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Kaiso overexpression promotes intestinal inflammation and potentiates intestinal tumorigenesis in $Apc^{Min/+}$ mice

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ABSTRACT

Constitutive Wnt/β-catenin signaling is a key contributor to colorectal cancer (CRC). Although inactivation of the tumor suppressor adenomatous polyposis coli (APC) is recognized as an early event in CRC development, it is the accumulation of multiple subsequent oncogenic insults facilitates malignant transformation. One potential contributor to colorectal carcinogenesis is the POZ-ZF transcription factor Kaiso, whose depletion extends lifespan and delays polyp onset in the widely used $Apc^{Min/+}$ mouse model of intestinal cancer. These findings suggested that Kaiso potentiates intestinal tumorigenesis, but this was paradoxical as Kaiso was previously implicated as a negative regulator of Wnt/β-catenin signaling. To resolve Kaiso's role in intestinal tumorigenesis and canonical Wnt signaling, we generated a transgenic mouse model ($Kaiso^{Tg/+}$) expressing an intestinal-specific myc-tagged Kaiso transgene. We then mated $Kaiso^{Tg/+}$ and $Apc^{Min/+}$ mice to generate $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice for further characterization. $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibited reduced lifespan and increased polyp multiplicity compared to $Apc^{Min/+}$ mice. Consistent with this murine phenotype, we found increased Kaiso expression in human CRC tissue, supporting a role for Kaiso in human CRC. Interestingly, Wnt target gene expression was increased in $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice, suggesting that Kaiso's function as a negative regulator of canonical Wnt signaling, as seen in *Xenopus*, is not maintained in this context. Notably, $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibited increased inflammation and activation of NFκB signaling compared to their $Apc^{Min/+}$ counterparts. This phenotype was consistent with our previous report that $Kaiso^{Tg/+}$ mice exhibit chronic intestinal inflammation. Together our findings highlight a role for Kaiso in promoting Wnt signaling, inflammation and tumorigenesis in the mammalian intestine.

1. Introduction

Uncontrolled Wnt signaling is a key contributor to CRC and identifying proteins or molecules that modulate this pathway have great potential for drug development for CRC treatment. In the past decade several new Wnt/β-catenin regulators and pathways that negatively or positively modulate Wnt signaling have been discovered but a thorough understanding of their roles in CRC remains unknown [1]. One novel Wnt/β-catenin regulator is the POZ-ZF transcription factor Kaiso that was first identified as a binding partner for the Armadillo catenin and cell adhesion cofactor $p120^{ctn}$ [2]. Kaiso is a unique dual-specificity POZ-ZF transcription factor that binds DNA at methylated CpG dinucleotides or a specific sequence known as the Kaiso Binding Site (KBS) [2–4]. Although few *bona fide* Kaiso target genes have been characterized to date, evidence from *Xenopus* embryos and mammalian cultured cells implicates Kaiso as a negative regulator of Wnt signaling, possibly via its interaction with members of the Tcf family of transcription factors and repression of Wnt target genes [5–8]. Surprisingly however, Kaiso depletion results in delayed polyp onset and prolonged lifespan in the $Apc^{Min/+}$ mouse model of intestinal neoplasia [9]. $Apc^{Min/+}$ mice carry a nonsense mutation in codon 850 of the *Apc* gene, which leads to a truncated and non-functional Apc polypeptide, nuclear accumulation of β-catenin and constitutive activation of Wnt target genes [10]. Given that Kaiso has been implicated as a negative regulator of Wnt signaling, the unexpected delayed polyp onset in Kaiso-null $Apc^{Min/+}$ mice may be independent of Kaiso's function in Wnt signaling. Indeed, Kaiso has been implicated in CRC progression via non-Wnt mechanisms [11], but no study has specifically examined the effects of Kaiso's overexpression on CRC development in mouse models.

Previously, we found that intestinal-specific $Kaiso^{Tg/+}$ mice exhibited several phenotypic abnormalities including hyperplasia, villi fusion

Abbreviations: CRC, colorectal cancer; APC, adenomatous polyposis coli; KBS, Kaiso binding site; CAC, colitis-associated cancer; IBD, inflammatory bowel disease

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and crypt expansion [12]. Interestingly, *Kaiso*$^{Tg/+}$ mice also exhibited increased leukocyte infiltration of the lamina propria and neutrophil activation, hinting that Kaiso may drive inflammation in the intestine [12]. This idea is further supported by the finding that mice with limited ablation of p120^{ctn} (i.e. unregulated Kaiso function) in the intestine developed chronic inflammation and adenomas [12]. Approximately 20% of all colorectal cancers can be attributed to genetic and familial syndromes, while the remainder are due to sporadic mutations influenced by environmental factors [13,14]. Colitis-associated cancer (CAC) is a CRC subtype that results from the occurrence of clinically detectable, chronic intestinal inflammation in patients with inflammatory bowel disease (IBD) [15–17]. IBD is characterized as the overactive immune response to intestinal microbiota and other environmental stimuli in genetically predisposed individuals, and increasing evidence indicates that IBD increases the risk of CRC by up to 20% [18–20]. Our findings in *Kaiso*$^{Tg/+}$ mice, combined with previous studies implicating Kaiso in CRC [9,11], led us to hypothesize that Kaiso may potentiate *Apc*$^{Min/+}$ tumorigenesis by predisposing the intestinal epithelium to inflammation in a mechanism akin to CAC.

In this study, we mated our *Kaiso*$^{Tg/+}$ mice with *Apc*$^{Min/+}$ mice to generate *Kaiso*$^{Tg/+}$*: Apc*$^{Min/+}$ mice. Ectopic Kaiso expression significantly shortened the lifespan of *Apc*$^{Min/+}$ mice and resulted in approximately 3-fold more polyps. *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice also presented with extensive regions of atypical hyperplasia, increased mitoses and focal crypt abscesses, which were largely absent or present at a lower frequency in age-matched *Apc*$^{Min/+}$ mice. Examination of Wnt target gene expression in *Kaiso*$^{Tg/+}$ mice revealed that ectopic Kaiso expression increases the expression of Wnt target genes, and this effect is further amplified in *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice. Lastly, *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice exhibited increased neutrophil activation and pro-inflammatory cytokine production, possibly through the induction of NFκB signaling. Together, our data suggest that Kaiso expression may induce intestinal inflammation, which then predisposes mice to intestinal tumorigenesis.

2. Materials and methods

2.1. Mouse husbandry and mating

All mouse work was performed with the approval of the McMaster Animal Research Ethics Board under Animal Utilization Protocol #10-05-32. *Kaiso*$^{Tg/+}$ mice were generated as previously described [12]. Briefly, Kaiso transgenic mice were generated by microinjection of a myc-tagged *villin-mKaiso* transgene into 1-cell C57BL6/CBA hybrid mouse embryos in vitro. *villin-mKaiso*-injected embryos were implanted into pseudopregnant foster mothers to produce transgenic founder Lines A and E, which were backcrossed with C57BL/6N mice (Taconic) for a minimum of 8 generations to obtain stable transgenic offspring. *Kaiso*$^{Tg/+}$ females were mated with *Apc*$^{Min/+}$ males (The Jackson Laboratory; C57BL6 background) to generate *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice. *Axin2*^{lacZ}*:Kaiso*$^{Tg/+}$ mice were generated by crossing *Axin2*^{lacZ} females (The Jackson Laboratory; C57BL6 background) with *Kaiso*$^{Tg/+}$ males. Pregnant females were fed a standard chow diet supplemented with transgenic dough once a week. 21 day-old pups were transferred to a Specific Pathogen Free (SPF) facility and fed a standard chow diet. All mating was performed in a clean, vented-rack room at the McMaster University Central Animal Facility (Hamilton ON). For *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ mice, health monitoring was conducted daily and mice were weighed every 3 days until they exhibited signs of the experimental endpoint, which was defined as the point at which animals lost at least 15% of body weight and exhibited two or more of the following symptoms: poor body condition, profuse rectal bleeding, slow movement, pale extremities. All animals were euthanized by CO_2 asphyxiation, followed by cervical dislocation. Measurements from both male and female mice were included in all analyses, except for body weight measurements where only male weights are depicted.

2.2. Mouse tissue harvest

Following euthanasia, spleen, liver, small intestine and large intestine tissues were immediately harvested. Intestinal tissues were flushed with cold 1× PBS, dissected longitudinally and either flash frozen or fixed in phosphate-buffered formalin (PBF) for 48 h at room temperature (RT). Spleen and liver tissues were rinsed in cold 1× PBS and fixed in PBF for 48 h at RT. Intestinal tissues harvested for histological and immunohistochemical (IHC) analyses were sectioned into 3 equal sections and rolled into "Swiss rolls" before fixation. Fixed tissues were paraffin-embedded, sectioned into 5 μm slices and mounted onto slides at the John Mayberry Histology Facility at McMaster. Hematoxylin and eosin (H&E) stains were also performed by the John Mayberry Histology Facility.

2.3. Polyp measurements

Fixed intestinal tissues were stained for 30 s in a 0.05% methylene blue solution at RT. Tissues were washed repeatedly in 1× PBS until polyps became easily distinguishable from surrounding intestinal tissue. Polyps in stained intestinal sections were counted and polyp area (mm^2) was measured using ImageJ software. A Student's *t*-test was performed to determine the statistical significance of any differences observed.

2.4. Immunohistochemistry

IHC staining for Kaiso and Ki67 was performed as previously described [12]. Staining for cleaved caspase-3 was performed using rabbit anti-cleaved caspase-3 antibody (Asp 175) (Cell Signaling) at a 1:200 dilution. MMP-7 staining was performed using rat anti-MMP-7 antibody (Vanderbilt Antibody Resource) at a 1:200 dilution. NFκB staining was performed using rabbit anti-NFκB antibody (Cell Signaling) at a 1:200 dilution. Colorectal cancer tissue microarrays (TMAs) were purchased from US Biomax (Catalogue #: CO951 and BC05115) and stained and scored for Kaiso expression. Immunostaining for cytoplasmic Kaiso and nuclear Kaiso were evaluated separately. Immunostaining was evaluated by determining the percentage of malignant cells in three random fields on a scale of 0–4. Scores of 0 (no staining), 1 (10%), 2 (10–50%), 3 (50–75%), and 4 (75%) were assigned as previously described [21,22]. F4/80 staining for macrophages was conducted by the John Mayberry Histology Facility, McMaster University. All images were acquired using the Aperio ScanScope and processed using ImageScope software.

2.5. β-Galactosidase staining

For β-galactosidase staining, "Swiss-rolled" intestines were flash frozen in OCT (Tissue-Tek). Mounted tissues were fixed in 0.2%

Table 1
qRT-PCR primer sequences and annealing temperatures.

Target	Annealing temperature (°C)	Primer sequences (5′–3′)
villin-mKaiso	55.0	*Forward: caacttcctaagatctcccaggt* *Reverse: caaggagttcagcagactgg*
Gapdh	55.0	*Forward: atgaccacagtccatgccatc* *Reverse: cctgcttcaccaccttcttg*
Axin2	57.0	*Forward: tgtgagatccacggaaacag* *Reverse: ctgcgatgcatctctctctg*
Mmp7	57.0	*Forward: gcggagatgctcactttgac* *Reverse: gcatctatcacagcgtgttc*
EphB2	65.2	*Forward: ctgtggtcgtcattgccatc* *Reverse: catgcctggggtcatgtgt*
CD44	56.4	*Forward: aacgagtgcaactacagcct* *Reverse: ctccgtaccaggcatcttcg*
Lect2	61.8	*Forward: cgactgtctggaagaggtttttg* *Reverse: ggtaaactttctgcaggggc*

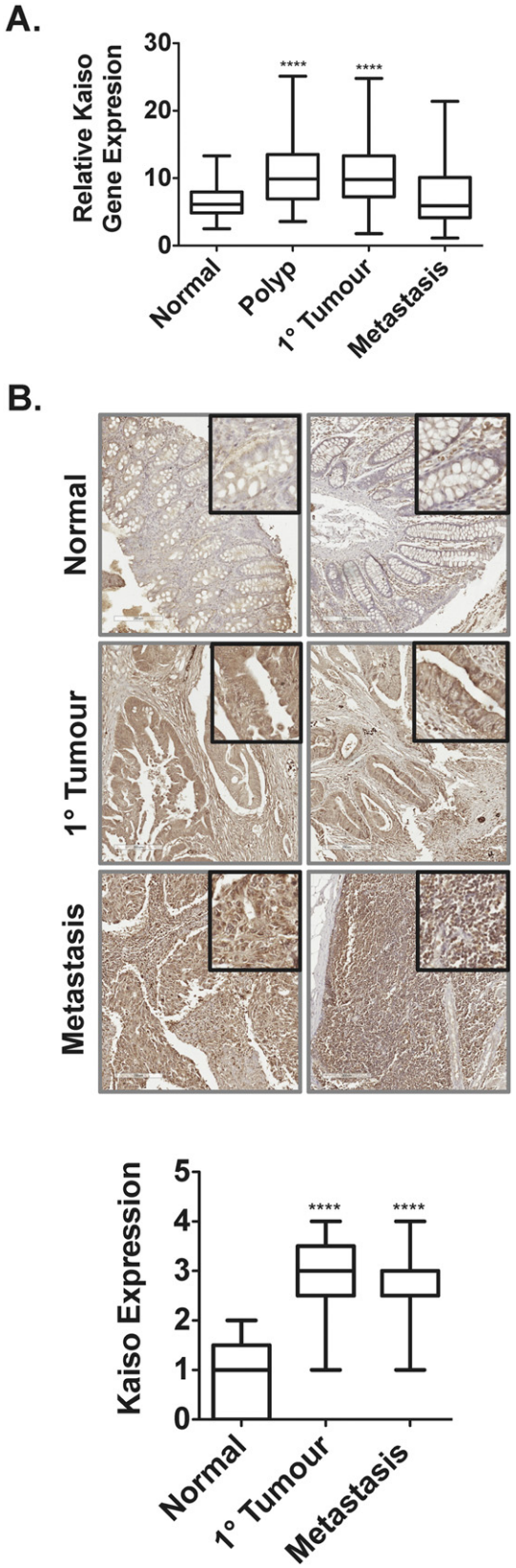

Fig. 1. Kaiso expression is increased in CRC patient biopsies and in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ polyps. A) Comparison of Kaiso mRNA expression in human normal colon tissues, polyps, primary tumors and metastases. B) IHC analysis and quantification of Kaiso protein expression in human primary colorectal tumors and metastases compared to normal colon biopsies (**** represents $p < 0.0001$. Error bars indicate standard error of the mean).

glutaraldehyde in 1× PBS for 10 min at 4 °C then washed in detergent solution (0.02% NP-40, 0.01% sodium deoxycholate, 2 mM $MgCl_2$) for 10 min at RT. Tissues were stained in a 1 mg/mL X-gal solution (Sigma) for 24 h at 37 °C in the dark and washed 3 times in 1× PBS for 2 min each. Post-fixation was performed by incubating tissues in 4% paraformaldehyde for 10 min. Tissues were counterstained with Nuclear Fast Red (Sigma) for 3 min at RT and rinsed under running ddH_2O for 5 min. Finally, tissues were dehydrated in an ethanol series and cleared with xylenes.

2.6. Myeloperoxidase (MPO) assay

50 mg of flash-frozen intestinal tissue was homogenized in a volume of HTAB buffer (0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer, pH 6.0) according to the formula V_{HTAB} = mg of tissue/50. Homogenates were cleared by centrifugation at 12,000 rpm for 5 min at 4 °C. 200 μL of o-dianisidine dihydrochloride solution (16.8 mg/mL o-dianisidine dihydrocholide in 5 mM phosphate buffer, pH 6.0 with 50 μL of 1.2% H_2O_2) was mixed with 7 μL of tissue homogenate and absorbance at 450 nm was measured 3 times at 30 second

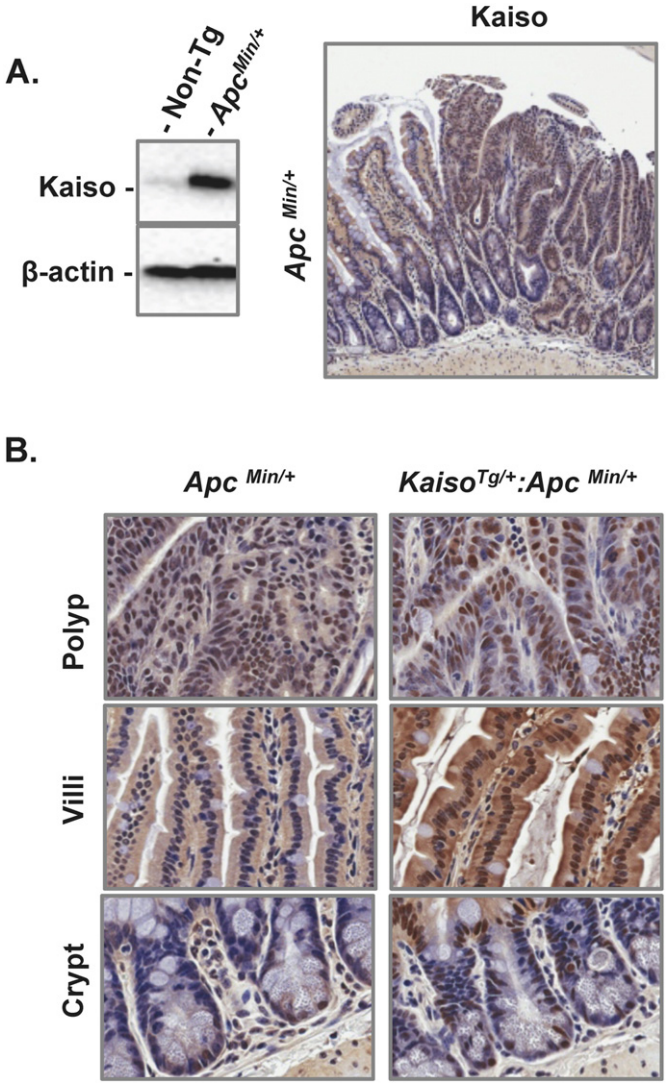

Fig. 2. Kaiso expression is increased in *Apc*$^{Min/+}$ mice. A) Western blot demonstrating high Kaiso expression in *Apc*$^{Min/+}$ versus Non-Tg mice intestinal tissues. Intense nuclear staining for Kaiso was observed in *Apc*$^{Min/+}$ polyps relative to adjacent villi and crypts where Kaiso was localized to both the cytoplasm and the nucleus. Data is representative of at least 3 independent trials performed with different mice. B) Kaiso expression in the small intestines of *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ mice.

intervals. The absorbance of each homogenate was measured in triplicate and the absorbances averaged to calculate MPO activity. MPO activity was calculated in units (U), where 1 U represents the amount of MPO required to degrade 1 μmol of H_2O_2/min at 25 °C, which gives an absorbance of 1.13×10^{-2} nm/min. MPO activity in each sample was determined as the change in absorbance $[\Delta A(t_2 - t_1) / \Delta min]/(1.13 \times 10^{-2})$.

2.7. Multiplex cytokine assay

Approximately 50 mg of fresh tissue was homogenized in buffer containing 20 mM Tris HCl (pH 7.5), 0.5% Tween 20, 150 mM NaCl and cOmplete Ultratablets (Roche) (1 tablet per 25 mL of buffer). Tissue homogenates were diluted to contain equal amounts of total protein (>400 μg/mL) and sent to Eve Technologies Corporation (Calgary, Alberta) where cytokine content was measured in pg/mL using the Mouse 32-Plex Cytokine Panel. A Student's *t*-test was performed to determine the statistical significance of any differences observed.

2.8. Western blot analysis

Western blot analysis was performed as described in [12]. Briefly, 50 mg of intestinal tissue was homogenized in RIPA buffer and the protein concentration of the resulting homogenates estimated using a Bradford assay. 25–50 μg of denatured protein lysate was electrophoresed on an SDS polyacrylamide gel, and then transferred to a nitrocellulose membrane. Antibodies used were as follows: rabbit anti-Kaiso polyclonal antibody at a 1:10,000 dilution, mouse anti-β-actin monoclonal antibody (Sigma Aldrich) at a 1:30,000 dilution, rabbit anti-NFκB monoclonal antibody (Cell Signaling) at a 1:1000 dilution, rabbit anti-pNFκB monoclonal antibody (Cell Signaling) at a 1:500 dilution, rabbit anti-iNOS polyclonal antibody (Cell Signaling) at a 1:1000 dilution, mouse anti-ICAM-1 monoclonal antibody (Santa Cruz Biotechnology) at a 1:100 dilution, mouse anti-plκBα monoclonal antibody (Cell Signaling) at a 1:500 dilution.

2.9. Gene expression analysis

Expression data from 123 primary colorectal cancer and 25 normal patient biopsies were obtained from Gene Expression Omnibus (GEO accession number GSE41258) [23]. Expression data were normalized using robust-microarray analysis (RMA), quantile normalized and background corrected using RMA background correction. A Student's *t*-test was performed to determine the significance of any differences observed between groups using GraphPad Prism 5 software.

2.10. Quantitative reverse-transcription PCR

Total RNA was isolated from flash-frozen tissues using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's protocol. 1 μg of RNA from each sample was treated with DNase I (Invitrogen) and reverse transcribed using the qScript cDNA SuperMix (Quanta BioSciences) according to the manufacturers' protocols. cDNA was amplified using the PerfeCta SYBR Green SuperMix, ROX (Quanta BioSciences). Each reaction was performed in triplicate using the Applied BioSystems Prism 7900HT sequence detection system. Primer sequences utilized for the targets and their corresponding annealing temperatures are listed in Table 1.

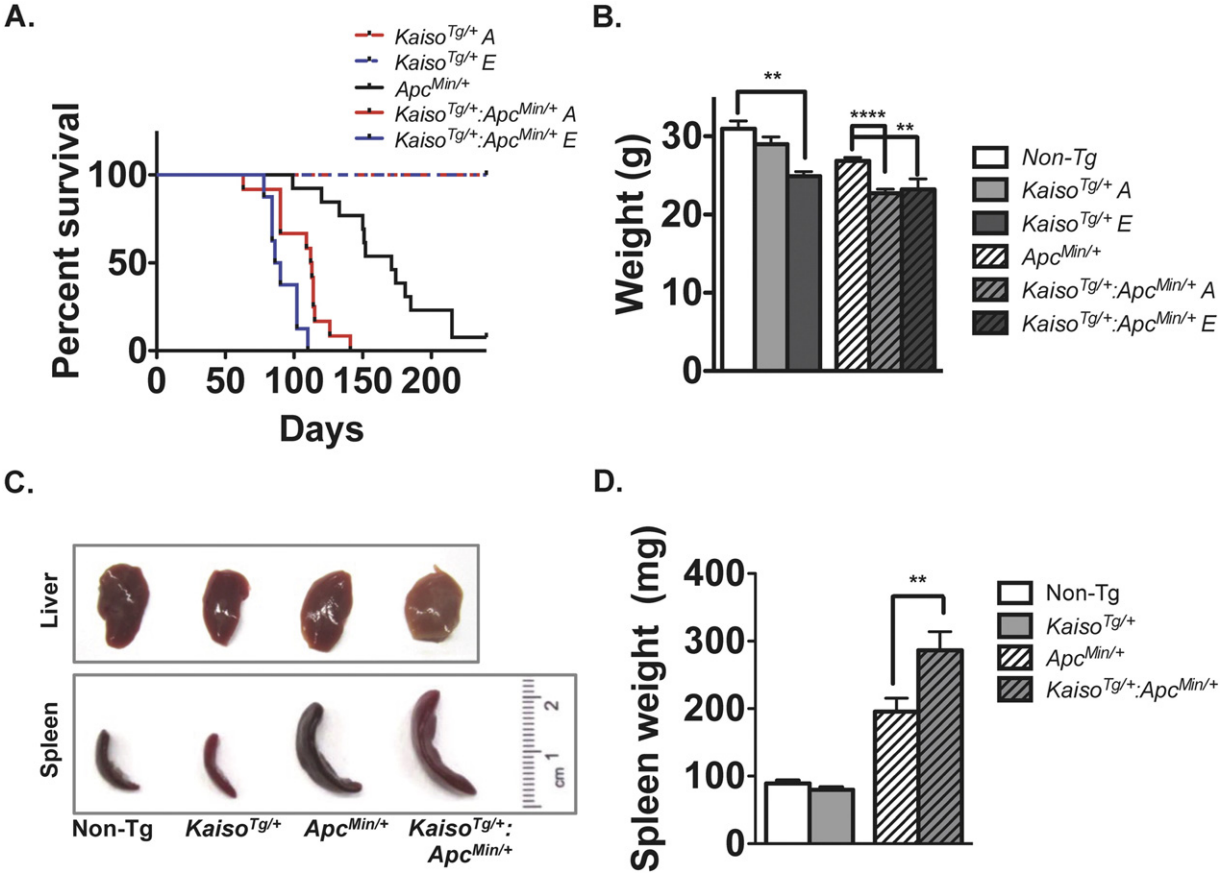

Fig. 3. $Apc^{Min/+}$ lifespan is attenuated by ectopic Kaiso expression. A) Kaplan–Meier survival curve comparing survival of $Kaiso^{Tg/+}$, $Apc^{Min/+}$ and $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice (median survival $Apc^{Min/+}$ = 161.5 days vs. $Kaiso^{Tg/+}$:$Apc^{Min/+}$ Line A = 112.5 & Line E = 88.5 days; n ≥ 5, Log rank test $p < 0.0001$). B) $Kaiso^{Tg/+}$:$Apc^{Min/+}$ Line A and Line E mice weigh significantly less than their $Apc^{Min/+}$ counterparts at 90 days of age. $Kaiso^{Tg/+}$ Line A and Line E mice also weigh less than their Non-Tg littermates at this age (n ≥ 5). C) Anemia and splenomegaly are enhanced in $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice as indicated by loss of liver pigment (top panel) and enlarged spleen (bottom panel). D) Spleen weight is significantly increased in 90 day-old $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice (n ≥ 18) (* represents $p \leq 0.05$; ** represents $p \leq 0.01$; **** represents $p < 0.0001$. Error bars indicate standard error of the mean).

The quantity of each target was determined using a standard curve and was normalized to *Gapdh* expression levels. The standard curve was constructed using 5-fold serial dilutions of cDNA reverse transcribed from a mixture of RNA from all mice. Statistical analyses were conducted using GraphPad Prism software. Statistical significance was calculated using a Student's *t*-test with measurements from at least 4 independent trials.

3. Results

3.1. Kaiso expression is increased in human colorectal tumor tissues and $Apc^{Min/+}$ polyps

Previous studies have implicated Kaiso in intestinal tumorigenesis utilizing murine models and cultured colorectal cancer cell lines [9,11]. However, no study has examined the expression of Kaiso in a large cohort of CRC patient tissues. We analyzed *Kaiso* mRNA expression in a gene expression data set from human colon biopsies, consisting of 74 normal colon tissues, 49 colonic polyp tissues, 186 primary colorectal tumor tissues and 69 metastases [23]. *Kaiso* expression was significantly increased in polyps and primary tumors compared to normal tissues ($p < 0.0001$ and $p < 0.0001$, respectively) (Fig. 1A). *Kaiso* mRNA expression was also increased in metastases, although this increase was not significant (Fig. 1A). To complement our bioinformatics studies, we examined Kaiso protein expression in two CRC tissue microarrays that consisted of 17 normal colon tissues, 60 primary tumor tissues and 40 metastases (US Biomax, MD, USA). In agreement with our bioinformatics analysis, Kaiso expression was largely absent or low in normal colon biopsies compared to primary and metastatic tumor biopsies ($p < 0.0001$ and $p < 0.0001$, respectively) where robust Kaiso staining was observed (Fig. 1B). While our analysis of Kaiso expression does not demonstrate a causal relationship between Kaiso and CRC progression, the analysis supports the idea that high Kaiso expression correlates with increasing malignancy.

We next examined Kaiso expression in $Apc^{Min/+}$ mice compared to non-transgenic (Non-Tg) mice. Since $Apc^{Min/+}$ mice develop polyps primarily in the small intestine [24], we focused our studies on the small intestine unless otherwise stated. Nuclear Kaiso expression was increased in polyps relative to the surrounding intestinal epithelium (Fig. 2A & B) and relative to that of Non-Tg mice (data not shown). Immunoblot analysis also confirmed increased expression of Kaiso in $Apc^{Min/+}$ intestinal homogenates compared to Non-Tg mice (Fig. 2A).

3.2. Kaiso overexpression significantly attenuates the lifespan of $Apc^{Min/+}$ mice

Previous studies investigating the effect of Kaiso depletion on murine development demonstrated that *Kaiso*-null mice exhibited no deleterious developmental defects [9]. Interestingly however, when *Kaiso*-null mice were mated with $Apc^{Min/+}$ mice the resultant progeny exhibited prolonged lifespan and decreased polyp size, although no change in polyp number was observed [9].

To confirm and extend the studies of Prokhortchouk et al., we generated and characterized a mouse model expressing an intestinal-specific, murine myc-tagged Kaiso transgene [12]. We opted for a transgenic approach to overcome the potential effects of functional redundancy between Kaiso and the Kaiso-like proteins ZBTB4 and ZBTB38 that may have precluded effective resolution of Kaiso's role in the Kaiso knockout model. Up to 12 months of age, $Kaiso^{Tg/+}$ mice did not develop polyps, but they displayed crypt hyperplasia and dysplasia of varying severities [12]. To facilitate our investigation of Kaiso's role in intestinal tumorigenesis, we utilized the $Apc^{Min/+}$ model of colon cancer as a sensitized background and mated these mice with two independently generated $Kaiso^{Tg/+}$ lines to generate $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice. $Kaiso^{Tg/+}$ Line E mice express more ectopic Kaiso than $Kaiso^{Tg/+}$ Line A mice, indicative of a higher transgene copy number [12]. As expected, $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibited robust nuclear Kaiso staining in both the polyps and the intestinal epithelium (Fig. 2B). However, Kaiso expression in $Kaiso^{Tg/+}$:$Apc^{Min/+}$ polyps was heterogeneous, with some cells expressing more Kaiso than others (Fig. 2B). Additionally, although Kaiso expression was increased in the crypts of $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice compared to $Apc^{Min/+}$ mice, crypt staining was not as pronounced as that observed in the villi, where both nuclear and cytoplasmic Kaiso was evident.

We recently reported that $Kaiso^{Tg/+}$ mice exhibited normal lifespan and that no intestinal polyps were detected in $Kaiso^{Tg/+}$ Line A mice up to one year of age although the mice exhibited crypt hyperplasia [12]. However, $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibited significantly reduced lifespan compared to $Apc^{Min/+}$ mice ($p < 0.0001$) in a dose-dependent manner, i.e. $Kaiso^{Tg/+}$:$Apc^{Min/+}$ Line E mice (high Kaiso expression) exhibited significantly reduced lifespan compared to $Kaiso^{Tg/+}$:$Apc^{Min/+}$ Line A mice (moderate Kaiso expression) ($p < 0.05$), suggesting that increased transgene dosage results in a more severe phenotype (Fig. 3A). Overall health, as indicated by weight and liver pigment, were also assessed in age-matched 90 day-old $Kaiso^{Tg/+}$:$Apc^{Min/+}$ and $Apc^{Min/+}$ mice since loss of 15% of body mass and anemia are often used as endpoint indicators in $Apc^{Min/+}$ mice. At 90 days of age, both $Kaiso^{Tg/+}$:$Apc^{Min/+}$ Line A and Line E mice weighed less than their $Apc^{Min/+}$ counterparts (Fig. 3B). No significant difference in weight between $Kaiso^{Tg/+}$:$Apc^{Min/+}$ Line A and Line E mice was observed, although the significantly diminished lifespan of $Kaiso^{Tg/+}$:$Apc^{Min/+}$ Line E mice precluded weight measurement of a larger sample size. Notably, $Kaiso^{Tg/+}$ Line E mice also weighed significantly less than their Non-Tg counterparts (Fig. 3B), suggesting that ectopic Kaiso expression results in decreased weight even in the absence of the Min mutation. At 90 days of age, $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibited decreased liver pigment, while $Apc^{Min/+}$ mice livers showed little to no loss of pigment (Fig. 3C, Top Panel). We also noted that $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice had grossly enlarged spleens, which weighed significantly more than spleens isolated from

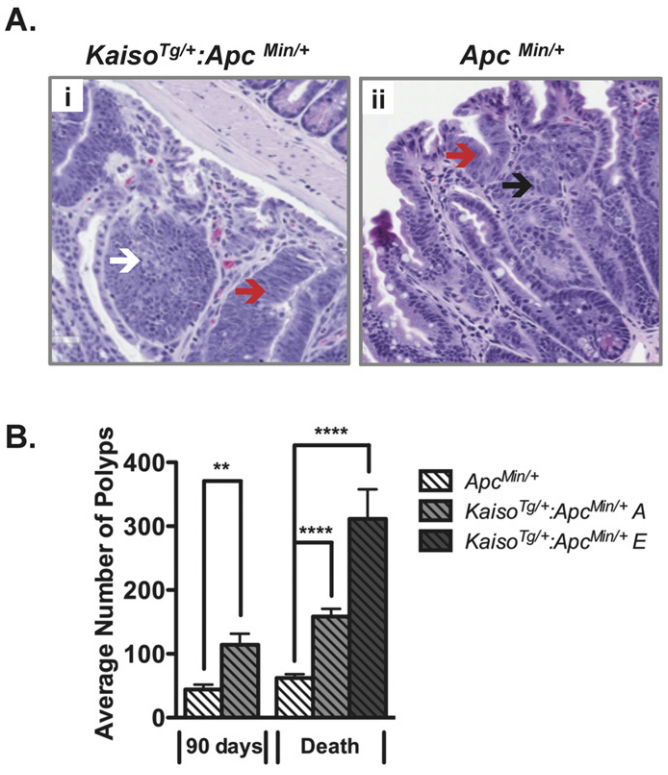

Fig. 4. Ectopic Kaiso enhances polyp formation in $Apc^{Min/+}$ mice. A) $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibit extensive regions of focal atypical hyperplasia (i, ii, red arrowheads) and formed adenomas (i, white arrowhead) compared to their $Apc^{Min/+}$ counterparts (ii), which exhibit mostly focal early adenomas (black arrowheads). Representative images from pathological examination of 3 mice/genotype. B) $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibit significantly more polyps than $Apc^{Min/+}$ mice ($n \geq 6$) (** represents $p \leq 0.01$; **** represents $p < 0.0001$. Error bars indicate standard error of the mean).

$Apc^{Min/+}$ mice (Fig. 3C, Bottom Panel, Fig. 3D). However the relevance, if any, of these enlarged spleens to the $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice phenotype or CRC is unknown.

3.3. Polyp burden is increased in $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice

We next examined the polyp burden of $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice. Gross histological examination of the small intestines from $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice revealed extensive regions of focal atypical hyperplasia (Fig. 4A-i) and increased mitoses. Regions of atypical hyperplasia were also evident in $Apc^{Min/+}$ mice, albeit at a much lower frequency (Fig. 4A-ii). $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice also exhibited increased numbers of early adenomas and numerous formed adenomas, which were largely absent in $Apc^{Min/+}$ mice (Figs. 4A-i & ii). More importantly, $Kaiso^{Tg/+}$:$Apc^{Min/+}$ Line A mice exhibited ~2.5-fold more and Line E mice ~5-fold more polyps at death than $Apc^{Min/+}$ mice. At 90 days of age, $Kaiso^{Tg/+}$:$Apc^{Min/+}$ Line A mice exhibited ~3-fold more polyps than age-matched $Apc^{Min/+}$ mice, suggesting that increased Kaiso expression accelerates polyp onset (Fig. 4B).

$Kaiso^{Tg/+}$:$Apc^{Min/+}$ polyps were significantly smaller than those of $Apc^{Min/+}$ mice at both 90 days of age and at death (~110 days), although this difference was more apparent at death (Fig. 5A-i). We postulated that the decreased polyp size in $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice may be attributed to the significantly shortened lifespan of these mice compared to their $Apc^{Min/+}$ counterparts. Indeed, the size distribution of polyps in $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice at 90 days of age was similar to that of $Apc^{Min/+}$ mice (Fig. 5A-ii). However, at death, $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibited fewer polyps of 3 mm^2 or larger, but a greater proportion of polyps less than 1 mm^2 and 1–3 mm^2 compared to their $Apc^{Min/+}$ counterparts (Fig. 5A-iii).

Although the reduced polyp size in $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice could be attributed to their shortened lifespan, it remained possible that the reduced polyp size could also be attributed to decreased proliferation or increased apoptosis. Staining for the proliferation marker Ki67 revealed no difference in proliferation between Non-Tg and $Kaiso^{Tg/+}$ mice or between $Apc^{Min/+}$ and $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice at 90 days of age (Fig. 5B). To measure apoptosis, intestinal sections were stained for cleaved caspase-3 [25] and the percentage of apoptotic cells/polyp determined. $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibited more cleaved caspase-3-positive cells than $Apc^{Min/+}$ *mice*, suggesting that ectopic Kaiso expression increases apoptosis in intestinal polyps, which may contribute to the decreased polyp size seen in $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice (Fig. 5C).

3.4. Wnt signaling is upregulated upon ectopic expression of Kaiso

Our finding that ectopic Kaiso expression increases polyp burden while Kaiso loss delays polyp onset in the $Apc^{Min/+}$ mouse model [9] is paradoxical in light of previous studies where Kaiso has been implicated as a negative regulator of canonical Wnt signaling [6,26]. Our findings in the $Kaiso^{Tg/+}$:$Apc^{Min/+}$ model thus raised the possibility that Kaiso's role as a negative regulator of Wnt signaling may be context dependent. To clarify Kaiso's function in regulating Wnt signaling in the intestine we

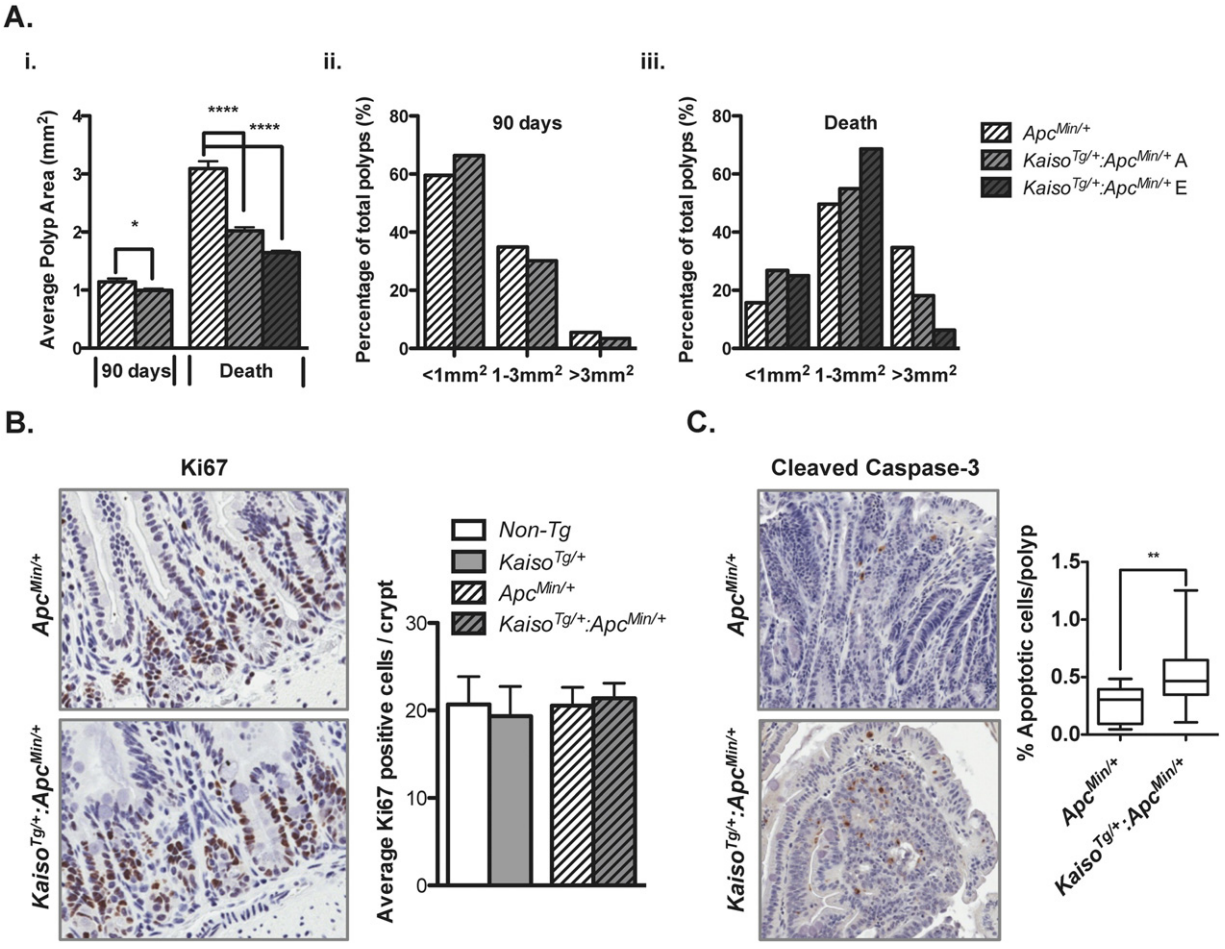

Fig. 5. $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice have smaller polyps than $Apc^{Min/+}$ mice at death. A) Intestinal polyps of Line A and Line E $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice are significantly smaller than those of $Apc^{Min/+}$ mice (n ≥ 292) (left panel). Age-matched $Apc^{Min/+}$ and $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice exhibit no difference in size distribution of polyps at 90 days of age (middle panel) but exhibit fewer polyps greater than 3 mm^2 at death (right panel). B) Staining for the proliferation marker Ki67 revealed no change in proliferation between $Apc^{Min/+}$ and $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice. C) Percentage of apoptotic cells per polyp is greater in $Kaiso^{Tg/+}$:$Apc^{Min/+}$ mice. Images depicted for Ki67 and cleaved-caspase 3 staining are representative of 3 independent trials (* represents $p \le 0.05$; ** represents $p \le 0.01$; **** represents $p < 0.0001$. Error bars indicate standard error of the mean).

examined the effect of ectopic Kaiso expression on Wnt target gene expression. *Kaiso*$^{Tg/+}$ mice were crossed with heterozygous *Axin2*^{lacZ} mice, a Wnt reporter line in which the DNA sequence encoding a nuclear-localized β-galactosidase (NLS-*lacZ*) is inserted in-frame into exon 2 of the endogenous Wnt target gene *Axin2* [27]. Surprisingly, *Axin2*^{lacZ}:*Kaiso*$^{Tg/+}$ mice exhibited increased expression of β-galactosidase compared to *Axin2*^{lacZ} mice (Fig. 6A). Consistent with this finding, *Kaiso*$^{Tg/+}$ mice and *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice exhibited increased expression of another Wnt target gene, MMP-7, compared to control mice (Fig. 6B). To confirm that Kaiso was positively regulating Wnt/β-catenin target gene expression in murine intestines, we examined the mRNA expression of four established Wnt target genes (*MMP7*, *Axin2, CD44* and *EphB2)* in the intestine using qRT-PCR. The expression of all four target genes was significantly increased in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice relative to control mice, ($p < 0.05$) (Fig. 6C). Interestingly, *Kaiso*$^{Tg/+}$ mice also exhibited increased expression of all four target genes, although these changes were not significant (Fig. 6C). Expression of β-catenin remained relatively unchanged across all 4 genotypes (data not shown), but expression of the Wnt pathway antagonist *Lect2* was significantly decreased in *Kaiso*$^{Tg/+}$ mice relative to Non-Tg mice (Fig. 6D). Collectively, these data suggest that Kaiso's transcriptional repression effects on Wnt signaling are context-dependent and are not maintained in the *Apc*$^{Min/+}$ model.

3.5. Kaiso induces inflammation in *Apc*$^{Min/+}$ mice

Previously, we found that 12 month-old *Kaiso*$^{Tg/+}$ mice exhibited signs of chronic intestinal inflammation, i.e. villi blunting, leukocyte infiltration and neutrophil activation [12] but it was unclear whether the Kaiso-induced inflammation occurred at younger ages. When we examined the ileum of 90 day-old Line A *Kaiso*$^{Tg/+}$ mice (when intestinal polyps are already well-developed in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice), an intestinal inflammation phenotype was evident albeit at a lower frequency than that observed in older mice (Fig. 7A). Extensive macrophage recruitment was also evident in inflamed epithelia overlying Peyer's patches in the ileum of Line A *Kaiso*$^{Tg/+}$ mice as measured by F4/80 staining (Fig. 7B). We also noticed several crypt abscesses in our *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice (Fig. 7C), reminiscent of those frequently observed in the colon of patients with IBD. However, abscesses were completely absent in both Line A *Kaiso*$^{Tg/+}$ and *Apc*$^{Min/+}$ mice (data not shown).

We next performed an MPO assay on intestinal homogenates to assess neutrophil activation as a surrogate marker for inflammation. No significant change in MPO activity was observed in 90 day-old *Kaiso*$^{Tg/+}$ mice relative to Non-Tg mice (data not shown), but a 6-fold increase in activity was observed in both the small and large intestines of 90 day-old *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice relative to *Apc*$^{Min/+}$ mice (Fig. 7D). Several pro-inflammatory cytokines and chemokines associated with innate immunity were also enriched in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ intestinal homogenates, namely granulocyte stimulating factors GM-CSF and G-CSF, eotaxin, IL-3, IL-7, LIF, MCP-1 and MIP1B (Fig. 7E).

Since the Kaiso binding partner p120^{ctn} has been implicated as an anti-inflammatory mediator in multiple tissues and was postulated to act through the NFκB signaling pathway, we examined NFκB expression in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ intestinal tissues. We detected increased nuclear NFκB in intestinal epithelial cells of *Kaiso*$^{Tg/+}$ and *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice (Fig. 7F). Immunoblot analysis further revealed activation of the NFκB pathway as evidenced by increased expression of iNOS, ICAM-1 and phospho-NFκB (Fig. 7G). Notably we also detected a modest decrease in expression of the NFκB inhibitor IκB-α (Fig. 7G). Collectively these data suggest that the increased polyp burden in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice may in part be due to Kaiso-induced intestinal inflammation that involves activation of the NFκB pathway.

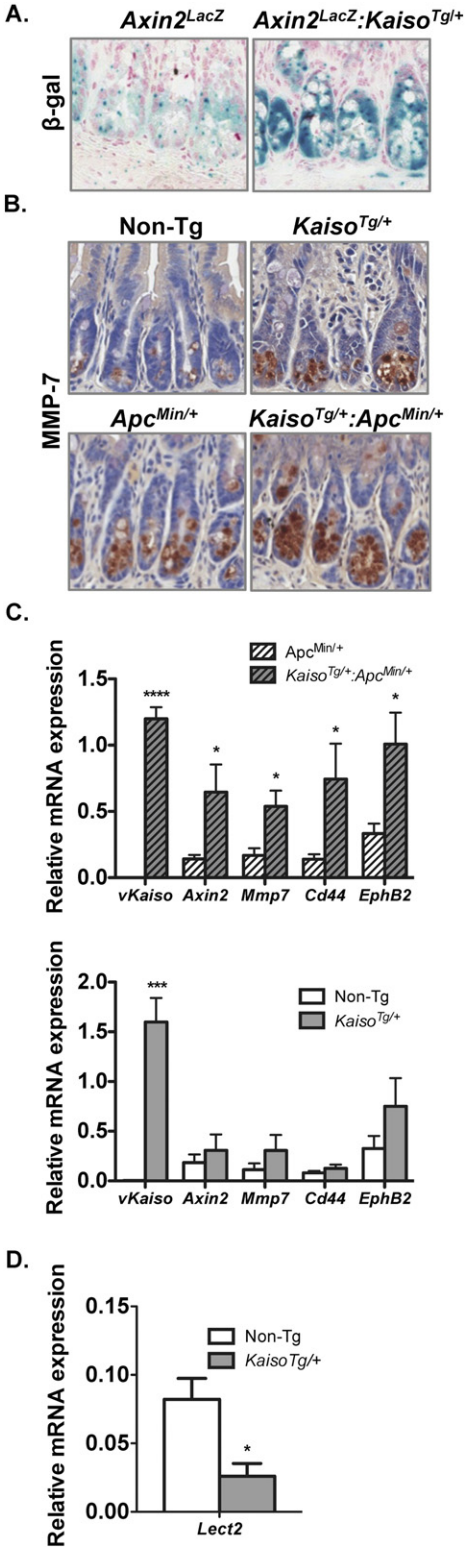

Fig. 6. Kaiso potentiates Wnt signaling in the intestine: A) *Axin2*^{lacZ}:*Kaiso*$^{Tg/+}$ mice exhibit increased Wnt reporter activity. B) MMP7 expression is increased in *Kaiso*$^{Tg/+}$ and *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice relative to their respective controls. Images depicting β-galactosidase and MMP-7 staining are representative of at least 3 independent trials. C) mRNA expression of Wnt target genes in *Kaiso*$^{Tg/+}$, *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ and control mice ($n \leq 5$). D) Expression of the Wnt antagonist Lect2 is reduced in *Kaiso*$^{Tg/+}$ mice ($n \leq 5$) (* represents $p \leq 0.05$; *** represents $p \leq 0.001$; **** represents $p < 0.0001$. Error bars indicate standard error of the mean).

4. Discussion

Kaiso has been implicated in the development and progression of several human cancers, including colorectal cancer [5,9,11,22,28–34]. To date however, most studies examining Kaiso's role in cancer have been largely correlative [9,29–31,33], while those offering mechanistic insight have been performed primarily in mammalian cultured cells [11,22,28,32,34]. Here we describe the first study to examine a potential mechanistic role of Kaiso in colorectal cancer using the *Apc*$^{Min/+}$ mouse model of intestinal cancer.

As previously reported, Kaiso depletion extends the lifespan of *Apc*$^{Min/+}$ mice [9], and therefore our finding that ectopic Kaiso expression decreases the lifespan of *Apc*$^{Min/+}$ mice was not surprising. However, unlike the Prokhortchouk et al. study, which reported no difference in the number of polyps between *Kaiso*-null *Apc*$^{Min/+}$ and *Apc*$^{Min/+}$ mice [9], we found increased polyp numbers in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ compared to *Apc*$^{Min/+}$ mice. Notwithstanding the disadvantages associated with the use of transgenic mouse models, our study highlights one advantage of the *Kaiso*$^{Tg/+}$ model; namely that certain effects arising from Kaiso depletion models may be masked

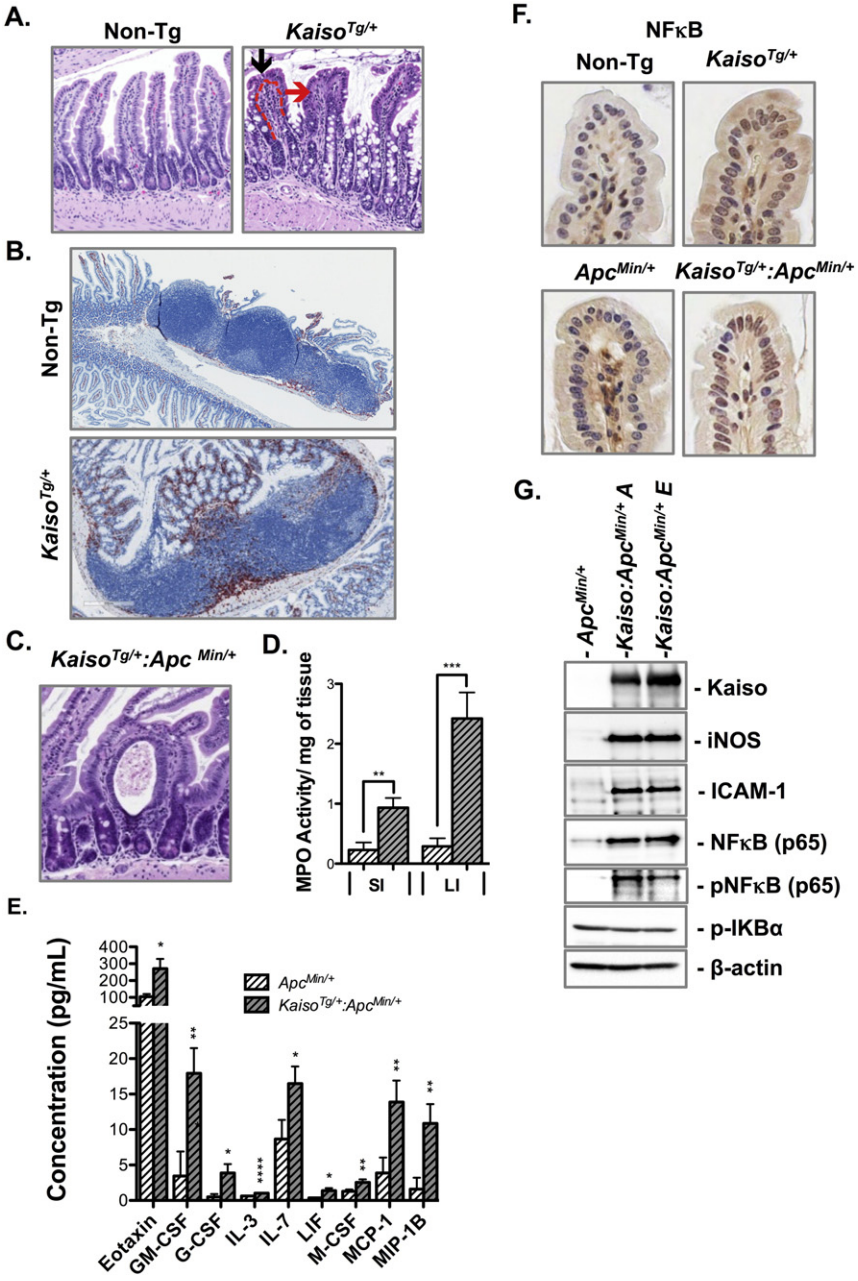

Fig. 7. *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice exhibit intestinal inflammation. A) H&E image showing villi blunting (black arrowhead), villi fusion (red arrow head) and immune cell infiltration of the lamina propria (red dotted line) in 90 day-old *Kaiso*$^{Tg/+}$mice, phenotypes that are absent in age-matched Non-Tg mice. B) F4/80 staining for macrophages and activated monocytes shows recruitment of these immune cells to an inflamed region in the ileum of 90 day-old *Kaiso*$^{Tg/+}$ through a Peyer's patch. C) H&E image of crypt abscesses observed in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice. All images representative of 3 independent trials. D) MPO activity is increased in small and large intestines (SI & LI) of *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice (n ≤ 11). E) Pro-inflammatory cytokines are upregulated in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ mice (n ≤ 10). F) NFκB localizes to the nucleus in *Kaiso*$^{Tg/+}$ and *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$tissues. G) NFκB signaling is activated in *Kaiso*$^{Tg/+}$:*Apc*$^{Min/+}$ tissues relative to *Apc*$^{Min/+}$ controls as evidenced by increased expression of iNOS and ICAM-1. All images representative of 3 independent trials (* represents $p \le 0.05$; ** represents $p \le 0.01$; *** represents $p \le 0.001$; **** represents $p < 0.0001$. Error bars indicate standard error of the mean).

due to the existence of the Kaiso-like proteins (ZBTB4 and ZBTB38) or other methyl-DNA-binding proteins that may function redundantly with Kaiso [35]. Indeed, this may explain why *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice exhibited more polyps than *Apc*$^{Min/+}$ mice, while *Kaiso*-null *Apc*$^{Min/+}$ mice did not exhibit the opposite effect (i.e. less polyps) [9].

The finding that ectopic Kaiso expression increases polyp number but decreases polyp size was surprising, since typically polyp number and size are positively correlated. *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice exhibit histologically more advanced tumors than age-matched *Apc*$^{Min/+}$ mice, although there was no difference in tumor proliferation rate, suggesting that *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice tumors form earlier. Although the significantly shortened lifespan of *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice may account for the smaller tumor size, we also observed a larger number of cleaved-caspase 3 positive cells in the polyps of *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice. Previous studies have demonstrated roles for Kaiso in cell cycle arrest and apoptosis [36,37]. Kaiso knockout mice exhibited increased expression of Bcl6, which in turn increased the expression of the cell cycle regulatory genes, *p27 (Cdkn1b)*, *p21 (Cdkn1a)* and *Gadd45a* [37]. Kaiso has also been shown to couple with p53 to promote the expression of p21 and the pro-apoptotic genes *BAX* and *PUMA*, and consequently, Kaiso depletion protects against etoposide-induced apoptosis in MEF cells [36]. These studies coupled with our observation of increased apoptotic cells in *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ polyps, supports the idea that the smaller polyps observed in *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice may be due, at least in part, to a Kaiso-induced increase in apoptosis.

Consistent with our previous findings that *Kaiso*$^{Tg/+}$ mice exhibited intestinal inflammation, *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice likewise exhibited enhanced intestinal inflammation. The onset of inflammation seems to be accelerated in the presence of the *Min* allele, consistent with reports that polyp formation is accompanied by increased inflammation in *Apc*$^{Min/+}$ mice [38]. Our initial investigation of the mechanism by which Kaiso promotes inflammation suggests that Kaiso's role in inflammation may be linked to its binding partner p120^{ctn} that has been characterized as an anti-inflammatory mediator in several tissues, including the intestine [39–43]. Conditional p120^{ctn} depletion in murine intestines trigger acute inflammation and lethality at less than 21 days of age [39], while limited p120^{ctn} ablation (~15% of the intestinal epithelium) results in chronic inflammation and adenoma formation [40]. While the cause of inflammation following p120^{ctn} depletion in the intestine is not fully understood, significant barrier defects due to decreased E-cadherin expression may be a contributing factor [40]. Remarkably, *Kaiso*$^{Tg/+}$ mice also exhibit a barrier defect, but there was no apparent decrease in p120^{ctn} or E-cadherin expression, suggesting that the barrier defect in *Kaiso*$^{Tg/+}$ mice may be a consequence rather than a cause of inflammation (our unpublished data). However recent studies suggest that p120^{ctn} pro-inflammatory effects may also be mediated through the NFκB pathway [40–44] and indeed, we found that NFκB pathway signaling is increased in *Kaiso*$^{Tg/+}$ and *Kaiso*$^{Tg/+}$*;Apc*$^{Min/+}$ mice. Ongoing studies are thus focused on characterizing the interplay between Kaiso and p120^{ctn} in regulating inflammation in the intestine.

One major goal of this study was to clarify whether Kaiso's postulated function as a negative regulator of the Wnt signaling pathway is maintained in the murine intestine. We found that contrary to findings in *Xenopus* embryos and mammalian cultured cells [6,8,26], ectopic Kaiso expression increased Wnt target gene expression. While this was initially surprising and unexpected, such an outcome could be explained by the fact that Kaiso has both transcriptional repression and activation roles [45,46]. Alternately, the active Wnt signaling in mammalian intestines may be promoting p120^{ctn}'s interaction with Kaiso and relieving Kaiso's inhibition of β-catenin/TCF transcriptional activation, as recently observed by del Valle-Perez et al. [8]. Such a possibility is also consistent with the *Xenopus* models proposed by Ruzov et al. [26], who suggest that the interaction between Kaiso and Tcf results in their mutual disengagement from Wnt target genes. Since Wnt signaling is most active in the intestinal crypts where it regulates the proliferation and differentiation of intestinal stem cells [47], it is conceivable that the active Wnt signaling in intestinal crypts promotes Kaiso's dissociation from Tcf4, thereby inhibiting Kaiso's repressive effects on Wnt target genes. However, since the majority of polyps in *Apc*$^{Min/+}$ mice have undergone a loss of heterozygosity at the *Apc* locus [13], the increased Wnt target gene expression observed in *Kaiso*$^{Tg/+}$*:Apc*$^{Min/+}$ mice may be also partially attributed to the significantly higher polyp burden.

An additional explanation for Kaiso's potentiation of Wnt signaling in the murine intestine may involve Kaiso's role in epigenetic or methylation-dependent gene silencing [48,49]. Multiple Wnt antagonists are downregulated through promoter hypermethylation in colorectal cancer [50–53]. For instance, MBD2 binds and regulates the modestly characterized Wnt pathway repressor *Lect2,* which functions to repress Wnt target gene expression at or below the level of TCF [53]. Our detection of decreased *Lect2* mRNA expression in *Kaiso*$^{Tg/+}$ mice suggests that Kaiso's stimulatory effect on Wnt signaling may be attributed to Kaiso-mediated methylation-dependent silencing of Wnt pathway antagonists.

In conclusion, the correlation between increased Kaiso expression and neoplastic progression in human colorectal cancer tissues, coupled with our findings that Kaiso overexpression potentiates Wnt-mediated polyp formation, Wnt target gene expression and inflammation in *Apc*$^{Min/+}$ mice, suggest that Kaiso may function in several distinct oncogenic capacities in colorectal cancer pathogenesis.

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