinery: Tight/apical junctions are comprised of claudin and occludin proteins, adherens junctions are comprised of assorted cadherins and catenins, and desmosomes, which are comprised of cytoskeletal components supported by cadherin proteins [12, 26, 50]. These intercellular protein complexes may also promote cell-cell signaling, which is typically facilitated by the communicatory intercellular structure: gap junctions. When an otherwise healthy epithelial cell loses its anchorage through malfunction of these structures, it undergoes programmed cell death known as anoikis [26, 27, 51]. Anoikis is a specific form of apoptosis that occurs when an adhered cell loses its anchorage, in a process known as the epithelial-to-mesenchymal (EMT) transition [45, 52]. EMT is a tightly regulated process that plays a key role in early vertebrate development [45, 50, 53]; fully transitioned mesenchymal cells generally do not employ cadherin-mediated adhesion, evading anoikis as they transition and thus are free to travel throughout the body in the vascular or lymphatic systems [54]. A cell may travel anywhere in a human body using these two transport systems, and if a tumor cell were to undergo EMT it would be able to leave the primary tumour site and metastasize to distant tissues that may go undetected in a patient. In fact, the majority of cancer fatalities occur due to complications related to secondary tumours in vital organs such as the lungs, liver or brain [45, 50, 52, 53].

Kaiso was originally discovered as a nuclear binding partner for cell adhesion cofactor $p120^{ctn}$ and has been linked to several other adhesion molecules (e.g. E-Cadherin, ZO1) in the years since [12, 42]. In addition to regulating these proteins by directly binding to their promoter regions, Kaiso indirectly destabilizes E-Cadherin by sequestering $p120^{ctn}$ in the nucleus where it cannot support the structural integrity of the Adherens junctions [40, 42]. In an even less direct method of regulation, Kaiso over-expression also stimulates Wnt signaling in cells, initiating a cascade that dissociates β-catenin from the cellular membrane and translocates the E-Cadherin-stabilizing protein to the nucleus where, much like $p120^{ctn}$, β-catenin cannot conduct its adherens stabilizing functions [22, 40, 41]. Indeed, there is mounting evidence that Kaiso can regulate cellular adhesion not only via protein-protein and transcription factor interactions, but through miRNA expression. Unpublished data from the Daniel Lab has found that miR-200c and miR-31 are both repressed by Kaiso in breast cancer cell lines (Daniel Lab, Unpublished miRNA Data). The miR-200 family of miRNA are known to directly inhibit EMT by stabilizing E-Cadherin via RNA interactions. This preliminary miRNA data suggests an alternate mechanism of action for Kaiso-mediated E-Cadherin destabilization, and further implicates Kaiso in the narrative of malignant EMT (Daniel Lab, Unpublished miRNA Data).

Kaiso's role in aberrant cell adhesion is further supported by the finding that *Kaiso*$^{Tg}$ mice exhibit progressive intestinal barrier leakage, which is thought to strongly contribute to their inflammatory phenotype [12]. This faulty epithelial barrier in the intestines is aggravated, and perhaps even caused, by compromised tight and *A*dherens junctions with insufficient expression of ZO1 and E-Cadherin proteins in the complex [12]. These findings in *Kaiso*$^{Tg}$ mice confirm that Kaiso's role in cell adhesion is consistent among *in vivo* mammalian models and lends credence to the theory that Kaiso plays an integral role in EMT.

## 1.5 Kaiso in Mammalian Signaling Pathways

Previously in *Xenopus* and cultured cell line models, it was observed that Kaiso over-expression inhibited Wnt signaling, reduced cell proliferation and promoted epithelial cell fate outcomes [49]. This provided the rationale for the generation of a Kaiso-overexpressing transgenic mouse model (*Kaiso*$^{Tg}$) through plasmid-introduced murine Kaiso DNA cloned under the regulation of the *villin* promoter, which is solely expressed in the intestine of the host C57bl/6 mice. It was reasoned that these mice would help discern if these observations were widely applicable to mammals. Surprisingly, the ***v****illin* **K**aiso (VK) genotype led to increased Wnt signaling, increased cell proliferation, over-representation of secretory cell fates, and severe tissue degradation [40]. VK mice also exhibited increased numbers of Goblet, Paneth and enteroendocrine cells relative to enterocytes, the absorptive epithelial cells, implying that Kaiso overexpression inhibited the Notch signaling pathway. These VK mice also exhibited intestinal inflammation [41], and while the exact mechanisms behind this innate immune response are unclear, we hypothesize that it may be due to the interactions between Kaiso and its binding partner p120$^{ctn}$ which is reported to play anti-inflammatory roles [40, 47]. Additionally, Kaiso's ability to destabilize E-Cadherin promotes faulty inter-cellular adhesion, which may allow pathogens and irritants to migrate past the intestinal barrier into the vulnerable interior of the human body, where the inflammatory immune response is initiated by local neutrophils [12, 20].

Additionally, when crossed with the *APC*$^{Min/+}$ mouse model for familial CRC, these *Kaiso*$^{Tg}$:*APC*$^{Min/+}$ mice exhibited a completely unexpected phenotype. It was hypothesized that Kaiso-mediated Wnt inhibition would rescue the tumour-forming phenotype of the *APC*$^{Min/+}$ mice. However, *Kaiso*$^{Tg}$:*APC*$^{Min/+}$ mice exhibited a significantly increased tumour burden, as well as a reduced life span of ~ 50% [41]. It is currently unknown what specific molecular mechanisms contribute to the synergistic cancer growth seen in *Kaiso*$^{Tg}$:*APC*$^{Min/+}$ mice.

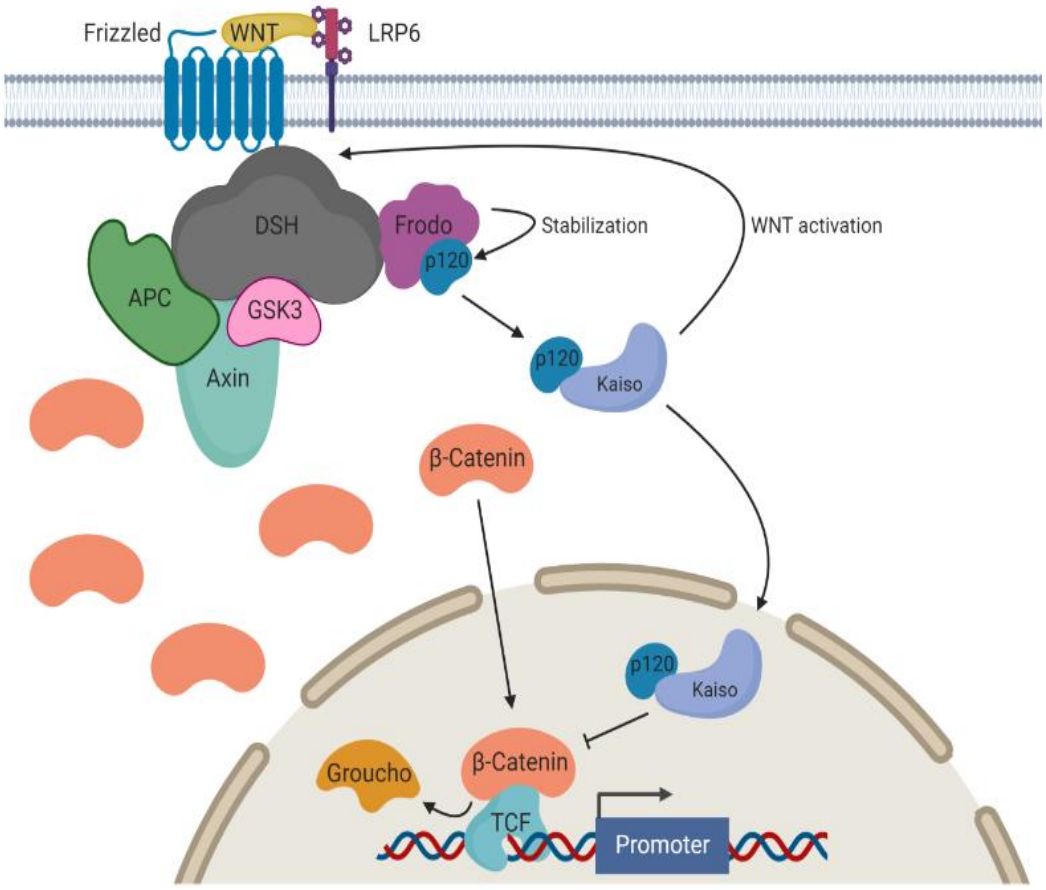



***Figure 4: The Wnt signaling pathway.*** *In the presence of WNT ligand, Dishevelled (DSH) is recruited to the cell membrane and the Glycogen synthase kinase 3 (GSK3)/APC/Axin complex is inactivated. WNT stimulus promotes p120$^{ctn}$ stability through the DSH functional regulator FRODO. Kaiso and p120$^{ctn}$ locate to the nucleus where Kaiso may regulate Transcription Factor (TCF) transcription.*

## 1.6 The Canonical Wnt Signaling Pathway

In ordinary tissue homeostasis and embryonic development, Wnt regulates cell polarity, differentiation, migration and proliferation through its two pathways: canonical and non-canonical [22, 55]. The canonical pathway is most commonly implicated in tumorigenesis, while the non-canonical pathway is more often associated with cell polarity and embryo development [30]. Wnt signaling has been associated with stem cell maintenance and activation, cell proliferation, cell fate, and differentiation; processes that are heavily influenced by the Wnt target genes *cMyc* and *Cyclin D1* [55]. For example, Wnt inhibition in mice results in the complete abolition of the intestinal crypts, the primary site of proliferation in the intestine, where the stem cells are housed and where they begin to differentiate as transit amplifying (TA) cells [55].

The canonical Wnt signaling cascade is responsible for deciding the fate of the cell adhesion protein and oncogene-activating transcriptional co-activator, β-catenin, which is an important factor in tumorigenesis [7, 22, 55]. In the absence of a Wnt signal (i.e. the Frizzled receptor is unbound) the multi-protein destruction complex, containing scaffolding proteins axis inhibition protein (Axin), APC and two kinases - glycogen synthase kinase 3 (GSK3) and casein kinase (CK1) which facilitate the ubiquitination of β-catenin, tagging it for degradation by the proteasome via serine/threonine phosphorylation [30]. When Wnt ligands bind and activate the Frizzled transmembrane receptor, the Wnt signaling cascade is initiated and the Dishevelled (DSH) protein is recruited, causing the creation of signalosomes that sequester Axin at the cell membrane. This results in β-catenin accumulation in the cytoplasm and subsequently its translocation to the nucleus where it displaces the transcriptional repressor Groucho from TCF/LEF transcription factors on target gene promoters [22, 29]. Once Groucho is displaced by β-catenin, Wnt target genes are activated and expressed at higher levels [29]. The Wnt pathway also displays auto-regulatory functions, as the expression of several Wnt regulators such as Axin2, LRP/HSPG, Frizzled and even TCF/LEF itself are all modulated by the β-catenin bound TCF transcription factor [22].

In 80% of CRC cases, the *APC* gene, which encodes the APC protein found in the destruction complex of the WNT pathway, contains a multiple intestinal neoplasia (MIN) mutation that results in a truncated APC protein; this prevents the destruction complex from degrading the intracellular signal transducer, β-catenin [22, 30, 55]. The resulting over-expression of downstream target genes promotes cell proliferation, over-abundance of secretory cell fates, and in the most extreme cases, oncogenesis. Oncogene activation and thus tumorigenesis also occurs in tissues exhibiting activating mutations in β-catenin and loss of function mutations in Axin1, both of which are key components of the destruction complex with APC [30]. Interestingly, Wnt signaling is highest at the base of the crypts in the stem cell compartment, and gradually decreases as cells travel upward toward the intestinal lumen. This phenomenon explains the levels of proliferation in the intestinal epithelium; stem cells in the crypt base replicate rapidly, driven by high Wnt signaling activity, producing complete epithelial turnover in less than a week [7, 22, 28, 30, 55]. Conversely, as cells ascend up the crypt and/or villi, they differentiate and arrest cell division, but maintain a state of senescence and remain essentially unchanged until they are replaced and die via anoikis [55]. Wnt signalling cooperates with multiple other signaling pathways, including the Notch pathway, to maintain sustainable levels of proliferation and ensure proper cell fate distribution in the epithelium [7, 56].

## 1.7 The Canonical Notch Signaling Pathway

The *Notch* gene was first discovered in *Drosophila melanogaster* by John Dexter over a century ago. Since then, four human Notch paralogues have been identified (Notch 1-4), all of which are single-pass type I transmembrane receptors [57]. As such, Notch contains both an extracellular and intracellular domain; the extracellular domain facilitates binding to the Notch ligands (Delta/Serrate/Lag-2 [DSL] family transmembrane proteins). Due to both the receptor and ligand being single-pass transmembrane proteins, Notch signaling is transmitted via interactions between

neighbouring cells [57]. Notch must be proteolytically cleaved at three separate sites to that ultimately results in the Notch intracellular domain (NICD) being released from the membrane and activation of the signaling cascade. The NICD then enters the cytoplasm before translocating to the nucleus to regulate transcription of the *Hes* family of transcriptional repressors [57, 58].

Like the Wnt pathways, Notch signal strength exists on a spectrum throughout the intestine, and results in different cellular responses in areas of differing stimulation. Notch signaling activity directly determines the fate of ISCs; increased Notch signaling promotes epithelial cell fates, increasing the representation of absorptive cell fates in these areas [8]. Conversely, decreased Notch signaling favours secretory cell fates, and decreases the absorptive cell concentrations. In the intestine, Wnt and Notch signals are typically inversely correlated: areas of low Notch signaling often correlate with areas of high Wnt signaling, causing ISCs to proliferate rapidly, producing disproportionate numbers of secretory cells (goblet, Paneth, enteroendocrine), as opposed to the equally disproportionate numbers of epithelial absorptive cells found in areas of high Notch and low Wnt signaling [7, 22, 55].

### 1.8 NF-κB Inflammatory Pathway

NF-κB is a heterodimeric protein complex comprised of a RelA/p65 subunit and a p50 subunit that acts as a potent immune activator with roles in nearly every inflammatory response in the human body [16, 48]. The NF-κB pathway is extremely complex and receives activating and repressive stimuli from multiple sources, including hormones and growth factors, as well as the TNFα, IL1 and the PI3K/Akt pathways [59]. All of these stimuli arise in response to tissue insult, and leads to the recruitment of neutrophils and macrophages in an inflammatory response. Another common feature of these pathways is the direct regulation of the proteins IKK and IκB [13, 48]. In the canonical NF-κB pathway, one of numerous membrane-bound receptors in the cell will phosphorylate the protein kinase IKK (facilitated by other signaling cascades such as Rho/ROCK and TGFβ), which in turn phosphorylates IκB, the inhibitory complex that

sequesters inactive NF-κB in the cytoplasm [60]. This phosphorylation targets IκB for processing via the proteasome and releases NF-κB from the inactivating complex, leaving NF-κB free to translocate to the nucleus where it may regulate genes related to mitogenesis, cell survival and the immune or inflammatory response [48, 59]. For these reasons, when visualizing NF-κB in a cell, it is important to realize that the nuclear protein pool represents the most active and significant population of the molecule, while the majority of cytoplasmic NF-κB are likely inactive molecules [48].

The NF-κB protein subunits themselves are heavily regulated in an attempt to prevent unnecessary inflammation throughout the body, even auto-inhibiting transcription when an inflammatory stimuli has not been received [13]. In fact unpublished data from our lab has identified at least 11 KBS sites in the RelA promoter, and multiple methylated CpG dinucleotide motifs throughout both the RelA and p50 promoter, implicating Kaiso as a potential regulator of NF-κB subunit gene expression (Daniel Lab, Unpublished Data). The comprehensive and widely applicable nature of NF-κB pathway makes it a difficult cascade to isolate and fully characterize. With at least nine known activating receptors (all belonging to completely distinct families), and innumerable points of cross talk between an extremely wide range of signalling pathways, it is very difficult to study the inflammatory response without encountering significant sources of confounding variables [15, 17]. Notably, the canonical NF-κB pathway is most heavily associated with a select few immune stimuli. NF-κB activation is most commonly induced by cytokine stimulus received from neutrophils and macrophages at the site of tissue damage and/or infection. This response activates the NF-κB pathway, allowing NF-κB itself to migrate to the nucleus where it regulates transcription. NF-κB generally targets genes that regulate additional interleukins, auto-regulatory signal transducers, and proteins that aid in cell survival/mitogenesis [13, 17].

While inflammation is meant to act as a short term measure to prevent infection and promote expedited immune response, over-stimulation sometimes occurs and

causes severe acute inflammation that may actually hinder the healing process, or causes chronic inflammation that puts tissues into a constant state of stress and aberrant gene expression [16]. Because the inflammatory response is meant to increase cell growth and survival, as well as to allow timely neutrophil recruitment rates, a situation where this environment becomes the norm also becomes increasingly more dangerous to the host. While permeabilized membranes may greatly increase the flow of white blood cells through an epithelial layer, it also exposes those cells to foreign molecules, leaving the cells vulnerable to chemical and osmotic imbalance, environmental pathogens, DNA/protein damage and even additional physical insult due to lack of cell rigidity [13, 16]. Additionally, while the inflamed cells are exposed to these constant threats, they are receiving mitogenic signals via the NF-κB pathway that promotes mitosis and proliferation. This increases the number of inflamed cells and causes an over-abundance of cells that may lead to hyperplasia [15-17, 59].

As the number of inflamed cells increase, so does the number of mutations found therein [5, 9, 14]. This promotes ectopic protein expression, gene regulation and intercellular interactions. Not surprisingly, if allowed to continue indefinitely, this environment becomes conducive to tumour formation [17]. Chronic inflammation greatly increases the mutation rate in cells, which will compound in every generation, increasing the probability of a tumor cell being born with every division, and promoting carcinoma through a lack of functional tumour suppressors [15]. Additionally, if this damaged tissue is exposed to an environment of carcinogens and irritants (such as found in the intestinal lumen, or epidermis) then the tumor formation is often accelerated even further [13, 15, 17, 59].

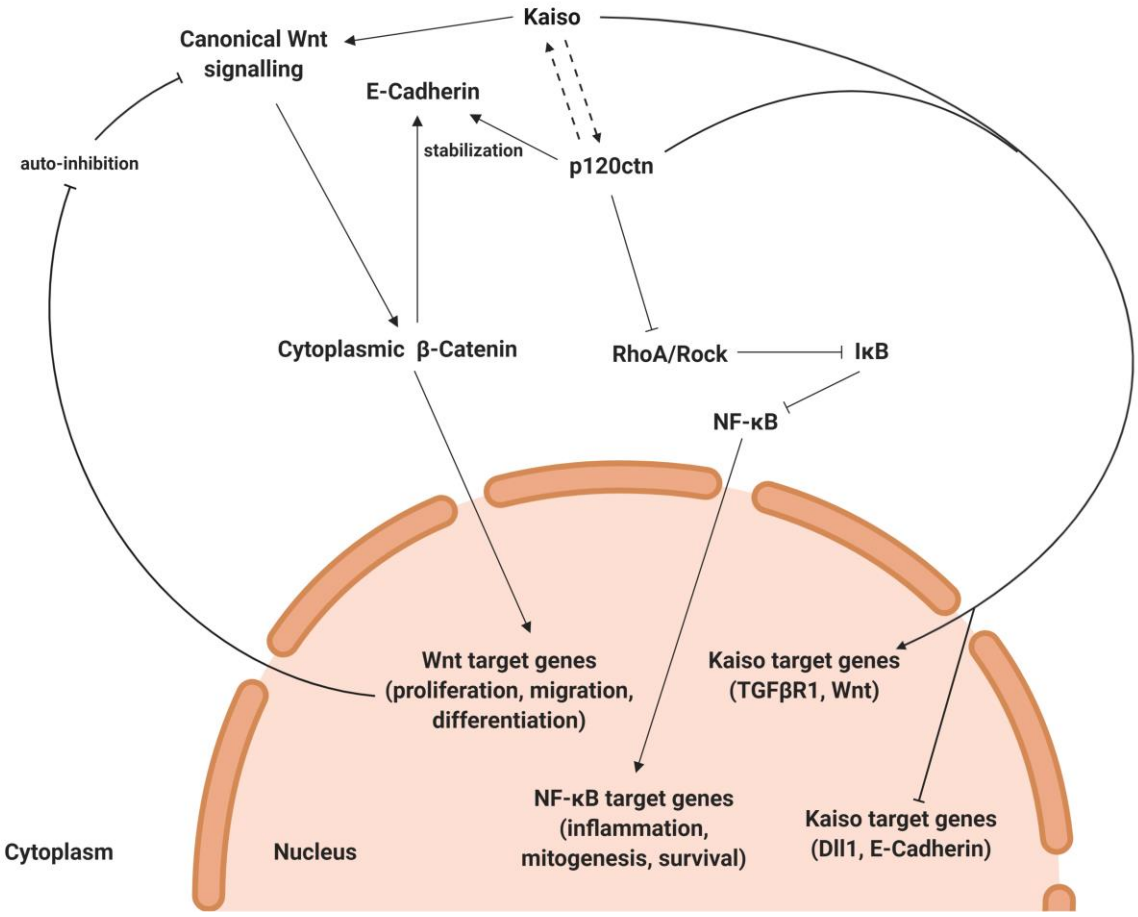



***Figure 5: Kaiso regulates cellular processes through direct and indirect modulation.*** *Kaiso interacts with signaling pathways through protein-protein binding and gene regulation.*

**1.9 Tumour Suppressor p53**

p53 is well known to be a lynchpin of the cell cycle, and its malfunction is implicated in all forms of cancer as it is the most commonly mutated protein in human cancer. p53 is a tumour suppressor that monitors DNA integrity throughout the cell cycle and is responsible for allowing cells to enter mitosis if they are healthy, and preventing damaged or compromised cells from dividing [32, 61, 62]. p53 acts in multiple capacities to ensure proper cell function and may prevent cancer by activating genetic repair mechanisms, stopping cell growth at routine checkpoints, and by initiating apoptosis if the cell is beyond repair [32, 61-63].

p53 can be activated by numerous factors, including but not limited to: genetic damage, osmotic shock, chemical/oxidative stress or abnormal oncogene expression [61]. Following the established "Vogelgram" model of CRC disease progression, in order for colonic hyperplasia to progress through the early, intermediate and late adenoma stages of tumour formation, at least 3 key tumour suppressor proteins (APC, KRAS, SMAD4) must be dysfunctional [64]. For a late adenoma to progress to carcinoma, a p53 mutation alone is sufficient; this displays the vital role that p53 plays in CRC [63, 64].

p53 is associated with many different regulatory pathways, but solely exists to prevent malignancy and unregulated cell growth. In a healthy cell, p53 is kept at very low concentrations via acetylation, ubiquitination and degradation by proteases. Kaiso was recently reported to be a regulator of p53, and can regulate acetylation of the protein at a minimum of 3 sites through interactions with p300-acetyltransferase and RelA/p65 [43, 44]. While the role of Kaiso regulating p53 remains unclear, it is intriguing that the two proteins interact in the nucleus [32, 43, 61].

### 1.10.1 Model Organisms

Even before the disciplines of science and research had been fully formed, philosophers of ancient civilizations used animals to learn more about life and the physiology of humans. Indeed, the practice of animal modelling continued to

revolutionize the way humans made discoveries, with Aristotle using embryogenesis in chicks to demonstrate that while there are many differences between the organisms on Earth, there are many similarities that we should focus on instead [6, 65].

In 1902, William Castle began breeding a relatively high throughput mouse animal model for genetic studies: *Mus musculus* [66]. Throughout the next century, mouse model technology advanced in leaps and bounds, with Clarence Little mastering murine inbreeding by the 1930s and the first transgenic and knockout mice being developed in 1976 and 1987 respectively [66]. Since then, the complete genomes of mice have been sequenced and the use of genetic manipulation technologies such as Cre/flox, plasmid and CRISPR have made the production of transgenic or mutant mice significantly easier than 30 years ago, which has facilitated unprecedented levels of *in vivo* research on mammalian physiological processes using mouse models [66, 67].

In fact, with the identification of murine homologues for specific genes, transgenic and mutant mice have been created to recapitulate innumerable human ailments and genetic disorders. For diseases or disorders that cannot currently be targeted genetically in mice, they may often be modelled through manipulation of food, water or via invasive procedures such as injection or gavage [66, 67]. For example, there are multiple methods for a researcher to model the phenomenon of intestinal inflammation, using both genetic and physical manipulation. The current gold standard for modelling familial colon cancer and intestinal inflammation is through the $APC^{Min/+}$ mouse model that utilizes truncating mutations in APC, a Wnt pathway protein, to mimic similar mutations that are commonly found in IBD and CRC patients [22]. While genetic models are consistent, reliable methods to model disease, there are models in which disease is initiated spontaneously through physical insult or exposure to environmental toxins, completely unrelated to the mutated gene, rendering these models ineffective [68, 69].

Currently, it is extremely popular to model colitis using the **d**extran **s**odium **s**ulfate (DSS) model [70]. DSS is a potent detergent that permeabilizes the epithelial intestinal barrier causing inflammation, and is very commonly used to recapitulate the effects of ingested inflammatory toxins [19, 20, 70]. DSS is typically dissolved in water and mice allowed to drink it naturally or via gavage [18, 70]. However, these types of studies are prone to error; the amount of DSS ingested cannot be completely controlled, oral gavage may cause undue stress, and the ingested chemical (DSS) may cause confounding off-target effects [69, 70].

To reduce these sources of error it is important to specifically plan an animal study, by choosing the exact genetic model needed, exposing the animal to treatment in as natural a way as possible, and appropriately combining genetic and exogenous intervention [66, 68]. In the world of IBD, the methods of inducing inflammation in mice are many, however they are notorious for the numerous off-target effects that may confound any data collected [18, 70]. When colitis is induced using current methods the inflammation caused by these mechanisms is often generalized throughout the body and throughout the lifespan, making these animals accurate models of inflammation, but giving them limited application for learning about the specific diseases that cause inflammation [10, 37, 41, 68, 69].

### 1.10.2 Kaiso$^{Tg}$ Mice

Fortunately, science and animal husbandry are constantly evolving, allowing for the production of increasingly more models that may accurately recapitulate the specific characteristics of individual diseases while decreasing the number of invasive procedures and off-target effects. An example of this is the Kaiso transgenic mouse model that was generated using Myc-tagged murine Kaiso (mKaiso-MT) cloned downstream of the murine intestinal-specific *villin* promoter in a pBluescript II vector [40]. The *Kaiso$^{Tg}$* mice exhibit both physiological and genetic evidence that they can specifically model the intestinal inflammatory disease Crohn's Disease, which is

currently modelled using NOD2 (an anti-bacterial immune protein) KO mice with very specific bacterial colonization in the gut [4]. However this model is not a widely applicable representation of human disease due to the off-target effects caused by a knockout involving a ubiquitous immune protein, and the necessary simultaneous co-colonization of the gut with *Mucispirillum schaedleri* [4, 71]. Human disease is extremely dynamic and any study that hopes to characterize such responses must endeavour to use the most applicable model, which usually means that it is inadvisable to rely on models utilizing complex mechanisms that do not apply universally to that disease. As such the development of an IBD model that does not require the stars to align would greatly improve the quality of research on IBD and throughout the field [4, 37, 41, 69, 71].

## Rationale and Aims

CRC is the 3rd most prevalent form of cancer worldwide, and represents a disproportionately large portion of all cancers diagnosed in the western world [72]. Individuals suffering from IBD for at least 30 years have a ~20% higher chance of developing CRC, and have an ~15% higher chance of dying from CRC [20, 73, 74]. It is currently unknown what molecular mechanisms enhance the risk of IBD patients to higher CRC incidence and mortality rates. Progress in IBD research has been slow, and has been hampered by a lack of public awareness, imprecise scientific interventions, and the indecipherable labyrinth of involved molecular signaling pathways with their innumerable interactions and crosstalk [19, 20, 23, 74].

The world of IBD research lacks a reliable model system that recapitulates the specific microenvironment seen in humans. Researchers currently rely on the inconsistent administration of chemical inducers of inflammation to mice, through invasive procedures; as well as the use of flawed genetic models that expose the study to confounding factors and off-target effects [69, 70]. To fill this gap in resources, we propose to use our *Kaiso*$^{Tg}$ mouse model in conjunction with exogenous carcinogens,

pro-inflammatory molecules and genetic manipulation to recapitulate the disease progression that occurs when patients with the relatively innocuous (albeit uncomfortable) IBD, transition into life-threatening CAC. We hope to provide a viable option for researchers who endeavor to delve deeper into the unknown etiology of IBD and its subsequent disease progression [9, 24, 69, 70].

To better characterize and elucidate the specific molecular mechanisms driving the endogenous *Kaiso*$^{Tg}$ intestinal inflammation, we propose to use chemical inducers of inflammation, general anti-inflammatories, carcinogens and the breeding of our *Kaiso*$^{Tg}$ mice with the genetic colon cancer mouse model, *APC*$^{Min/+}$. This will allow us to study the effects of exacerbated inflammation, anti-inflammatory (aspirin) treatment on endogenous inflammation, and additional genetic insult on these mice. Since our *Kaiso*$^{Tg}$ mice exhibit signs of severe intestinal inflammation, which has been linked to CRC in humans [40], this implicates Kaiso as a key player in gastrointestinal immune dysfunction and led us to hypothesize that **Kaiso transgenic mice will develop intestinal polyps if treated with carcinogens and pro-inflammatory chemicals, or if mated with the *APC*$^{min/+}$ mouse model for familial CRC.**

We specifically aimed to:

1. Characterize intestinal tissues of *Kaiso*$^{Tg}$ mice treated with the carcinogen Azoxymethane (AOM) and/or the pro-inflammatory detergent DSS
2. Characterize the intestinal tissues of *Kaiso*$^{Tg}$ and Kaiso$^{Tg/+}$/APC$^{min/+}$ treated with the anti-inflammatory and chemo-preventative aspirin for 10 weeks
3. Elucidate a role for Kaiso in the regulation of the pro-inflammatory proteins p120$^{ctn}$ and NF-κB

## 2.0 Materials and Methods:

### 2.1 Ethics:

All mouse work was conducted following the guidelines of the McMaster University Animal Research Ethics Board (AREB). All protocols for mouse breeding, husbandry, genotyping and euthanasia were approved by AREB under Animal Utilization Protocol (AUP) 18-09-39. Euthanasia was conducted via $CO_2$ asphyxiation followed by cervical dislocation.

### 2.2 Immunohistochemistry:

Tissues were fixed in 10% phosphate buffered formalin, paraffin embedded and cut into 1 µm sections and adhered to glass slides. During the IHC experiment, de-paraffinization of the slides was achieved by two separate 5-minute xylene washes. The tissue samples were then rehydrated via decreasing ethanol gradient washes (100%, 95% and 70% ethanol) for 3 minutes each, followed by transient immersion in double distilled water. The tissue was then permeabilized in 0.05% 1X tris buffered saline (TBS)-Tween for 20 minutes, followed by 3x 5 minute 1X TBS washes, and 3x 1 minute dH20 rinses. Antigen retrieval was achieved by boiling the tissues in a pH 6.0 sodium citrate solution-tween (1 mM) (or Tris EDTA-Tween). The boiling was conducted in a microwave on “high power” for 4 minutes, followed by 16 minutes at 40% power. Slides were allowed to cool at room temperature and the tissues were washed 3 x 5 minutes each in 1X TBS. Tissues were then blocked for one hour in a 5% normal donkey serum (NDS) or normal goat serum (NGS), 1% bovine serum albumin (BSA) (10% solution) in 1X TBS solution. Avidin blocking solution (Vector Labs) was added at a concentration of 4 drops/mL of total blocking solution and slides were incubated in a humidifying chamber. After the tissues were blocked, they were incubated overnight in a humidifying chamber at 4°C with primary antibody solution that consisted of the blocking solution and the following primary antibodies with respective dilutions: Ki67 (BD Bioscience [550659], 1:1000), NF-κB (Abcam [AB86299], 1:500), E-Cadherin (BD Biosciences [610181], 1:200) and $p120^{ctn}$ (Gift from Dr. A. Reynolds [15D2], 1:500). The following day, the slides were

washed 3x 5 minutes in 0.05% 1X TBS-Tween (TBS-T) and 3x 5 minutes in 1X TBS before the endogenous peroxidases were blocked using 3% H2O2 for 10 minutes at room temperature in the dark. Following the H2O2 step, slides were once again washed in 1X TBS 3x 5 minutes. Secondary antibody was then applied, using blocking solution augmented with Goat anti-Mouse biotin-conjugated secondary antibody (Vector Labs [ZA0425], 1:1000) and Donkey anti-Rabbit biotin-conjugated secondary antibody (Abcam [AB205718], 1:1000) when appropriate. The slides were incubated with the secondary antibody at room temperature for 2 hours. Following the secondary antibody incubation, the tissues were washed 3x 5 minutes in 0.05% 1X TBS-T and 1x 5 minutes in 1X TBS. Vectastain Elite ABC (Vector Labs) was added to each tissue and the tissues were incubated for 30 minutes in a humidifying chamber at room temperature. The tissues were then washed 3x 5 minutes in 1X TBS. The tissues were then stained with DAB solution (Vector Labs), which was prepared using 2 drops of buffer, 2 drops of H2O2, 4 drops of 3,3'-Diaminobenzidine (DAB), and 5 mL of ddH2O as per the standard DAB protocol. The DAB solution was left on the tissues simultaneously for up to 10 minutes or until there was visible staining of the positive control tissues. The tissues were then washed simultaneously under running H2O for 5 minutes. The tissues were then counterstained with filtered hematoxylin for 1 minute. Slides were washed under running H2O for 5 minutes. Acid Alcohol was added for 25 seconds, followed by a 5-minute wash under running H2O. Slides were then immersed in Scott's Tap Water for 45 seconds, followed by a 5-minute rinse under running H2O as before. The tissues were then dehydrated in a gradual increasing ethanol series of 70%, 95% and 100% each 1x 2 minutes then cleared in Xylenes, 2x 5 minutes. Finally, the slides were mounted using polymount (Polysciences Inc., Warrington, PA) and scanned and imaged using the Aperio ScanScope (Leica Biosystems, Concord, ON).

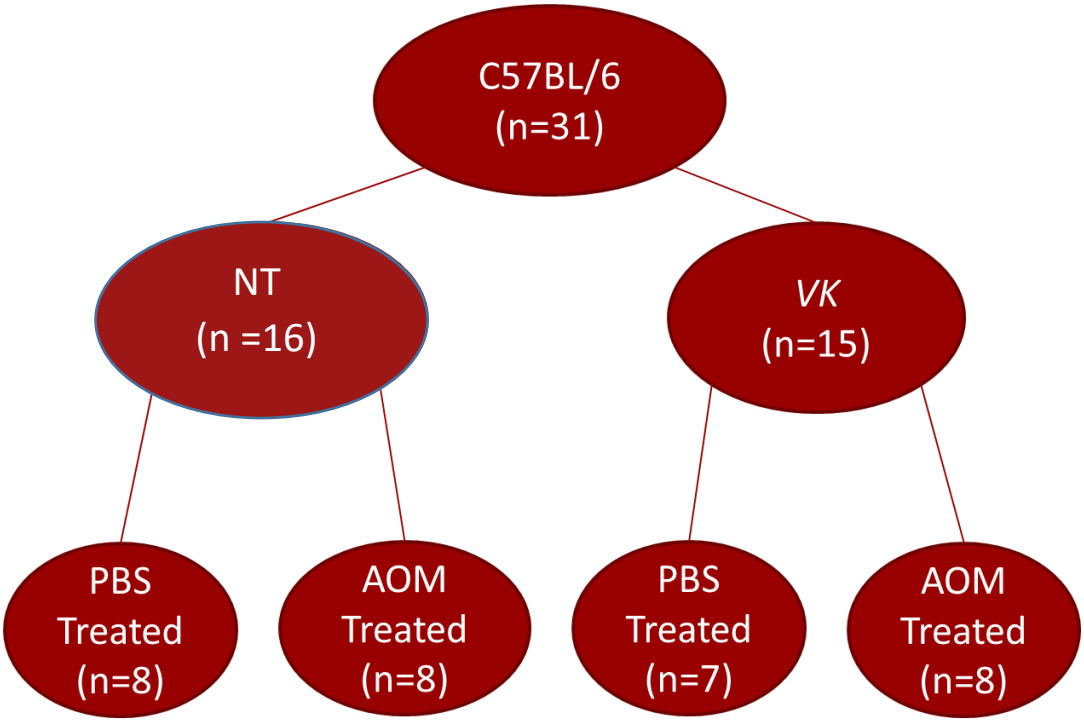
## 2.3 Tissue Culture:

The colorectal cancer cell lines HCT116, HT29 and SW480 were purchased from the American Tissue Culture Collection (ATCC) and cultured in Dulbecco's Modified

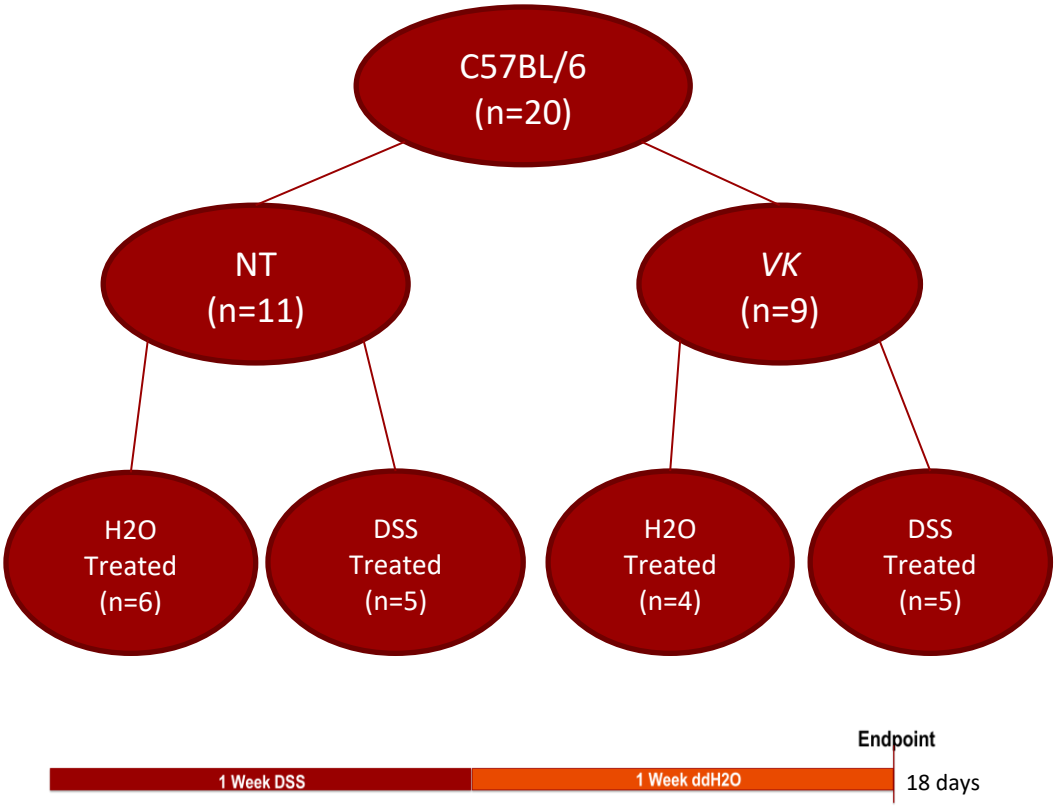
Eagles Medium (DMEM; Lonza) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA) and 1% penicillin/ streptomycin (Gibco, Waltham, MA). Stable pRS-shKaiso-scrambled and pRS-shKaiso HCT116 and HT29 cell lines were selected with 2 μg/mL puromycin, while stable SW480 pRS-shKaiso-scrambled and pRS-shKaiso cell lines were selected with 4 μg/mL puromycin [12]. Cells were grown to 80-100% confluency before they were lysed and total protein was isolated.

## 2.4 Protein Isolation and Western Blot:

Cultured cells were washed once with cold 1X PBS. 1 mL trypsin was then added to the plate and cells were scraped off the plate using a cell scraper. The solution of PBS and cells were then transferred to an eppendorf tube and centrifuged at 3000 RCF at 4°C for 3 minutes to form a pellet. The PBS was aspirated off and the cell pellets were lysed using NP-40 sample buffer containing 1x mini protease inhibitor cocktail tablet (Roche) and sodium orthovanadate (Sigma-Aldrich [89H0938]) on ice for 15 minutes, periodically agitating the solution using a vortex. Once denatured, lysates were centrifuged at 13.3 RPM at 4°C for 10 minutes. Protein concentration was quantified using a standard Bradford Assay, according to the manufacturer's protocol (BioRad). Equal amounts of protein lysate (50 μg) were loaded and electrophoresed on a 7% SDS-PAGE gel for 2 hours at 34 mAmp. Proteins were transferred onto nitrocellulose membrane using Hoeffer wet transfer system at 22 mAmp for 2 hours. Membranes were blocked with 4% milk in 1X TBS for 1 hour at RT before incubation with primary antibodies with the following respective dilutions overnight at 4°C (β-actin, Sigma Aldrich [A5441] 1:10,000; Kaiso, rabbit polyclonal, 1:10,000; NFκB, Abcam [AB86299], 1:5000). Membranes were then washed 5x for 5 minutes each with TBS on a Belly Dancer rocker, incubated with each respective HRP-conjugated secondary antibody (Abcam [AB6820], 1:10,000) (1:10,000), for 2 hours at room temperature and then washed again washed 5x 5 minutes with TBS on a Belly Dancer rocker. Protein bands were visualized using Western Lightning enhanced chemiluminescent reagent (Perkin Elmer), and images were acquired using the ChemiDoc MP Imaging System (Bio-Rad).




***Methods Figure 1: AOM study treatment plan***




***Methods Figure 2: DSS study treatment plan***

## 2.5 AOM Mouse Study:

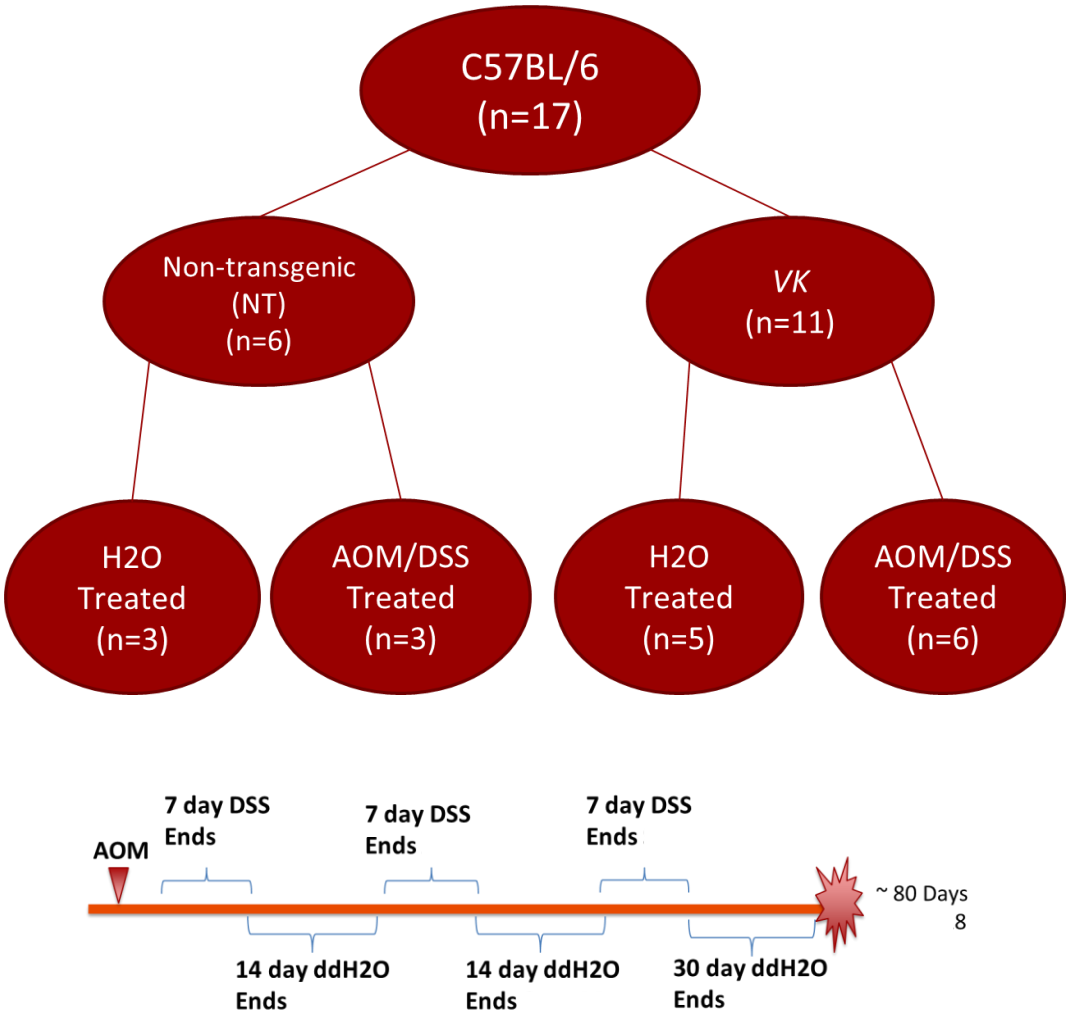
During the azoxymethane (AOM) animal study, 16 non-transgenic (NT) mice and 15 heterozygous (villin-Kaiso or *Kaiso*$^{Tg}$) VK mice [40] were injected intraperitoneally (IP) with either 10 mg/kg bodyweight of AOM, or an identical concentration of PBS (vehicle control) once a week for five weeks. The mice were monitored daily for weight loss and signs of physical & behavioural distress. Mice were sacrificed 52 weeks after the initial injection, dissected and the tissues harvested for IHC analysis. The tissues were fixed in phosphate-buffered formalin (PBF) for 48 hours then transferred to 70% EtoH before being paraffin-embedded and mounted on slides. **(Methods Fig. 1)**

## 2.6 DSS Mouse Study:

During the dextran sodium sulfate (DSS) study, 8 NT mice and 9 VK mice were exposed to either 2% DSS in their drinking water, or normal dd$H_2O$ drinking water for one week, followed by one week of dd$H_2O$ for both the experimental and control groups. Mice were sacrificed and dissected immediately at the end of the second week, and their tissues harvested for IHC analysis. The tissues were fixed in PBF for 48 hours, transferred to 70% EtOH and then paraffin-embedded and mounted on slides. **(Methods Fig. 2)**

## 2.7 AOM + DSS Mouse Study:

During the AOM + DSS study, 8 NT and 10 VK, eight-week old mice were exposed to either 1% DSS in their drinking water or normal dd$H_2O$ as a control, for three 7 day exposures, with 14 day dd$H_2O$ rest periods in between. Similarly half the mice were injected IP with either 12.5 mg/kg AOM, or an equivalent volume of PBS as a vehicle control on the first day of the first DSS cycle. The entire study lasted for ~80 days, with three 7-day DSS cycles, two 14-day dd$H_2O$ rest periods, and a final 30-day dd$H_2O$ rest period before intestinal tissue collection. Mice were sacrificed and dissected at approximately 80 days, and their tissues harvested for IHC analysis. Tissues were fixed in PBF for 48 hours and transferred to 70% EtOH and then paraffin-embedded and mounted on slides. **(Methods Fig. 3)**



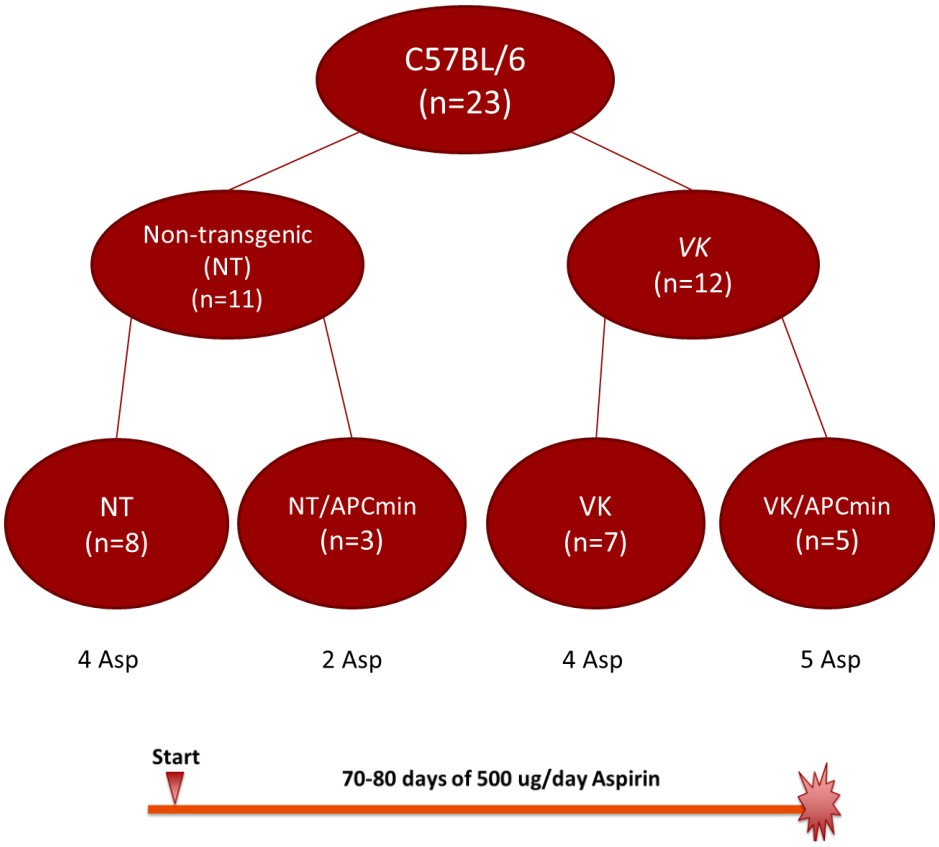

***Methods Figure 3: AOM + DSS study treatment plan***

## 2.8 Aspirin Mouse Study:

For the aspirin study, *Kaiso*$^{Tg}$ mice were mated with the *APC*$^{Min/+}$ mouse model for colorectal cancer in order to create a *Kaiso*$^{Tg}$:*APC*$^{Min/+}$ mouse colony. From this colony, 15 NT, 13 VK, 4 NT/*APC*$^{Min}$ and 11 VK/*APC*$^{Min}$ twelve week old experimental mice were exposed to ~70 days of 500 µg of acetylsalicylic acid (Aspirin) daily in their water, while the controls received dd$H_2O$ for an equivalent period. Mice were sacrificed and intestinal tissues dissected and harvested at 10 weeks after first exposure for IHC analysis. Tissues were fixed in PBF for 48 hours and transferred to 70% EtOH, before paraffin-embedding and mounting on slides. **(Methods Fig. 4)**

## 2.9 Statistics:

All statistical analyses were performed using GraphPad software. Results are presented as mean ± SD of three experiments. P-values were determined by unpaired students t-tests and one-way ANOVA. A p value of $<0.05$ is considered to be statistically significant.




***Methods Figure 4: Aspirin study treatment plan***

## 3.0 RESULTS:

### VK mice exhibit an impeded weight gain phenotype

A ubiquitous endpoint for lab animals is the loss of bodyweight of 20-30% depending on the mouse [66, 68]. When the mice received their regular weighing, it became apparent that *Kaiso*$^{Tg}$ mice were experiencing impeded weight gain. Growth charts of VK mice compared to their NT siblings, for all treatment and control groups in their respective studies **(Figure 6)** display a significantly impeded weight gain phenotype that presents consistently among VK mice. **Figure 6A** shows the growth of VK and NT mice that were treated with AOM or PBS to examine the effects of carcinogen treatment on VK mice. The graphs show that while the impeded weight gain phenotype exists in both the treatment and control groups, the impeded weight gain is strongly exacerbated by the presence of AOM. **Figure 6B** depicts the inability of VK mice to recover and gain weight after 1 week of DSS exposure compared to their NT siblings that recover at a significantly accelerated rate, surpassing the highest growth speed seen in the study. The non-treated mice of both genotypes gained weight at the same standard rate.

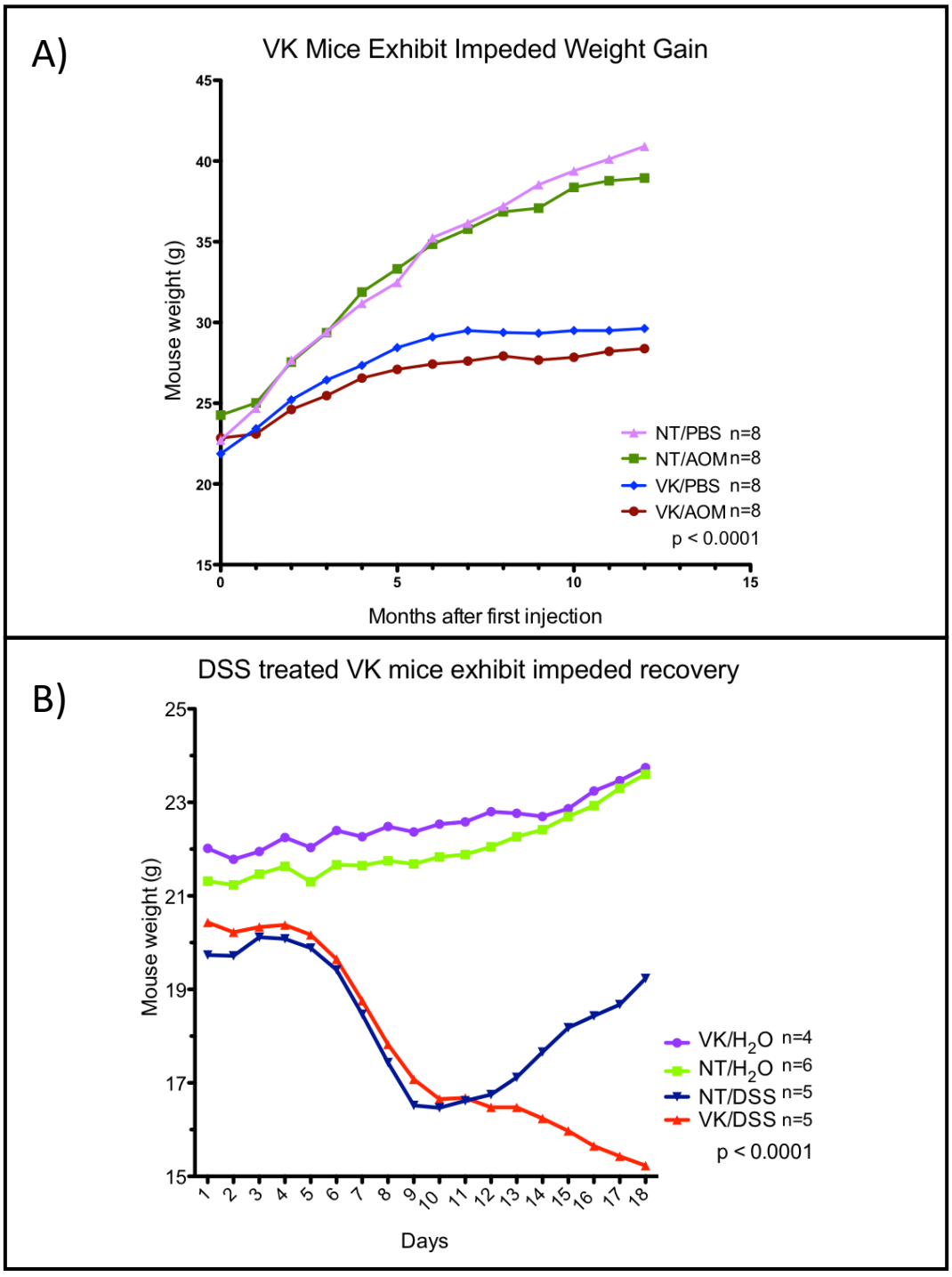



***Figure 6: VK mice exhibit an impeded weight gain phenotype (A)*** *Kaiso-overexpressing mice (VK) exhibit reduced weight gain when compared to non-transgenic (NT) controls over a 12-month period.* ***(B)*** *8 wk old VK mice exhibit a lack of recovery from transient DSS treatment. Statistical tests conducted using one-way ANOVA.*

Kaiso overexpression promotes aberrant expression and localization of cell adhesion proteins in DSS-treated mice

DSS is a potent detergent that when ingested, destabilizes the intestinal epithelium and compromises the robust physical barrier created by trillions of adhesive bonds between cells [11, 18].When the intestinal barrier is destabilized and becomes "leaky", pathogens and environmental toxins may then pass between cells into the underlying LP (See Figure 2). VK mice have been previously shown to develop intestinal barrier defects, driven in part by Kaiso-mediated repression of integral adhesion proteins.

To determine the effects of ingested pro-inflammatory stimuli on the endogenous inflammatory intestinal microenvironment of VK mice, 37 C57BL/6 mice (18 VK and 19 NT) were split into four groups (n>7) and treated with either 2.5% DSS in their drinking water for one week followed by one week of regular ddH2O, or two weeks of regular ddH2O as a control . The intestinal tissues of these mice were analyzed via IHC, to determine the subcellular localization and relative expression of inflammatory and tumorigenic proteins. **Figure 7A** depicts decreased E-cadherin expression in Kaiso overexpressing VK mice and is consistent with Kaiso's well-documented repression of E-Cadherin expression [12, 41], an important cell adhesion protein. Interestingly, regardless of treatment received, VK mice exhibited reduced E-Cadherin expression compared to their NT siblings. DSS exposure did not noticeably alter E-Cadherin expression, but it did appear to slightly alter the localization of E-cadherin from the membrane to the cytoplasm.

$p120^{ctn}$ is an adhesion cofactor and regulator of E-Cadherin stability, however it also has anti-inflammatory functions, and transcriptional inhibition functions [12, 47]. In **Figure 7B**, we observe that VK mice express slightly more total $p120^{ctn}$ compared to NT mice that received identical treatment. However, VK mice express relatively low nuclear

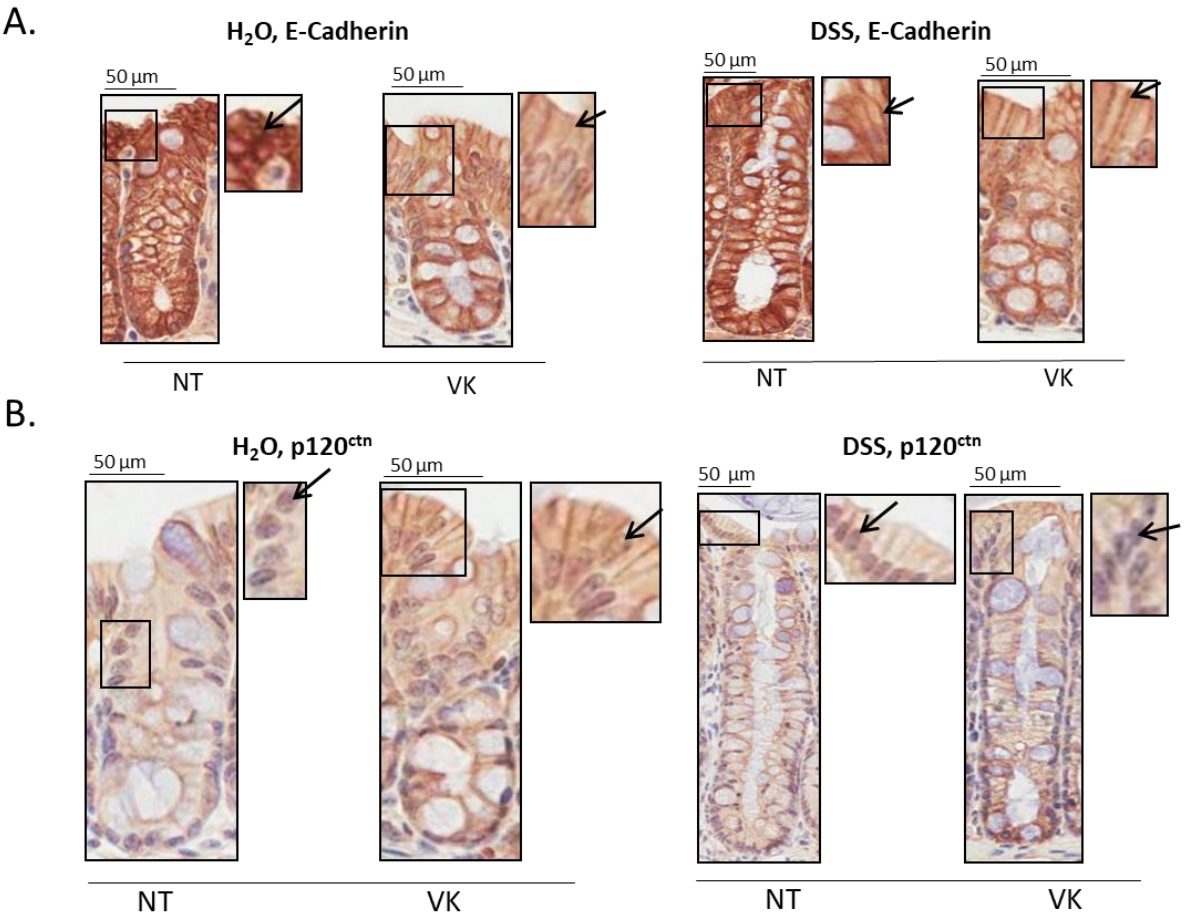



***Figure 7: Kaiso overexpression reduces E-Cadherin expression in the large Intestine.***

***(A)*** *Kaiso transgenic mice exhibit decreased E-Cadherin expression in cell membranes.* ***(B)*** *Kaiso overexpression causes decreased nuclear p120$^{ctn}$ expression in the DSS-treated mice, and higher nuclear p120$^{ctn}$ expression in the $H_2O$-treated mice.*

p120$^{ctn}$ levels in the presence of DSS while the $H_2O$ treated mice exhibit strong basal nuclear expression and strong membranous localization.

**Kaiso overexpression and AOM treatment synergistically promote excessive cell proliferation**

Azoxymethane (AOM) is a carcinogenic chemical that, when metabolized into the potent mutagen methylazoxymethanol (MAM) inside a cell, induces widespread structural damage to DNA, which leads to accelerated tumour formation in an inflamed microenvironment [75]. The AOM/DSS model has become the gold standard for recapitulating spontaneous CRC; the DSS induces intestinal inflammation, which in turn promotes cell survival, allowing the mutations caused by AOM to accumulate unhindered by cell cycle arrest, until hyperplasia develops into intestinal polyps.

To observe the effects of environmental carcinogens on Kaiso-induced inflammation, and to determine whether VK mice possess an inflammatory environment that is sufficient for AOM to initiate tumorigenesis, 31 C57BL/6 mice (16 NT and 15 VK) were divided into four treatment groups (n>7 for all groups) and injected IP with either AOM or a PBS vehicle control at 12.5 mg/kg bodyweight, once a week for 5 weeks, and then observed for 60 weeks before the mice reached endpoint and were culled for tissue analysis via IHC.

IHC analysis of the intestinal tissues confirmed the treatment-independent nature of Kaiso-mediated decreases in E-Cadherin expression (**Figure 8A).** There was a marked decrease in E-Cadherin expression in VK mice compared to their NT siblings, regardless of treatment group. p120$^{ctn}$ also showed negligible change in expression of subcellular localization between AOM and PBS-treated mice but maintained the characteristic nuclear localization in VK mice **(Figure 8B)**.

To assess malignancy changes in the intestinal epithelium, tissues were stained for the proliferation marker Ki67, which positively stains nuclei of actively dividing cells. **Figure 8C** displays a progression of increased proliferative migration between NT and VK

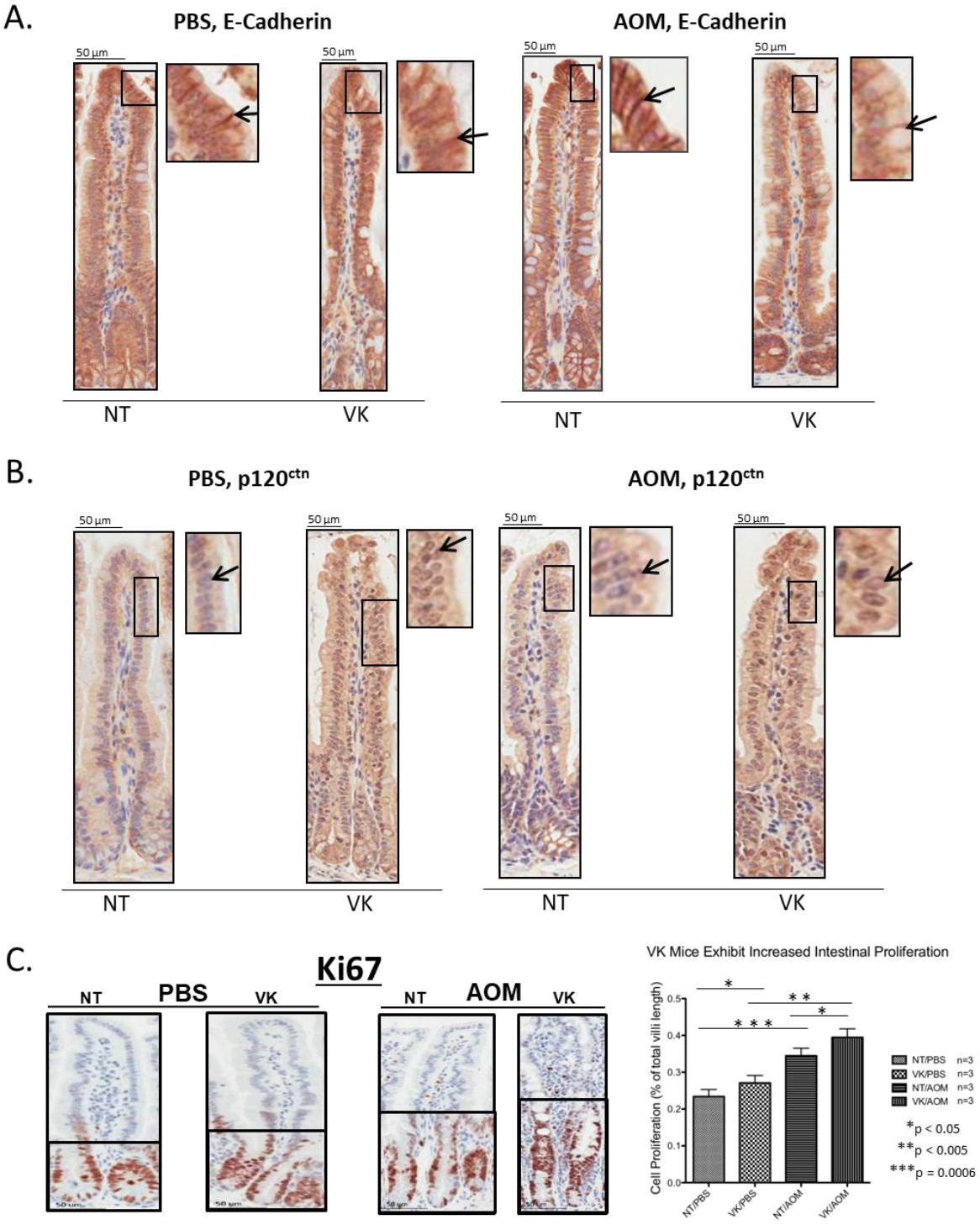



***Figure 8: Kaiso overexpression promotes aberrant expression of cell adhesion proteins and proliferation machinery: (A,B)*** *VK mice exhibit noticeably less E-Cadherin expression, and markedly higher p120$^{ctn}$ expression than controls after AOM treatment. AOM treatment appears to decrease E-Cadherin expression, and increase nuclear p120$^{ctn}$ expression.* ***(C)*** *VK mice and AOM-treated mice exhibit an expanded crypt phenotype, which facilitates cell proliferation in a higher percentage of intestinal epithelium when compared to controls. P values determined using standard t-tests.*

mice, and again between PBS and AOM-treated groups. NT mice treated with PBS exhibit basal levels of proliferation, which are significantly increased in their VK counterparts. AOM-treated NT mice exhibit a significantly higher level of cellular migration than control VK mice, but are in turn surpassed by the AOM-treated VK mice, which exhibit a synergistic phenotype, with the highest percentage of their intestinal tissues existing in a state of active cell division.

Kaiso overexpression promotes ectopic adhesion protein behaviour in AOM/DSS treated mice

To determine whether there was any interplay between Kaiso's inflammatory and oncogenic mechanisms with the disease progression of the spontaneous colon carcinoma modeled by AOM and DSS, thirty-five 8-week old (15 NT and 20 VK mice) mice were injected with an initial dose of 12.5 mg/kg bodyweight AOM, followed by three separate week-long treatments of 1% DSS water, interspersed with 14-day periods of $ddH_2O$ and ending with a 30 day recovery period of $ddH_2O$ before dissection and tissue collection .The intestinal tissues of these mice were analyzed via IHC visualization of cell adhesion proteins. IHC analysis revealed the consistent treatment-independent decrease in E-Cadherin expression found in previous studies, but we also observed an AOM+DSS induced decrease in E-Cadherin expression in NT mice **(Figure 9A)**. Interestingly, $p120^{ctn}$ is aberrantly expressed in AOM+DSS treated tissues; we observed high nuclear localization of $p120^{ctn}$ in AOM+DSS-treated VK mice, while NT mice expressed low nuclear $p120^{ctn}$. Additionally, $p120^{ctn}$ is expressed at similar levels in NT and VK control treated mice **(Figure 9B)**.

VK mice exhibit histological hallmarks of inflammation

To verify observations made regarding intestinal pathology in VK mice, 70> H&E stained tissues from multiple studies were scored by veterinarian pathologist Dr. Geoff Woods (Guelph Univ.) for specific inflammatory criteria such as tissue degradation, neutrophil infiltration, and thickened intestinal mucosa. According to the criteria, the

tissues were given a score of 0 (no inflammation), 1 (low inflammation), 2 (moderate inflammation) or 3 (high inflammation). Mice were also scored for the number of regions of hyperplasia present in their intestines based on signs of cancer formation, i.e. aberrant crypt foci (ACF), increased cell proliferation, etc.

Mice exposed to the pro-inflammatory chemical DSS exhibited high inflammatory scores, while VK mice exhibit a moderate inflammatory score **(Figure 10A-i)**. **Figure 10B** depicts the increased inflammatory levels in VK mice compared to the NT mice. Interestingly AOM-treated VK mice show a trend of increased inflammation (**Figure 10B-iii)** when compared to their control group counterparts. While the inflammation increased with AOM treatment, the number of hyperplasias remained unchanged, implying that AOM and Kaiso do not promote CRC in the same way that AOM+DSS does. Although AOM-treated mice did not exhibit signs of hyperplasia, 50% of DSS-treated mice did, implying a potentially tumorigenic property of the detergent.

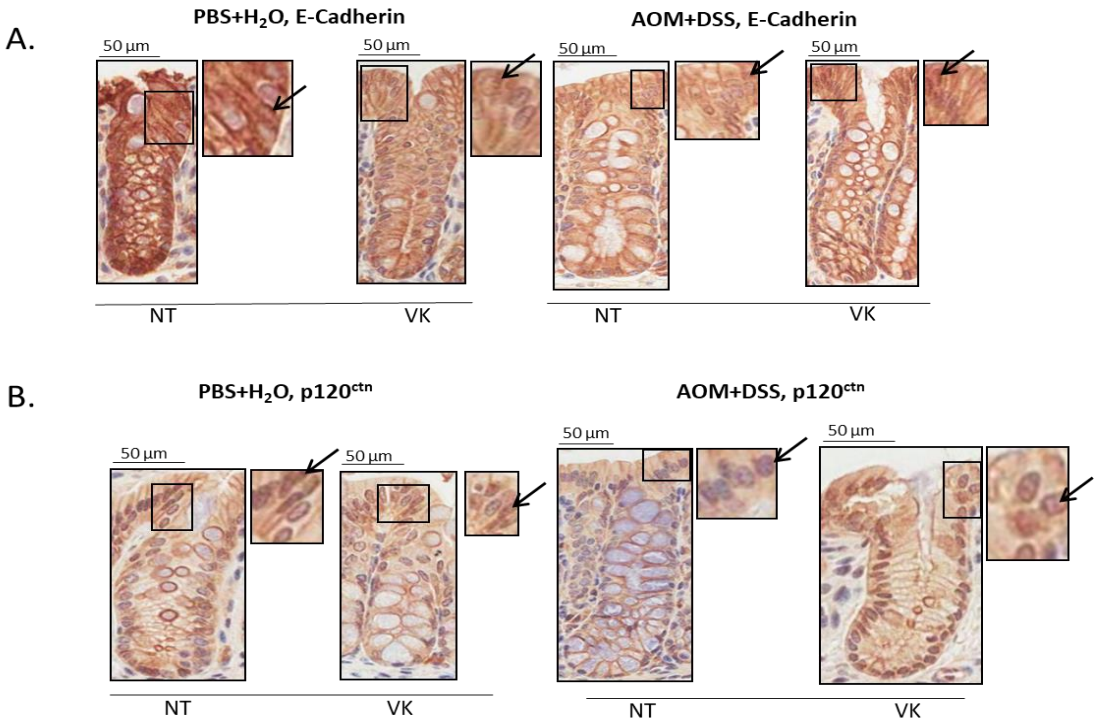
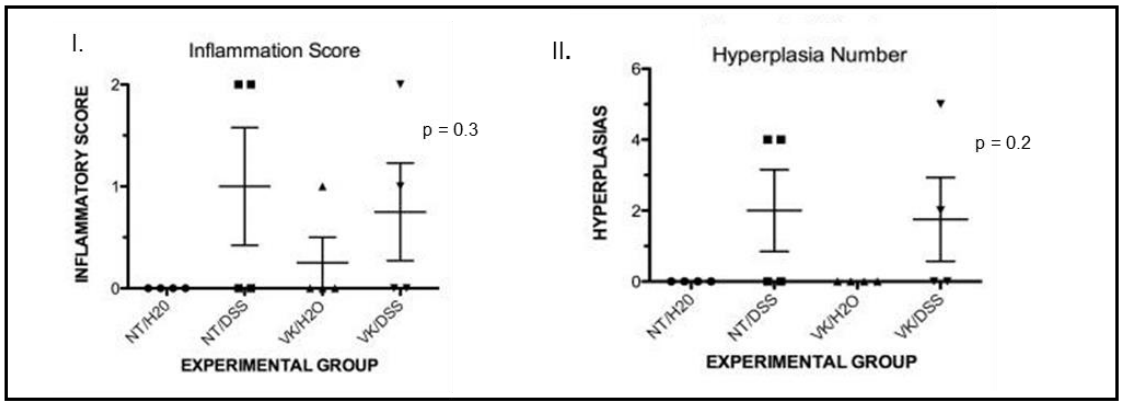



***Figure 9: AOM+DSS treated Kaiso$^{Tg}$ (VK) mice exhibit reduced membranous E-Cadherin expression; while Kaiso also increases nuclear p120$^{ctn}$ in an inflammatory microenvironment: (A)*** *Kaiso$^{Tg}$ mice exhibit low E-Cadherin expression, while AOM+DSS appears to mask all differences with its heavy reduction of E-Cadherin expression.* ***(B)*** *AOM+DSS treated Kaiso$^{Tg}$ mice exhibit higher nuclear p120$^{ctn}$ localization, and similar expression levels in controls.*

Kaiso promotes inflammation through interactions with NF-κB and p120$^{ctn}$

To better understand the inflammatory actions of Kaiso in mammalian systems, multiple experiments were conducted to evaluate NF-κB and Kaiso/p120$^{ctn}$ expression in cell lines, mice and human IBD tissue respectively. **Figure 11A** shows the strong correlation between high Kaiso expression and the pro-inflammatory molecule NF-κB in HCT116 CRC cells representing WT and shK (Kaiso depleted) genotypes. Preliminary western blot and densitometry data (Trials = 1) confirm the depletion of Kaiso in the HCT116 shK cells **(Figure 11A-ii)** and display the subsequent loss of NF-κB, which remains present in WT cells with basal Kaiso expression **(Figure 11A-iii)**. **Figure 11B** shows intestinal biopsy cores taken from a CD patient that have been stained for Kaiso and p120$^{ctn}$. These samples demonstrate strong nuclear Kaiso localization in CD patients which inversely correlates with weak p120$^{ctn}$ expression. Lastly, in order to apply the previous NF-κB findings to the VK mouse model, IHC analysis was conducted for the inflammatory protein, and revealed markedly increased NF-κB staining and nuclear localization in VK mice compared to their NT siblings **(Figure 11C)**.

A.




B.

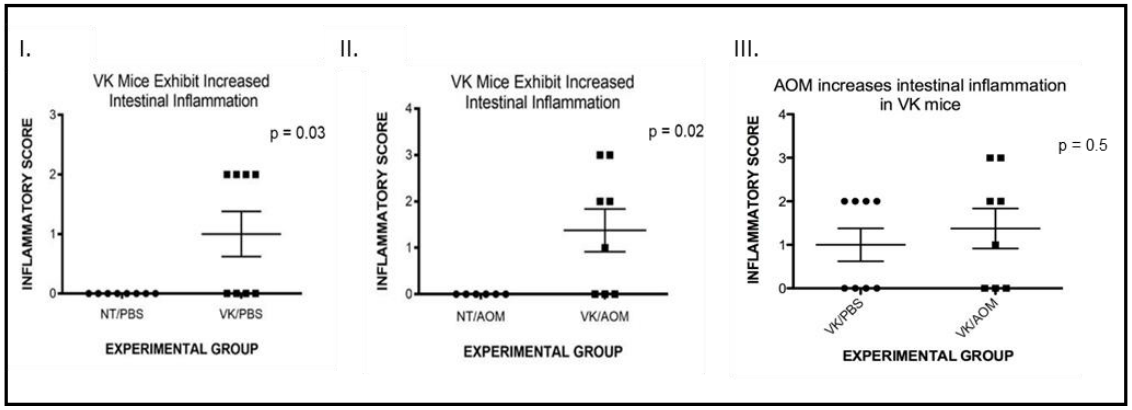



***Figure 10: VK mice present with higher inflammatory scores than their NT siblings:*** ***(A)*** *DSS treatment results in increased inflammation and hyperplasia in all treatment groups.* ***(B)*** *VK mice exhibit high inflammatory scores indicating higher levels of tissue degradation, neutrophil infiltration and goblet cell presence. Mouse tissues were scored by veterinary pathologist Dr. Geoff Wood, University of Guelph. Outliers identified using ROUT Q=1%.*

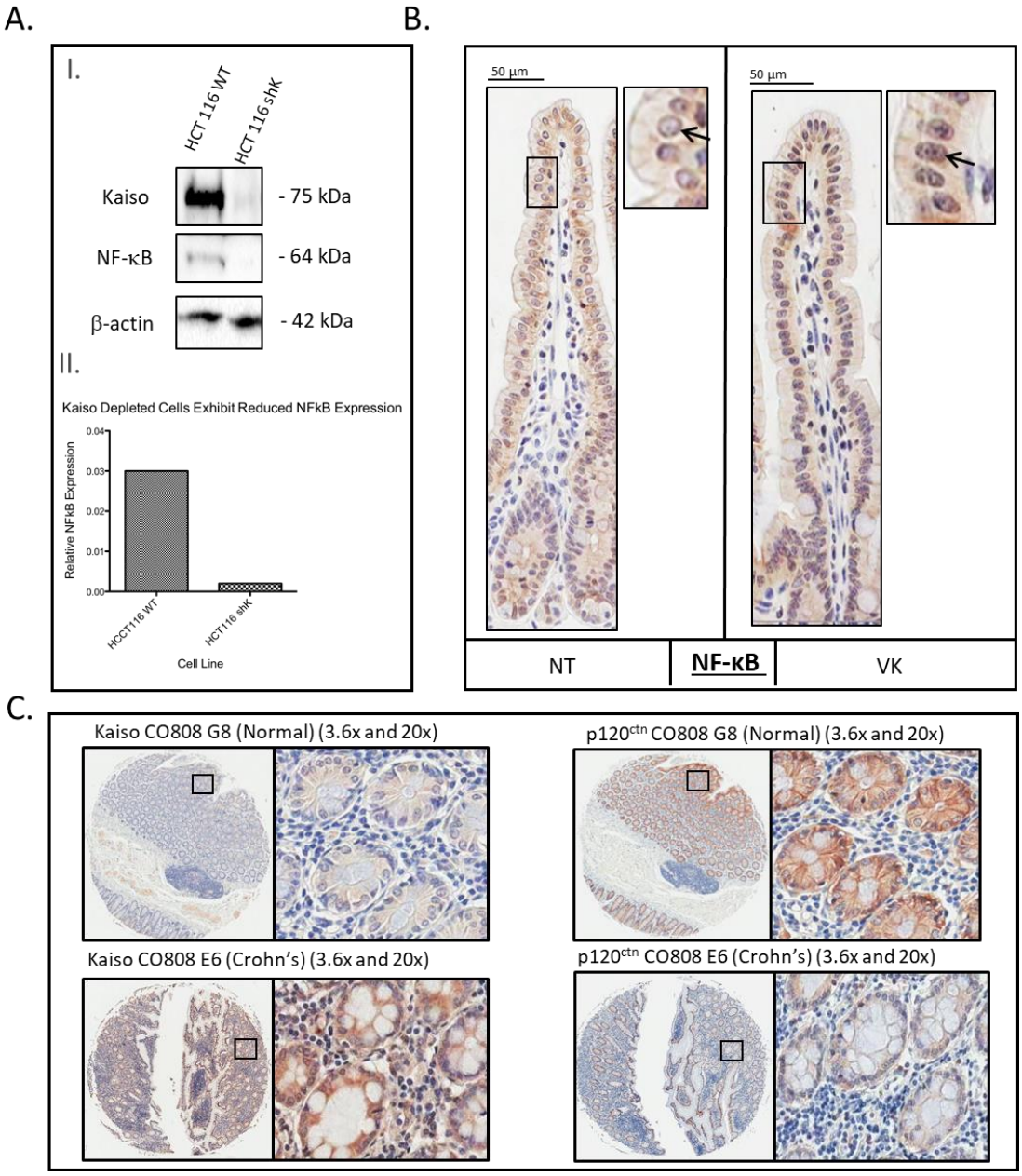



***Figure 11: Kaiso Promotes Inflammation By Altering Protein Expression: (A) (I)*** *Immunoblot of HCT116 colon tumor cell protein lysates show an inverse relationship between Kaiso and NF-κB expression in parental (WT) and Kaiso-depleted (shK) cells.* ***(II)*** *Densitometry analysis of the decreased NF-κB in shK cells.* ***(B)*** *Nuclear NF-κB expression is higher in VK mice than their NT siblings.* ***(C)*** *Human normal and Crohn's Disease (CD) tissue from U.S. Biomax TMA C0808 show an inverse expression profile for Kaiso and p120$^{ctn}$, with high Kaiso expression observed in the inflamed tissues, while p120$^{ctn}$ expression was higher in normal tissues.*

**DISCUSSION:**

VK mice exhibit an impeded weight gain phenotype

The VK mice in **Figure 6** display a consistent impeded weight gain phenotype, which is seen to act synergistically with other sources of health deterioration to further reduce body growth. **Figure 6A** shows the growth of VK and NT mice treated with AOM or PBS; the results showed that while the phenotype exists in both the treatment and control groups, the impeded weight gain is strongly exacerbated in the presence of AOM **(Figure 6A)**. The cause of the impeded weight gain phenotype of VK mice **(Figure 6)** is currently unknown but is likely related to the progressive inflammation these mice exhibit. Inflammation reduces the absorptive capabilities of the intestinal epithelium it affects, reducing nutrient uptake and fluid recycling, and often inflicting diarrhoea which may reduce body fluids dangerously fast. In addition, inflamed tissues typically exhibit an ATP production deficit [5, 20]. Hence, the ulcers and polyps formed in VK mice are most likely preventing their hosts from efficiently deriving energy and water from ingested food, and forcing a potentially large portion of the intestinal epithelium into an inflamed state of innate immunity, using up resources that could have otherwise been converted into adipose tissue.

DSS-treated mice exhibit a similar phenotype. The mice used in this study were 8 weeks old and treated either with DSS or $H_2O$ **(Materials Fig. 2)**. Typically, when mice are treated with DSS and then allowed to recover with no more DSS, the weight loss induced by acute colitis is rapidly reversed once DSS treatment halts. While this holds true for the NT/DSS mice in **(Figure 6B)**, the VK/DSS mice, did not recover from their weight loss. Interestingly this occurs at 8 weeks of age, ~3 months before the impeded weight gain phenotype manifest in untreated VK mice (~5 months). This finding suggests that VK mice are already experiencing growth and nutritional pressure at a young age.

Kaiso overexpression promotes aberrant expression and localization of cell adhesion proteins in DSS-treated mice

As recently reported by Robinson *et al.* [12], the well-known Kaiso-mediated repression of E-Cadherin in prostate and breast tumor cells is also present in CRC, and even begins in the pre-inflammatory stages of the intestinal epithelium [12, 39, 46]. This phenomenon is also confirmed in our model as seen in **Figure 7A**, where VK mice are seen to express noticeably less E-Cadherin; it should be noted that this interaction is independent of DSS treatment. DSS treatment itself did not seem to change E-Cadherin expression, but did reduce membrane localization, most likely a result of the detergent disrupting cell membrane lipids and cell adhesion interactions; which cause E-Cadherin to dissociate from the cell membrane, leading to the characteristic DSS-induced barrier infiltration. Likewise, Kaiso overexpression results in aberrant E-Cadherin function, disrupting the epithelial barrier and promoting inflammation[12].

The nature of Kaiso's "dance" with the adhesion molecule $p120^{ctn}$ is currently unknown; it is well documented however, that Kaiso and $p120^{ctn}$ heterodimerize via strategically placed $p120^{ctn}$ binding sites that allow the cell-adhesion stabilizer to bind and repress Kaiso's DNA-binding abilities by blocking the C-terminus ZF motifs [34, 35]. Additionally, an abundance of nuclear Kaiso is thought to recruit $p120^{ctn}$ away from the cell membrane and into the nucleus where it regulates Kaiso transcriptional activity, at the expense of the E-Cadherin stabilizing and anti-inflammatory properties that $p120^{ctn}$ usually exhibits when it is localized to the membrane or cytosol [42, 47]. If Kaiso over expression sequesters $p120^{ctn}$ in the nucleus, then it is noteworthy that VK mice exhibit low nuclear $p120^{ctn}$ expression when treated with DSS **(Figure 7B)**, another inflammatory initiator. In fact, we see the hallmark nuclear localization of $p120^{ctn}$ in the VK mice treated with control ddH2O, implying that DSS itself is causing a reduction of total $p120^{ctn}$ expression, or inhibiting the nuclear localization in VK mice. Indeed, total

p120$^{ctn}$ concentrations appear decreased by DSS treatment; perhaps via the destabilized cell adhesion complexes that p120$^{ctn}$ works to support.

Kaiso overexpression and AOM treatment synergistically promote excessive cell proliferation

Although AOM generally requires co-treatment of DSS to initiate tumorigenesis, we hypothesized that the endogenous inflammation previously observed in VK mice [12] would provide a sufficient environment for AOM-induced tumour initiation, and DSS would not be needed for AOM to act in an effective manner. Additionally, the AOM/DSS model recommends a single AOM injection at the beginning of the study, followed by DSS treatment. In order to boost the carcinogenic hit, the mice in the AOM study received five separate IP injections of AOM, starting at 8 weeks of age. Despite the additional AOM injections, the mice did not develop disease at the rate we expected. Indeed, the majority of the mice survived the entire 60 weeks, a surprisingly long time for mice with progressive colitis and five carcinogenic exposures. This unusual longevity is the product of multiple known factors, in conjunction with any other unknown influencers. Firstly, VK mice belong to the C57BL/6 subspecies of lab mice which are reported to possess moderate resistance to AOM-induced mutations [68]; this genetic background would undoubtedly hinder the progression of malignancy in AOM models. Secondly, Kaiso overexpression does not induce the same level of inflammation as DSS treatment, and thus does not provide an adequate replacement for the detergent. Kaiso-induced colitis is a chronic, progressive disease that does not manifest until 12 weeks of age, at which point these mice will be receiving the last of their AOM injections; DSS treatment however causes severe acute inflammation that manifests within days of exposure, providing an ideal transient opportunity for AOM to mutate DNA before the tissues recover enough to regain regulatory processes. Furthermore, VK mice exhibit a very specific type of intestinal inflammation that presents discontinuously throughout the intestinal tract, inhibits TH1 cytokines and progresses at a relatively

sluggish rate [5, 11, 33]. These symptoms are reminiscent of CD, a subset of IBD that produces a relatively small percentage of global CAC cases. If VK mice recapitulate CD, then not only will their cancer form at a slower rate, but cancer will form in the small bowel as well as the large intestine. This theory would represent a revision to the prevailing model of Kaiso induced CAC; While the initiation would be slower, the cancer would undoubtedly behave in a manner more similar to human disease. Additionally, if VK mice indeed recapitulate CD, then they will instantly become an invaluable tool for research on Crohn's mechanisms, and this will highlight Kaiso as a potential therapeutic target for treating CD patients.

In line with this theory, **Figure 8A** and **Figure 8B** display VK-mediated repression of E-Cadherin, and nuclear localization of $p120^{ctn}$, which appears to occur independently of the ectopically disorganized expression of both E-Cadherin and $p120^{ctn}$ in the presence of AOM. This suggests that AOM does not directly influence the pathway by which Kaiso regulates these proteins.

When treating mice with AOM, it is logical to analyze the slides for uncontrolled cell growth, a typical precursor to polyp formation. Also, when dealing with the intestines it is important to remember that the stem cells are housed solely at the base of the crypt where they produce daughter "transit amplifying cells" that ascend the crypt-villus access as they differentiate. In order to quantify the levels of epithelial proliferation, tissues were stained for the proliferation marker Ki67, which is a highly conserved protein among all mammalian dividing cells that will never leave the nucleus due to inextricable DNA interactions. These properties make Ki67 an ideal target for identifying levels of cellular multiplication. Every brown-stained nuclei represents an actively dividing cell, and these cells can be used to quantify the "percent positive cells" which indicates the specific number of dividing cells compared to the total epithelium. Alternatively, the distance the cells migrate from the stem cell compartment, up the crypt-villi axis can be used to quantify the migratory distance of these dividing cells.

In an epithelium such as the intestines, the total number of cells does not generally change until EMT is achieved and cells are able to free themselves from the extracellular matrix. In the intestines, the cells move in a "conveyer belt" pattern, with each new daughter cell displacing the previously produced progeny cell, and pushing it towards the intestinal lumen and along the crypt villi axis (See **Figure 1**). Once cells reach the villus tip, their adhesive anchors are released and they are targeted for destruction by anoikis. For this reason, it is beneficial to observe the migratory distance of proliferating cells, as opposed to the sheer amount of cell division. As seen in **Figure 8C**, proliferating cells migrate significantly farther in VK mice than their NT siblings (As supported by Robinson et al. [12]), and this phenotype is even further exacerbated by AOM treatment, resulting in ~15% higher percentage of their tissues multiplying than the negative controls. Interestingly, the VK mice treated with AOM exhibit significantly increased proliferative migration, but do not seem to exhibit a significant increase in individual replicating cells. This finding suggests that Kaiso overexpression may modulate the rate of cellular differentiation and turnover. An increase in intestinal apoptosis would cause an increase of cellular migration, while leaving the total number of cells unchanged, due to the "conveyor belt" mechanisms of the intestinal epithelium.

This result was entirely expected, as AOM and Kaiso have both been implicated in accelerated tumour progression individually. These results show a synergistic relationship between Kaiso and AOM-mediated proliferation, implying unique mechanisms of action. It should be noted that AOM treated VK mice exhibit markedly higher amounts of immune cell recruitment and tissue degradation, forming blunted and fused villi, with extended crypt abscesses. The physical insults to the tissue and over-abundance of immune cells are indicative of inflammation, and represent a precursor to polyp formation, which will progress into a tumour if left untreated.

Kaiso overexpression promotes ectopic adhesion protein behaviour in AOM/DSS treated mice

AOM and DSS are often used together to simulate human spontaneous colon carcinoma using mice; generally utilizing 2-3% DSS water and 10-13 mg/kg bodyweight AOM dosage. As Kaiso-overexpression had proved an insufficient source of acute inflammation, we hypothesized that adding an unconventionally low dose of DSS to the treatment would be enough to push the inflammation past the threshold required for optimal progression of AOM induced malignancy. Using 1% DSS we conducted several cycles of transient exposure following an IP administered AOM or PBS dose, which extended significantly longer than the DSS study displayed in **Figure 7**. As expected, E-Cadherin expression remained consistently lower in VK mice, with additional repression exhibited by the AOM+DSS treated group in **Figure 9A**. However, $p120^{ctn}$ behaviour continues to complicate the matter.

$p120^{ctn}$ expression in AOM+DSS treated mice appears to act inversely to $p120^{ctn}$ expression in mice treated with DSS alone **(Figures 9B and 7B)**. NT mice in the treatment group present with relatively low nuclear $p120^{ctn}$ localization, while the VK mice exhibit robust nuclear staining for $p120^{ctn}$ in the presence of AOM+DSS. This localization is the exact opposite of the results seen in the DSS study, where NT mice favoured nuclear $p120^{ctn}$ and VK mice prevented nuclear translocation **(Figure 9B)**. This ectopic $p120^{ctn}$ localization only occurs in the presence of AOM and DSS together, suggesting that the potent carcinogenic result of combination treatment drives this context dependent response. This interaction is unexpected and warrants additional study to rule out artefacts; despite steps taken to ensure result replication (all results presented are validated by at least three consistent IHC trials from tissues of three separate mice representing each treatment group). This localization is unexpected due to Kaiso's ability to recruit $p120^{ctn}$ to the nucleus, despite the strong affinity of $p120^{ctn}$ for the membrane localized processes. $p120^{ctn}$ should only enter the nucleus as a binding

partner for specific POZ-ZF proteins, and such strong nuclear expression **(Figure 9B)** hints at the existence of additional nuclear $p120^{ctn}$-binding partners. Or perhaps this is due to unrelated AOM+DSS induced stimuli [34, 35, 42].

Continuing the trend of unconventional $p120^{ctn}$ cellular localization, we observed that control treated NT and VK mice exhibit comparable concentrations of $p120^{ctn}$ in the nucleus, cytosol and cell membrane; once again countering previous reports that Kaiso overexpression increases nuclear $p120^{ctn}$ localization. This unexpected finding sis likely related to the previous unexpected results seen in the treatment group of this study but cannot be attributed to confounding AOM+DSS factors. Interestingly, unpublished data from IHC analysis of human samples in our lab suggests that Kaiso either reduces total $p120^{ctn}$ expression, or masks $p120^{ctn}$ antigens in inflamed tissues, rendering the protein difficult to visualize and giving the appearance of ectopic expression (Unpublished CRC TMA Data, Daniel Lab). Naturally, variance may also occur due to a plethora of potential sources of error including the processes of: tissue processing, embedding and preparation, as well as antibody contamination and insufficient blocking (leading to excessive unspecific staining).

VK mice exhibit histological hallmarks of inflammation

Inflammation is typically accompanied by a host of genetic, cellular, and histological mechanisms that form a pattern easily recognized by a trained eye, especially those of a pathologist who use their expertise and experience to recognize these patterns and convert qualitative observations into quantitative data. This is possibly due to the remarkably uniform appearance of the many inflammatory hallmarks such as neutrophil infiltration, an immediately obvious physiological feature characterized by the excessive accumulation of immune cells in the LP. Other ubiquitous inflammatory markers include: thickened intestinal sub-mucosa, degraded tissues (blunted villi, expanded crypts, fused epithelial superstructure), and the presence of genetic markers such NF-κB, TNFα and secreted TH2 cytokines [5, 10, 11, 20, 33].

Using these criteria, a pathologist blindly analyzed DSS and AOM-treated intestinal segments stained with the general tissue dyes haematoxylin, a basic dye used to stain DNA and RNA and eosin, an acidic dye used to stain cytoplasmic and cytoskeletal proteins (H&E). The pathologist provided inflammatory scores which were then used to produce **Figure 10**. Unsurprisingly, DSS was the greatest contributor of inflammation in **Figure 10A-i**, which overpowered the relatively low inflammatory effects of Kaiso, which achieved a score of 1 in the H2O treated cohort of VK mice. In the AOM and PBS treated groups, VK mice exhibit high inflammation across both treatments' groups, and NT mice achieved no positive scores for inflammation as expected **(Figure 10B)**. Interestingly, when comparing PBS and AOM treated VK mice, it is apparent that AOM exacerbates the endogenous inflammation of VK mice, which is notable due to the fact that AOM does not generally initiate inflammation, and tends to instead initiate tumorigenesis in a pre-inflamed micro-environment such as VK mice. AOM's interaction with Kaiso-mediated inflammation is corroborated by the synergistic cellular migration observed in **Figure 8C** and together these findings support a model of AOM-accelerated VK inflammation.

Even more surprising than the observation of AOM-driven inflammation, is the observation that AOM-treated mice exhibit no signs of hyperplasia. This finding supports the hypothesis that VK is not a replacement for DSS treatment, and both promote carcinogenesis through different pathways. While AOM-treated mice developed no visible hyperplasia, we found that DSS mice develop oncogenic traits in a fraction of the expected time. DSS-mediated hyperplasia appears to develop independently of Kaiso, and the cancer forming properties of VK were dwarfed when measured against the harsh detergent.

Kaiso promotes inflammation through interactions with NF-κB and $p120^{ctn}$

Kaiso-mediated inflammation is a previously documented affliction, and yet very little is known about the mechanisms by which this inflammation is induced and

maintained [12, 40, 41]. In all likelihood the inflammation is the result of a labyrinthine combination of signaling pathways and their surfeit of crosstalk; new protein-protein and promoter binding targets are discovered daily, muddying the water but opening new avenues of Kaiso research. Two of the avenues most frequently traveled are those of the $p120^{ctn}$ and NF-κB interactions, which are two proteins that each interact with Kaiso, as well as with each other. Independently of Kaiso, $p120^{ctn}$ acts as a cytosolic inhibitor of NF-κB's pro-inflammatory functions. This regulation is mediated through the Rho/ROCK pathway and results in stabilization of IκB, the cytoplasmic NF-κB inhibitor, which continues to sequester inactive NF-κB outside of the nucleus. When nuclear Kaiso concentrations increase, $p120^{ctn}$ localization is switched from the cytoplasm into the nucleus where $p120^{ctn}$ binds to Kaiso, inhibiting certain promoter interactions. When $p120^{ctn}$ leaves the cytoplasm, it can no longer inhibit NF-κB via the Rho/ROCK pathway, and more importantly it cannot perform its primary function of E-Cadherin stabilization, which results in epithelial barrier defects and allows for inappropriate EMT.

In support of the aforementioned mechanism, **Figure 11A-i** depicts a preliminary immunoblot for Kaiso and NF-κB using cell lysates from WT and shK (Kaiso depleted) HCT116 CRC cells. Furthermore, using densitometry (n=1) we confirmed a preliminary correlation between Kaiso and NF-κB expression, demonstrating strong Kaiso and NF-κB expression in WT cells, that is absent in Kaiso depleted cell lines **(Figure11A-ii/iii)**. This Kaiso and NF-κB relationship is supported by evidence seen in the VK mouse model. **Figure 11B** exhibits a noticeable increase of nuclear NF-κB expression in the villi of VK mice in contrast to their NT siblings. This relationship between Kaiso and NF-κB in intestinal cells that we see in our *in vivo* mouse model and in our *in vitro* cultured CRC tissues is likely regulated on multiple levels, including the $p120^{ctn}$ mediated Rho/ROCK stabilization of IκB [47], the p300/p53/Kaiso-RelA/p65 protein complex [44] and potential direct promoter binding that is teased by multiple KBS and methylated CpG islands in the p65/RelA and p50 promoter regions (Unpublished Data, Daniel Lab).

With extensive data from VK murine models and preliminary data from CRC cell lines, the last unexplored avenue in the Kaiso-mediated inflammation puzzle lay with clinical research. While clinical research extended beyond the scope of this project, we decided to analyze human CRC tissues by staining a human intestinal disease TMA for proteins of interest, including $p120^{ctn}$, Kaiso and NF-κB. The results of $p120^{ctn}$ and Kaiso IHC staining in normal and CD tissues are displayed in **Figure 11C** where inflamed cells appear to express abnormally high Kaiso levels and correspondingly low $p120^{ctn}$ levels in the same regions. Inversely, $p120^{ctn}$ expression is relatively high in normal, un-inflamed tissues, while Kaiso expression is quite low, supporting the notion of an inverse relationship between Kaiso and $p120^{ctn}$ interaction in the inflammatory microenvironment.

Reduced nuclear $p120^{ctn}$ expression in the presence of Kaiso could result from Kaiso masking the target antigen of $p120^{ctn}$, which is often phosphorylated, and may be remedied by the identification of alternative epitopes and clones that may maintain access throughout the binding event, or by using a more specific primary antibody (phospho-$p120^{ctn}$). This observation of aberrant Kaiso expression in CD patients supports the findings of Robinson *et al.* [12] who observed the same results in a different TMA of CD biopsy cores. High Kaiso expression in CD patients supports the hypothesis that VK mice recapitulate the specific inflammatory progression of human Crohn's Disease. This hypothesis is supported by the physical presentation of VK inflammation (discontinuous ulcers throughout the GI tract), as well as genetic data from unpublished RNA-seq results that links Kaiso to the inhibition of important anti-inflammatory TH1 cytokines that are directly manipulated in human CD (Daniel lab, unpublished data).

Current models of inflammation and CRC include the genetic model $APC^{min/+}$, which contains a truncating mutation in the Wnt pathway tumour suppressor; APC. This causes excessive Wnt stimulation and rapid tumour formation. Previously, our lab has crossed the VK mouse lines with $APC^{min/+}$ mice and found that the progeny had a much

higher tumour load and tumour size, and experienced a short survival. To determine if the endogenous inflammation in VK mice was exacerbating the tumour growth in the VK/ $APC^{min/+}$ mice, we treated these mice with the general anti-inflammatory aspirin, and hypothesized that tumour burden would be rescued/reversed by aspirin treatment. The tissues are currently under analysis for IHC and histological scoring, but the mouse weights throughout the study may be found in **Appendix Fig. 2**, which shows no effect of aspirin treatment on mouse growth.

Our original hypothesis stated that VK mice would aberrantly express tumorigenic and pro-inflammatory proteins, in addition to forming intestinal polyps, when treated with carcinogens, inflammatory inducers, or when mated to the $APC^{min/+}$ model of spontaneous CRC. The null hypothesis stated that VK mice would express target proteins in a normal range and that polyps would not form with exposure to aforementioned conditions. The null hypothesis has been disproven by the findings that carcinogenic and inflammatory exposure altered expression level in proteins of interest, and hyperplasia developed in DSS treated mice, which represent the precursors of polyps. The validity of the VK murine model of colitis depends on the elucidation of specific mechanisms and signaling pathways involved in the endogenous inflammatory response. Interestingly, it was recently discovered that VK mice very accurately recapitulate the specific clinical, molecular, and genetic conditions seen in CD patients. VK mice present with relatively slow growing, discontinuous lesions throughout the intestine, as is seen in CD patients. Additionally, the exact anti-inflammatory TH1 cytokines inhibited in CD tissues are repressed by Kaiso over-expression. Lastly, human CD biopsies have shown markedly higher Kaiso concentrations than normal intestine controls, verifying the presence of Kaiso over-expression in patients suffering from CD. Further research into the relationship is warranted, but it could potentially provide a therapeutic target for a chronic, incurable disease like CD, providing symptom relief and peace of mind previously unknown to these patients. Even if Kaiso does not provide an

effective therapeutic target, the VK model could potentially provide the most accurate *in vivo* model ever seen in the field of gastro-enterology. With accurate and useful animal models to study, researchers worldwide would benefit from the resources, accelerating CRC, CAC and IBD research exponentially.

## Conclusion, Significance and Future Directions:

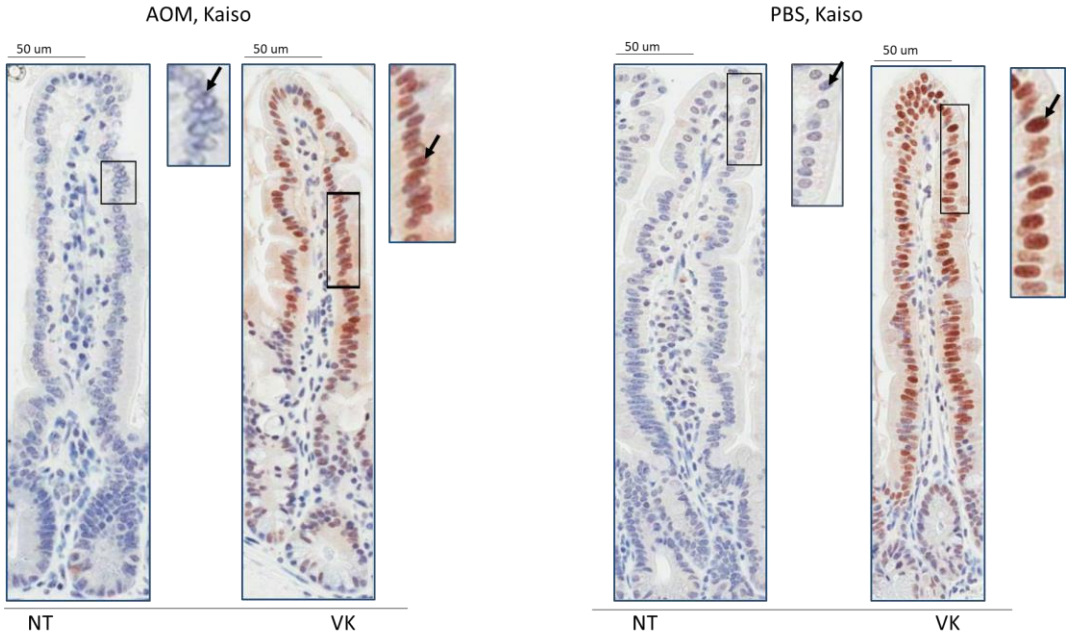
Kaiso's role in mammalian systems is strikingly complex, and even when isolated to the arena of intestinal inflammation, the promiscuous TF does not idly her secrets tell, which often leaves researchers with more questions than they started with. To minimize confounding factors and study Kaiso in the isolated environment of the gut, VK mice were generated and characterized through exposure to carcinogens, pro-inflammatory molecules, and genetic mutation. In less than a decade, VK mice have been identified as a promising model of IBD-to-CAC transition and could fill an empty niche and supply a source of reliable, specific colitis and CRC modelling.

Indeed, the research outlined in this thesis supports the claim of an integral role for Kaiso in colitis through NF-κB regulation, $p120^{ctn}$ interaction and loss of epithelial barrier integrity. However it is rarely that simple and contradictory results regarding $p120^{ctn}$ further complicate the picture. To further understand the interaction between Kaiso and $p120^{ctn}$ it would be wise to investigate the effects of Kaiso over-expression in a low $p120^{ctn}$ expression environment. $p120^{ctn}$ ablation should be generated in the VK mouse model, or $p120^{ctn}$ KO mice should be mated with the VK model, with an accompanying RNA-sequencing assay to identify any affected gene expression. Several additional assays may be conducted to further characterize the Kaiso- $p120^{ctn}$ relationship: immunofluorescence would allow researchers to visualize protein co-localization, re-ChIP would detect any DNA interactions with the protein complex, and immunoblot analysis may of protein lysates from intestinal epithelial cells of the $p120^{ctn}$ null VK mice may determine any changes to protein concentrations. Additionally, results from this study and the presence of potential KBS and methylated promoter binding

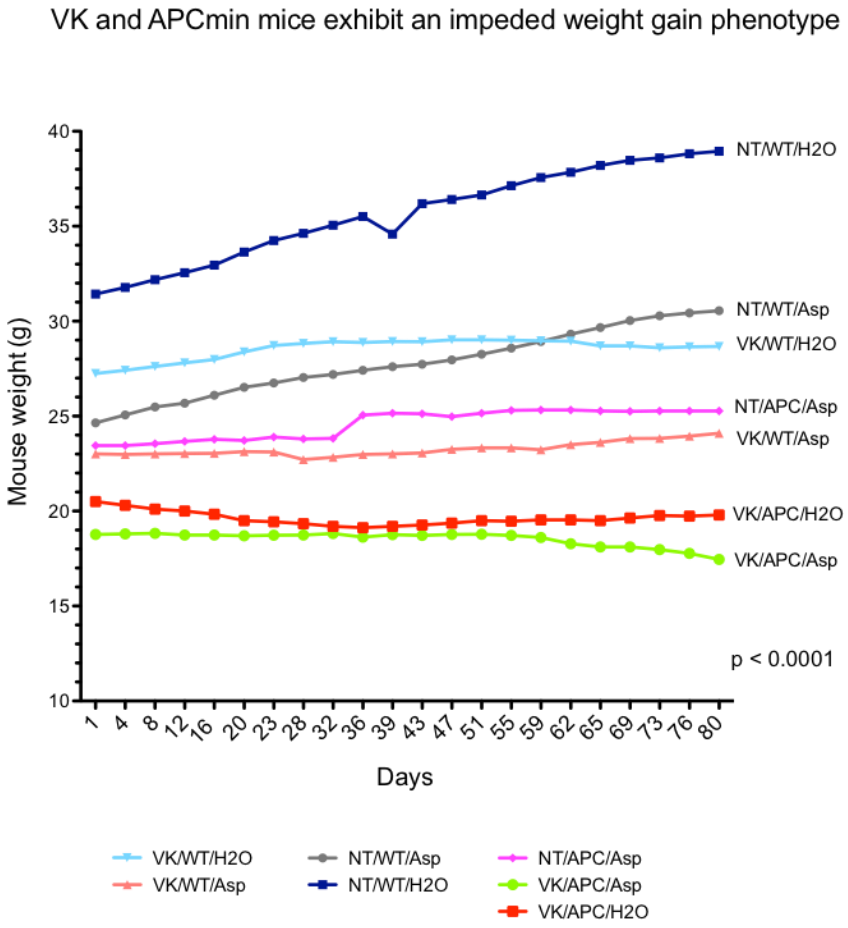
regions in the regulatory regions of NF-κB subunit encoding exons provides rationale for a ChIP study to elucidate the nature of Kaiso-NF-κB interplay. NF-κB staining of human tissues and co-localization staining in murine tissues may help characterize the relationships between NF-κB, Kaiso and $p120^{ctn}$.

These results implicate a role for Kaiso in gut immunity, and maybe lead to the characterization of a novel murine IBD model for a research field bereft of suitable *in vivo* research subjects. Kaiso's interactions support logical theories that provide numerous methods of action for this inflammation, and further investigation is necessary for proper characterization.

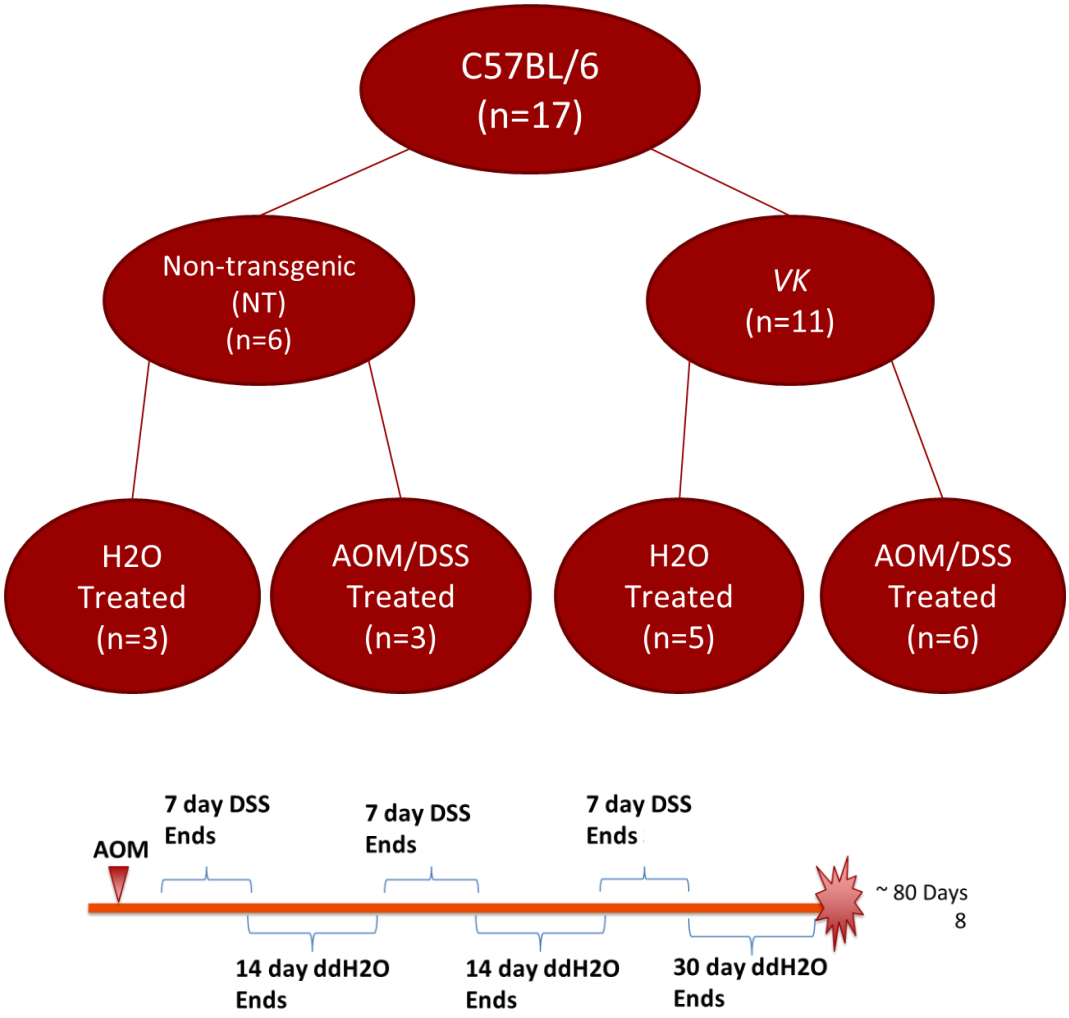
# Appendix



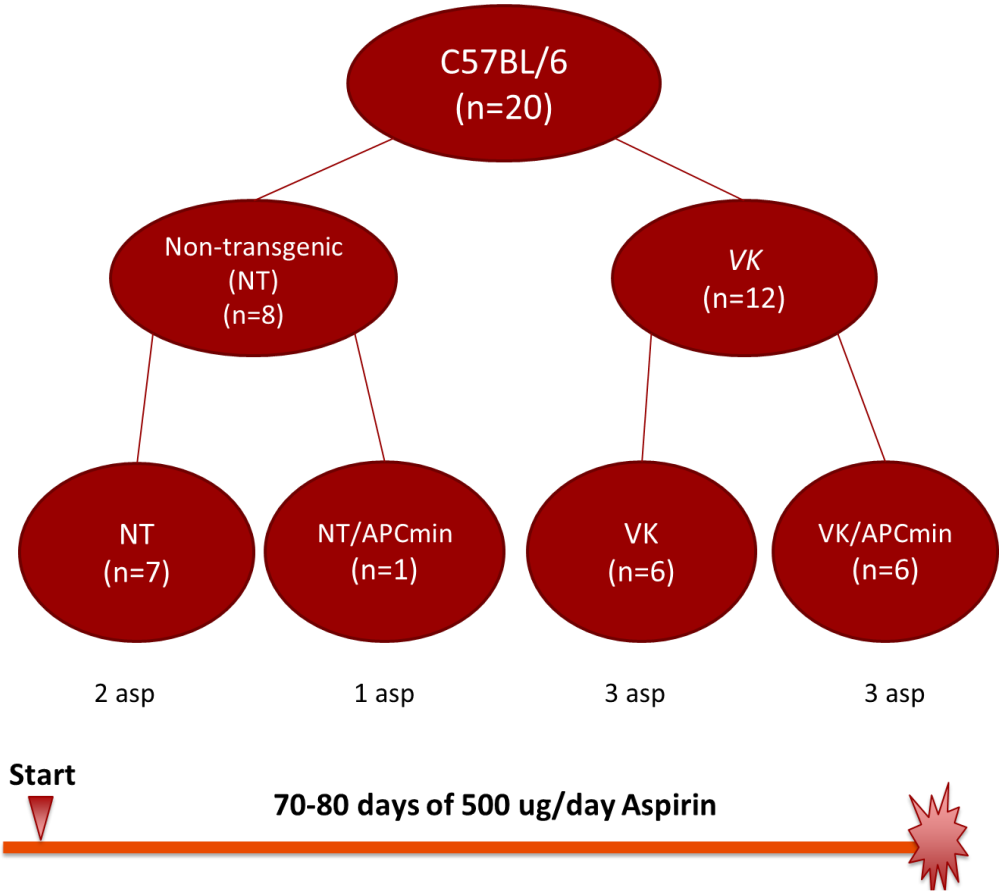

*Appendix Figure 1: Kaiso expression is increased in VK mice*




*Appendix Figure 2: Aspirin treatment does not affect VK growth*




*Appendix Figure 3: AOM + DSS treatment plan trial 2*




*Appendix Figure 4: Aspirin treatment plan trial 2*

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